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Chemical And Morphological Characteristics Of The Calcification System Inrana Catesbeiana Skin.

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CHEMICAL AND MORPHOLOGICAL CHARACTERISTICS OF THE CALCIFICATION SYSTEM IN *RANA CATESBEIANA* SKIN

by

Luther T. Cale, Jr., D.M.D.

A DISSERTATION

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TO MY WIFE, LINDA

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INTRODUCTION

The chemical and physical principles responsible for the deposition, maintenance, and removal of calcium salts in biological calcification systems have been studied for many years, but many basic questions remain unsolved. In regard to the composition and crystalline structure of the mineral in bone and other calcifying tissues, recent data suggest that hydroxyapatite, the major mineral component of most systems, is not deposited directly. Amorphous calcium phosphate, octacalcium phosphate, hydrated tricalcium phosphate, and secondary calcium phosphate all have been proposed as the initial mineral phase which later is converted to hydroxyapatite. Another question receiving major emphasis concerns the location and identity of the specific chemical radicals which are responsible for the property of biological calcification. These nucleating radicals have been suggested to exist in the fibrous organic matrix, in the amorphous ground substance and in the cytoplasm. Other studies have been concerned with the role of enzymatic, hormonal, and dietary influences on calcified tissue.

Much of our present knowledge concerning calcified tissues has been derived from studies on bone and teeth. However, useful information has been gained from studies of calcification systems in unicellular animals, mollusc shells, the gastrolith

and exoskeleton of the lobsters, the leg tendon of turkeys, the otolithic organ of rats, and soft tissues. From these studies it has been shown that various organic matrices are capable of nucleating hydroxyapatite crystals. These matrices include the cytoplasm of some unicellular organisms, the epidermal fibrous tissue of enamel, the mesenchymal fibrous tissue of bone and dentin, and various matrices associated with intracellular and extracellular pathological calcification including that of salivary calculi (microorganisms and their secretions). Also from these studies, crystals other than hydroxyapatite have been found which include calcite, aragonite, calcium sulfate, and calcium oxalate. All of these studies have and will continue to strengthen our basic understanding of how and why a tissue will or will not mineralize.

Amphibian skin was shown to contain granular mineral deposits some 100 years ago. However, relatively little is known about their basic characteristics and mechanisms of formation. The purpose of the experiments described herein was to characterize the biochemical and physiological nature of the skin calcification system in the North American bullfrog, Rana catesbeiana. Specific objectives included the definition of its histological and histochemical characteristics, the changes, if any, which occur during maturation, the physical and chemical nature of the mineral, and the function of the ground substance. The data acquired suggest

that R. catesbeiana skin represents a valuable model in which some of the basic problems in calcification research can be studied.

Section I consists of a review of the pertinent literature relating to amphibian skin calcification systems and a brief description of anuran maturation. Section II gives the experimental approach, results, and discussion of the several subdivisions of this investigation. And finally, Section III presents the conclusions of this investigation and a discussion of how the calcification system in R. catesbeiana skin relates to bone calcification.

SECTION I - LITERATURE REVIEW

THE CALCIFICATION SYSTEM IN ANURAN SKIN

The general structural characteristics of the granular deposits in amphibian skin as well as an excellent review of previous investigations on these skin deposits were reported by Guardabassi and Sacerdote (1951). The dermis of amphibians was observed to consist of two zones (Figure 1): a superficial zone, the stratum spongiosum, containing very thin fibers irregularly interlaced and rich in cells, vessels, and glands; and an internal zone, the stratum compactum, characterized by lamina of collagenous fibers which are parallel to the surface of the skin. Bands of subcutaneous connective tissue were reported to traverse vertically the stratum compactum carrying vessels, nerves, and collagen and elastic fibers. Upon reaching the stratum spongiosum the collagenous fibers of these perforating bands spread out, contacting the superficial border of the stratum compactum, the fibers of the stratum spongiosum, and finally, the epidermis.

Guardabassi and Sacerdote (1951) stated that as early as 1822, Heusingler[1] reported the presence of "kreideartige Kornchen" between the dermal layers in Pipa americana. However, Eberth (1869)[1] was credited as being the first to observe that a fine granular mass existed between the two layers of Rana fusca dermis.

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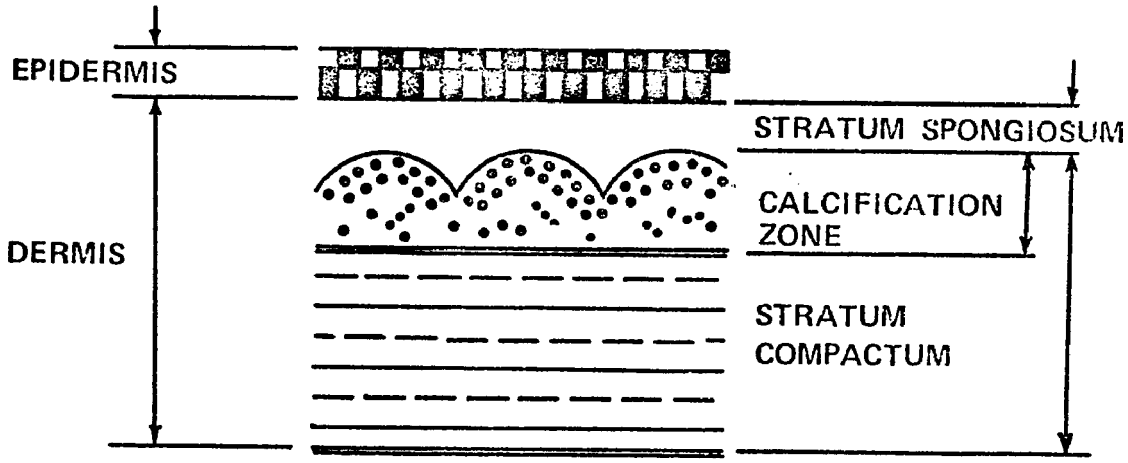
[1]Cited by Guardabassi and Sacerdote (1951).

Figure 1. A Diagram of the Anatomical Divisions in Rana catesbeiana Skin.


EPIDERMIS
DERMIS
STRATUM SPONGIOSUM
CALCIFICATION ZONE
STRATUM COMPACTUM

Numerous reports followed which confirmed Eberth's observation. Kastschenko (1882)[1] fed madder to Rana esculenta and observed a stained layer between the spongy and compact portion of the dermis. Kastschenko observed that the madder stained layer was granular with the granules arranged "disorderly". Confirmation of this latter report was made by Gaup (1896)[1] who also observed the granular layer in Rana esculenta skin and noted the existence of a space between the stratum spongiosum and compactum. Flattened nuclei were observed in this space and Gaup proposed that they were endothelial cells associated with lymphatic vessels, but Guardabassi and Sacerdote speculated that these were fibroblastic nuclei.

Kressman (1912)[1] and Phisalix (1912)[1] noted in Siren lacertina large granules toward the lateral aspect of the stratum compactum. Krause (1921)[1] reported in Rana esculenta skin the presence of large basophilic granules. However, Zepp (1923)[1] denied the presence of granules in the superficial aspect of the compactum and explained the previously described report of granules as only the tasseling of very fine fibrils. Tretjakoff (1922)[1] studied the skin of Rana with "Wood lamp" and reported that much fluorescence was produced in the granular layer. He proposed an inorganic substance existing in the superficial aspect of the

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[1]Cited by Guardabassi and Sacerdote (1951).

compactum but failed to define its chemical nature. Porto (1936)[1] reported that Bufo arenarum also exhibited the granular layer and that it showed great affinity for the basic colorants of analine and hematoxylin. Also, he reported nuclei amidst the granular substance.

Although the previous reports would suggest that the granular deposits contained calcium, Sacerdote (1942) was the first to define the granular deposits as inorganic salts of calcium. While studying the beginning and evolution of the dermoliths in the stratum spongiosum of Bufo vulgaris, calcium salts were identified between the stratum spongiosum and compactum averaging 20 microns in thickness. In a more detailed study, Guardabassi and Sacerdote (1949) described the nature of these calcium deposits in Rana esculenta skin. The calcified layer, "lamina cribosa", located in the superficial part of the stratum compactum, was of variable thickness ranging from 20 to 50 microns and had an "undulated" appearance. The "lamina cribosa" was completely perforated by the presence of many funnel-shaped indentations which burrow through the layers of the compactum 50 to 70 microns deep. The area of the "lamina cribosa" that was adjacent to the stratum spongiosum and the funnel-shaped indentations contained more calcium salts than other parts of the mineral

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[1]Cited by Guardabassi and Sacerdote (1951).

zone. According to these authors, the calcium deposits appear first in the tadpoles at the level of the otic region, simultaneously with the ossification of the first phalanges of the posterior limb, extend next to the dorsal and finally to the ventral skin. The calcified layer in the skin of the tadpoles was reported to be similar to that in the adults.

In a later publication, Guardabassi and Sacerdote (1951) described the skin of 26 species of Anura, 10 species of Urodela, and 1 species of Apoda. The granular calcium deposits in the superficial aspect of the stratum compactum were found in 23 species of the Anura but the skins of Urodela and Apoda showed no evidence of the granular calcium layer. Also, no apparent relationship existed between the presence of the granular calcium deposits and the habitat or way of life of the various species of anurans.

The calcium salts were noted to be present in tadpoles at various stages of development and generally to increase with the age of the animal. Specific cells responsible for the calcification granules were not evident. The authors speculated that the granules are mainly influenced by humoral factors. They observed that the granules were more abundant in the "revolted" surface toward the stratum spongiosum and in areas adjacent to the "perforating bands". Also, metachromasia was observed in the granular zone before and after demineralization.

In a study initially designed to measure Na-K-ATPase in R. catesbeiana frog and tadpole skin, Taylor, Taylor, and Barker

(1966) encountered an unexpectedly high skin content of inorganic phosphate. Their study showed that the previously described calcium salts existed as granular calcium phosphate deposits located in a "granular layer" at the junction of the stratum spongiosum and the stratum compactum. Showing considerable variability, the mineralized area was generally composed of an upper narrow layer of very densely packed granules which became progressively less dense in the deeper zone of the stratum compactum. The "granular layer" varied between 10 and 50 microns in thickness and was relatively free of collagen and reticular fibrils. Electron photomicrographs demonstrated a clear demarcation of the mineral granules from the stratum spongiosum by what appeared to be "a narrow rim of cytoplasm". No distinct border for the medial aspect of the granular layer was observed. Instead, the granules decreased in number and were progressively more closely associated with the deeper collagen fibers.

The calcium phosphate granules were associated with an amorphous matrix which was essentially devoid of substructure. No blood vessels or nerves were observed within the granular layer proper. No intracellular crystals and no association of the mineral granules with external cell surfaces were evident. Individual granules varied from 0.1 to 1.5 microns in diameter and were composed of randomly arranged crystal-like elements ranging from 10 to 20 $\mathring{A}$ wide and up to 500 $\mathring{A}$ long.

Adult ventral skin contained between 68 and 168 millimoles of phosphate per kilogram of wet skin while that of tadpole skin at various stages of development ranged between 85 and 325 millimoles/kg. No significant correlation was observed between ventral skin concentration of phosphate and developmental age of the Rana catesbeiana. The mean molar relationship of calcium to phosphate was 1.5.

Concerning the organic matrix of the granular zone, two reports are of special interest. First, Imamura, Takeda, and Sasaki (1965) examined ventral skin from adult Rana nigromacurata. Using radioautography, they observed that an area at the periphery of the stratum compactum characteristically bound ^{22}Na and ^{45}Ca. However, they were apparently unaware of the presence of granular calcium deposits in this region of frog skin. This sodium and calcium accumulating area (corresponding anatomically to the granular calcium deposits) was further shown to stain metachromatically with both toluidine blue and azure A suggesting the presence of acid mucopolysaccharides. The presence of chondroitin sulfate was not suggested since the metachromasia was observed only in the wet state above pH 3.0, and $^{35}SO_4$ was not bound in the granular area. Neither neuraminidase or hyaluronidase activity altered the metachromasia. Alcian blue-periodic acid-Schiff staining produced a purple-blue color in the sodium and calcium binding area which changed to red upon mild methylation and returned to the original purple-blue color after demethylation.

Secondly, tail fins and dorsal skin from Rana catesbeiana tadpoles were assayed for acid mucopolysaccarides by Lipson and Silbert (1965). Approximately one milligram of mucopolysaccharide per gram of wet tissue was recovered. The mucopolysaccharide was non-sulfated, degraded by testicular hyaluronidase, and at least ninty-six per cent of the recovered mucopolysaccharide was chemically similar to hyaluronic acid.

The physiological significance of the calcium phosphate deposits in anurans is unknown. However, since the major onset of skeletal ossification occurs during metamorphic climax when tadpoles do not eat (Simkiss, 1967), Taylor et al. (1966) suggested that the granular deposits function as a reservoir for calcification of the skeleton. This would be similar to the proposed function of the paravertebral "lime sacs" in certain anurans (Guardabassi, 1960; Pilkington and Simkiss, 1966). Finally, Imamura et al. (1965) suggested that the zone which binds ^{22}Na and ^{45}Ca possibly influences sodium transport in R. nigromacurata skin.

A review of the calcification system found in amphibian skin would be incomplete without mention of the mineral concretions found closely associated with the granular glands in the stratum spongiosum of certain species. These concretions are different morphologically from the previously described granular calcium deposits. Faraggiana (1938)[1], in a study of skin glands of the Bufo vulgaris, described numerous 20 to 300 micron concretions in

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[1]Cited by Taylor et al. (1966).

in the spongy layer of the dermis. Sacerdote (1942) reported that these dermoliths were normal constituents in the skin of the Bufo vulgaris and represented "dead-like masses" which grew up to 400 microns in diameter. Sacerdote gives Davy (1826)[1] credit for first reporting these calcareous formations and then Leydig (1876)[1] for proposing that calcium and magnesium salts help form the dermoliths in Bufo vulgaris. Leydig observed these deposits in adult animals but not in tadpoles.

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[1]Cited by Sacerdote (1942).

ANURAN MATURATION - i.e., GROWTH AND DEVELOPMENT

The studies to be described in this dissertation utilized R. catesbeiana at various maturation (developmental and growth) stages. In order to more clearly understand the significance of chemical and morphological observations, a brief review of anuran maturation is required.

Etkin (1964) summarized the major changes during maturation of a typical anuran, R. pipien. Following the end of an embryonic period, the three major periods of larval development and growth are called premetamorphosis, prometamorphosis, and metamorphic climax. The premetamorphic or larval period is characterized principally by much growth and very little differentiation of the post-embryonic animal which has assumed the definitive larval form with an oval head-body region and a muscular tail nearly twice the head-body length. In the first metamorphic period, called prometamorphosis, growth continues but differential development of tissues to adult forms begins. The most conspicuous change is the accelerated growth of the relatively small hind-legs, reaching lengths comparable to the head-body length. Finally, after emergence of the forelegs through openings developed by degeneration in the opercular skin, a rapid and profound morphological change, called metamorphic climax, occurs in which the tail undergoes complete resorption, the wide frog mouth develops,

the tongue increases to more than double its initial length, and the gills and remaining operculum are resorbed. Following climax, the young frogs grow to their adult size over variable periods of time depending on the species and, partly, on climatic conditions.

In R. catesbeiana, the process of metamorphosis may extend over periods up to three years in length depending on environmental temperature. However, the last phase, metamorphic climax, generally is completed within a few weeks and usually occurs during late summer.

The initiation and control of metamorphosis in amphibians is mediated via the thyroid gland and may be accelerated by the administration of exogenous thyroid hormone, thyromimetic substances, or thyroid stimulating hormone.

SECTION II - EXPERIMENTAL STUDIES

CHAPTER 1

THE HISTOLOGICAL AND HISTOCHEMICAL CHARACTERISTICS OF THE SKIN CALCIFICATION SYSTEM IN RANA CATESBEIANA

Introduction

In order to understand more completely the nature and natural history of the calcification zone in anuran skin, histological and histochemical observations were made principally on the skin from three R. catesbeiana, each at a different maturation stage. Experiments were designed to observe the initial deposition of the calcium phosphate granules during larval stages and the changes, if any, in the calcification zone during maturation. Additionally, a study of the calcification zone after chemical demineralization was made to determine the nature of the associated matrix. Finally, the skin was examined histochemically for glycosaminoglycans since metachromasia of the calcification zone had been previously reported (Imamura et al., 1965; Guardabassi and Sacerdote, 1951). If glycosaminoglycans (previously called mucopolysaccharides) were found in the calcification zone of Rana catesbeiana skin, then their histochemical identification was planned.

Materials and Methods

R. catesbeiana tadpoles and frogs were obtained from Lemberger and Company, Oshkosh, Wisconsin, and maintained at 22 ± 2° C in rooms artificially illuminated between the hours of 6 A.M. and 6 P.M. The tadpoles were kept in artificial pond water (Alvarado and Johnson, 1966) or dechlorinated tap-water and fed twice weekly with canned spinach. The frogs were not fed during the one-week period that they were held in the laboratory. The tadpoles and frogs were killed (pithing and decapitation, respectively) and their ventral and dorsal skins were carefully removed, fixed for 24 hours in 10% formalin buffered at pH 7.0 with 75 mM sodium phosphate.

After the skin samples were embedded in paraffin and cut into sections of approximately 5 microns, the tissue sections were stained with hematoxylin and eosin, alcian blue (for acidic carbohydrates), periodic acid-Schiff (for carbohydrates), and von Kossa (for inorganic phosphate or carbonate).

For studies on the development of the skin deposits, skin samples were taken from a small and very young premetamorphic tadpole (less than 5 cm in length), a newly metamorphosed frog, and a large adult frog. Skin from the premetamorphic tadpole was cut so that each section represented the complete excursion from the cephalic to the caudal ends of the body. The skin samples from the older animals were taken from the more caudal ends of both ventral and dorsal body skin.

In order to examine the demineralized calcification zone, contralateral skin samples from each animal (with the exception of the premetamorphic tadpole) were fixed in 10% neutral buffered formalin and then, demineralized for 17 hours in 10% neutral buffered formalin containing 5% disodium ethylene-diaminetetraacetic acid. The skin samples were subsequently prepared for histological examination as described above.

In another early prometamorphic tadpole, India ink was injected into the aorta. The ink was allowed to circulate until it was visible in the small arteries beneath the dermis of the ventral skin. The ventral skin was then removed and prepared for staining with PAS.

The histochemical identification of the glycosamino-glycans associated with the mineral zone in ventral skin of the newly metamorphosed frog was based on three alcianophilic tests. The first was the reversal of the glycosaminoglycan - alcian blue complex by increasing electrolyte concentrations as described by Quintarelli and Dellovo (1965). Tissue sections were stained for 2 hours with 0.05% alcian blue buffered to pH 5.6 with 0.025 M sodium acetate and containing $MgCl_2$ at concentrations ranging from 0 to 1.0 molar. Secondly, the tissue sections were tested for alcianophilia at a very low pH by contrasting sections stained with 1% alcian blue at pH 1.0 for 30 minutes with control sections stained for this period with 1% alcian blue at pH 2.6 (Spicer et al., 1967). PAS was used as a counterstain.

Finally, the susceptibility of the alcianophilia to testicular and bacterial hyaluronidase was tested by the methods of McManus and Mowry (1960) and Quintarelli (personal communication), respectively. Control skins were exposed to sodium acetate buffer only (0.1 M, pH 5.5) while the same buffer containing 0.5 mg/cc testicular hyaluronidase (Calbiochem) or 0.5 to 2 mg/ml bacterial hyaluronidase (Organon) was used for the enzyme-treated tissue sections. After 6 hours incubation the tissue was stained with alcian blue (pH 2.6). Each of the three histochemical procedures included experimental and control sections of human umbilical cord in order to check each procedure with a well characterized tissue.

Results

In the cephalic end of the premetamorphic ventral skin samples, areas were found in which the skin had not differentiated into a histologically distinct epidermis and dermis (Figure 2). Fibers resembling collagen were observed subjacent to the basal layer of epithelial cells. Examination of the more central portions of this skin sample revealed a separation between the basal epithelial cells and their subjacent fibers. A basophilic border was observed at the base of the epithelial cells and the fibers which were previously adjacent to the basal epithelial cells appeared to have separated, forming a zone containing granular mineral deposits (Figure 3). The granular material was von Kossa

Figure 2. Premetamorphic Ventral Skin before Differentiation of the Dermis and Calcification Zone.

Hematoxylin-eosin; x 400.

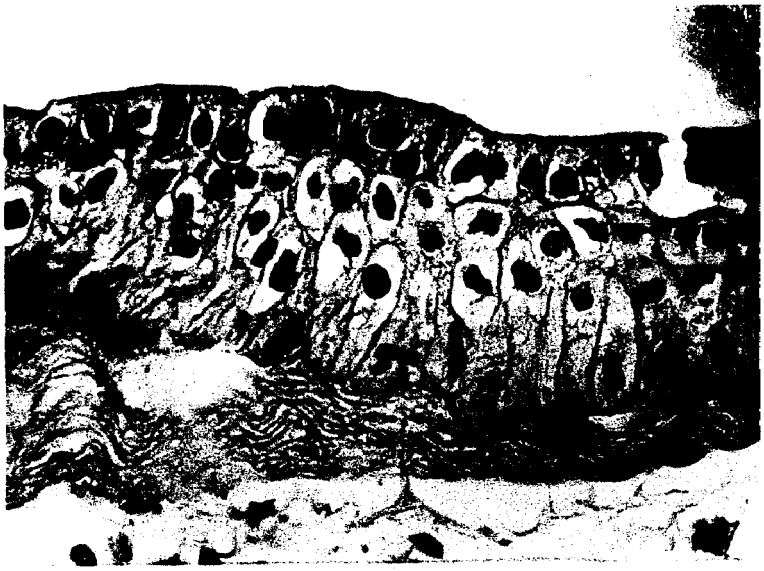
Figure 3. Premetamorphic Ventral Skin with Developing Calcification Zone.

The arrow designates the site of early granule formation.
Hematoxylin-eosin; x 1000.

Figure 4. Premetamorphic Ventral Skin with Developing Calcification Zone and Stratum Spongiosum.

Hematoxylin-eosin; x 400.

2



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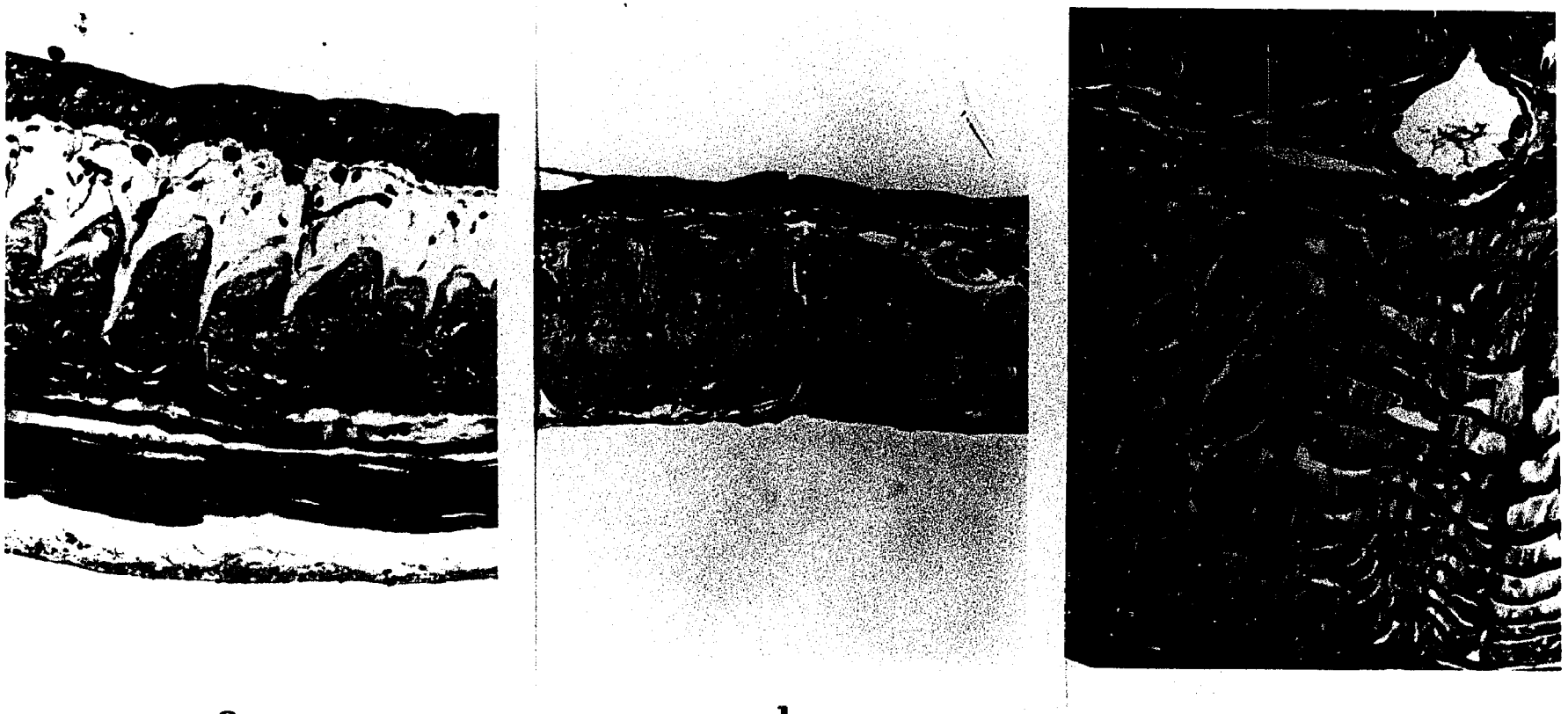
and alcian blue (pH 2.6) positive. Further examination toward the central area of the skin sample showed areas in which the granular material had more extensively separated from the epidermal cells, leaving a definitive space containing sparse fibers and nuclei (Figure 4). This space may represent the developing stratum spongiosum. Dorsal skin of the premetamorphic animal contained a similar, but thinner, granular layer at the peripheral region of the stratum compactum.

Changes with maturation in Rana catesbeiana skin were dramatic and included the entire dermis. The stratum compactum of the prometamorphic tadpole consisted of the two histologically distinguishable zones as shown in Figure 5. First, and most superficial was the calcification zone characterized by the presence of the von Kossa positive granules (Figure 6). The immediately subjacent deep compactum zone consisted of thick collagenous fiber bundles which were parallel to the epidermis. Based on three R. catesbeiana, each at a different maturation stage, the thickness of the total stratum compactum in ventral skin was 170 microns in the prometamorphic tadpole, 180 microns in the newly metamorphosed frog, and 450 microns in the large adult. The thickness of the calcification zone was approximately 70 microns in the prometamorphic tadpole, 110 microns in the young frog, and 34 microns in the large adult frog. The deep compactum zone measured 100 microns, 70 microns, and 416 microns in the

Figure 5. The Morphology of the Ventral Skin of Rana catesbeiana.

(a) Early Prometamorphic Tadpole,
(b) Newly Metamorphosed Frog, and
(c) Adult Frog; Hematoxylin-eosin, x 100.



a b c

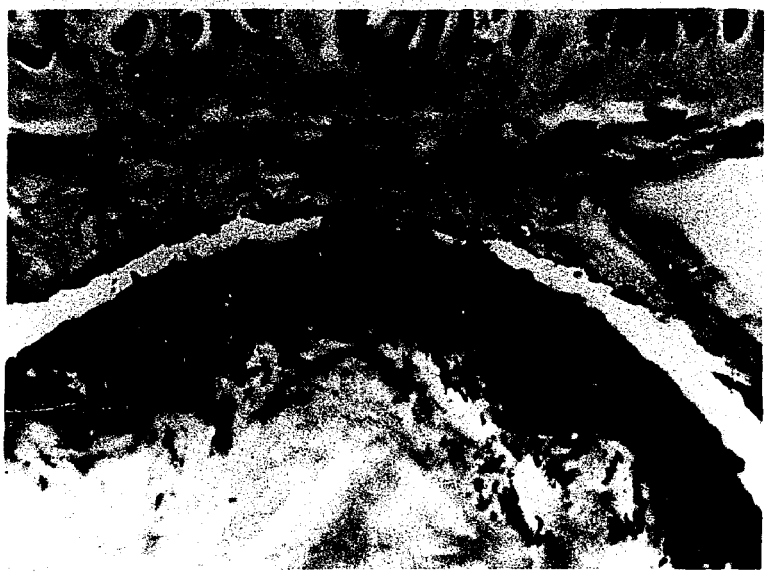
Figure 6. The Calcification Zone as Shown by von Kossa Stain.

(a) Early Prometamorphic Tadpole,
(b) Newly Metamorphosed Frog, and
(c) Adult Frog; x 400.

a

b

c



prometamorphic tadpole, newly metamorphosed frog, and adult frog, respectively.

In dorsal skin the thickness of the stratum compactum was 100 microns in the prometamorphic animal and 135 and 690 microns in the newly metamorphosed and adult frogs, respectively. The calcification zone measured 30, 60, and 40 microns in the prometamorphic tadpole, newly metamorphosed frog, and adult frog, respectively. The deep compactum zone was approximately the same thickness in the prometamorphic and newly metamorphosed animal (60 and 75 microns, respectively). However, the deep compactum zone of the adult measured 650 microns.

As shown in Figure 6, the peripheral border of the calcification zone as defined by von Kossa stain was highly convoluted in larval skins (Figures 6a and 6b) but not in adult skin (Figure 6c). In all maturation stages, this border of the calcification zone was well defined and clearly separate from the stratum spongiosum. Von Kossa positive granules were more numerous at the periphery of the calcification zone and became less numerous in the central and medial areas of this zone. Frequently, the samples from the early prometamorphic tadpole and newly metamorphosed frog had mineral deposits so densely packed at the peripheral aspect of the calcification zone that the von Kossa staining area appeared solid, rather than granular. In adult frog skin, the entire calcification zone was characterized by this continuous, less granular-appearing type of von Kossa positive area. Although

less densely packed, the medial border of the calcification zone was also defined by the von Kossa stain and appeared to end adjacent to the thick collagenous fiber bundles which are parallel to the epidermis.

Demineralized ventral skin revealed more clearly the fibrous matrix of the calcification zone (Figure 7). A definite basophilic limiting structure with closely associated nuclei was observed at the peripheral border and was stained with alcian blue (pH 2.6) and periodic acid-Schiff. Just medial to the limiting structure (where the most densely packed mineral granules were found), an area characterized by very thin, randomly oriented fibrils was observed. Toward the more central portion of the zone, the fibers progressively increased in size but were still loosely packed and irregularly arranged. Cell nuclei were observed throughout the calcification zone. In ventral skin from the large adult the zone contained many thick fibers which spread laterally in progressively thinning tufts until they contacted the external border of the compactum.

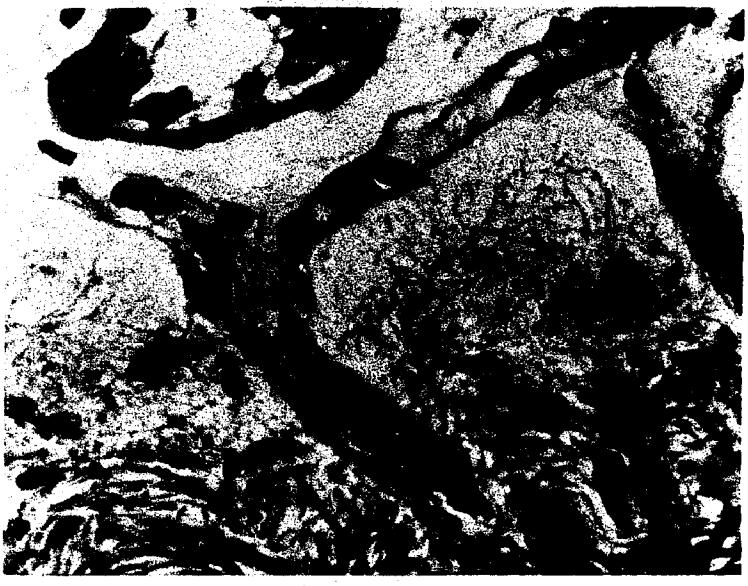
The peripheral border of the calcification zone folded in medially and, in many cases, vascular elements penetrated via these folds toward the epithelium (Figures 7, 9, 11b). In skin from the tadpole perfused with India ink, carbon particles were seen in vessels at the base of the compactum and within channels penetrating the compactum (Figure 8). High concentrations of mineral granules were observed adjacent to these vascular channels

Figure 7. The Demineralized Calcification Zone in Ventral Skin of Rana catesbeiana.

(a) Early Prometamorphic Tadpole,
(b) Newly Metamorphosed Frog, and
(c) Large Adult Frog: Demineralization with 5% EDTA in 10% neutral buffered formalin; Hematoxylin-eosin, x 400.

a

b



c

Figure 8. A Vascular Channel Penetrating the Stratum Compactum in Ventral Skin of a Prometamorphic Tadpole.

The animal was perfused with India Ink in order to demonstrate the vascular channels; (a) and (b) represent two consecutive sections of the same vessel; the arrow designates the Periodic Acid-Schiff positive granules; x 400.

a

b

which were very apparent in skin of tadpoles and newly metamorphosed frogs but less obvious in skin from the large adults.

The organic matrix of the calcification zone stained moderately with PAS (Figure 8) and strongly with alcian blue pH 2.6 (Figure 10a). Both the PAS and alcian blue staining in the granular zone appeared distributed in a pattern similar to that of the von Kossa positive granules; that is, sharply demarcated and highly concentrated on the peripheral border of the calcification zone with a less dense but clearly defined medial border suggesting that the organic matrix was intimately associated with the granular deposits.

The glycosaminoglycans of the calcification zone stained selectively with alcian blue in 0.05 M sodium acetate buffer at pH 5.7. However, the addition of 0.1 M or 0.2 M $MgCl_2$ blocked alcianophilia of the calcification zone (Figure 9). According to (Spicer et al., 1967), hyaluronic acid, and some weakly acidic sulfomucins are not stained at or above 0.1 M $MgCl_2$. In contrast, most sulfated mucosubstances including those metachromatic with azure A at pH 0.5 stain strongly and selectively at 0.2 M $MgCl_2$ levels.

Alcian blue selectively stained the glycosaminoglycans of the calcification zone at pH 2.6 (Figure 10) but not at pH 1.0 indicating that sulfated mucopolysaccharides are not a major constituent of the calcification zone's organic matrix (Spicer et al., 1967).

Figure 9. The Effect of Increased Electrolyte Concentration on the Alcianophilia (pH 2.6) of the Calcification Zone in Ventral Skin from a Newly Metamorphosed Frog.

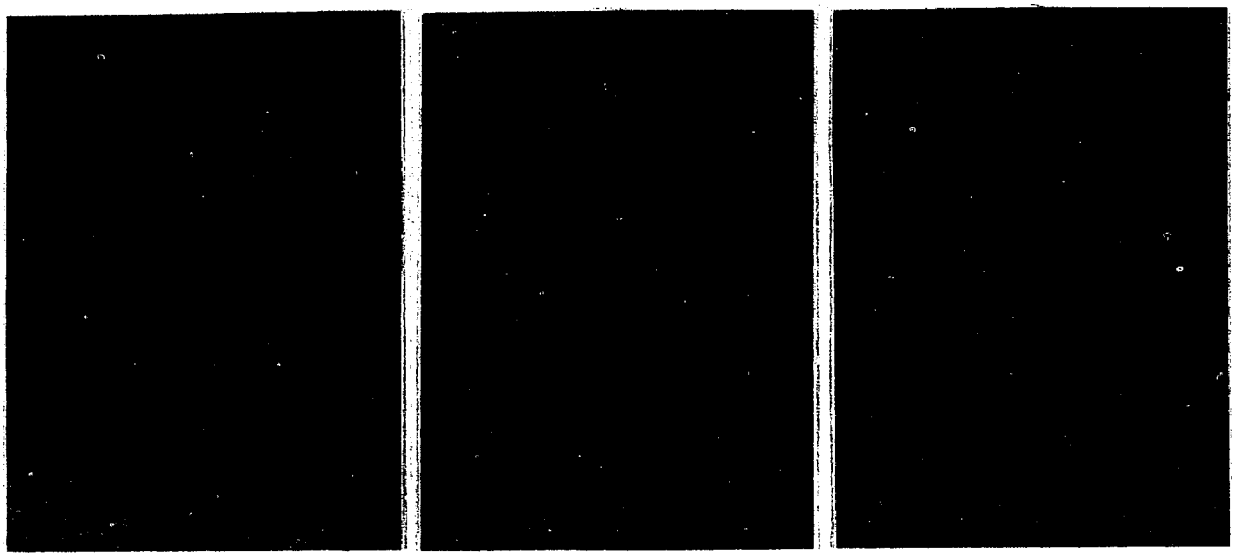
0.05 M Sodium Acetate Buffer at pH 5.7 and containing (a) 0.0 M $MgCl_2$, (b) 0.1 M $MgCl_2$, and (c) 0.2 M $MgCl_2$; x 400.

Figure 10. The Effect of pH on the Alcianophilia of the Calcification Zone in Ventral Skin from a Newly Metamorphosed Frog.

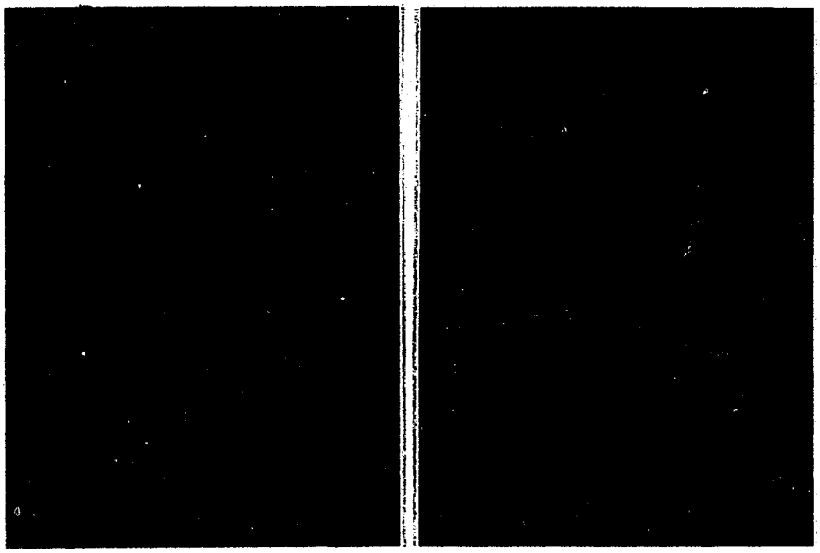
(a) pH 2.6, and (b) pH 1.0; Counterstained with periodic acid-Schiff, x 400.

Figure 11. The Effect of Hyaluronidase on the Alcianophilia (pH 2.6) of the Calcification Zone in Ventral Skin from a Newly Metamorphosed Frog.

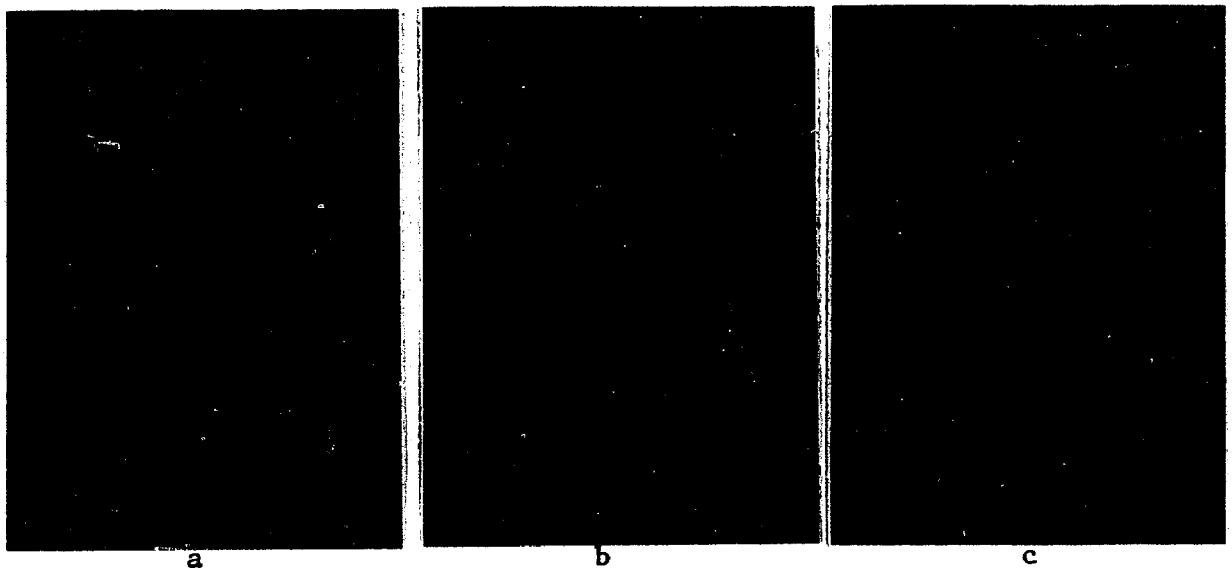
(a) Control, (b) Treated with Testicular Hyaluronidase, and (c) Treated with Bacterial Hyaluronidase; x 400.



a b c



a b



a b c

Incubation with testicular hyaluronidase eliminated the alcianophilia of the calcification zone (Figure 11) suggesting the presence of hyaluronic acid, chondroitin-4-sulfate, or chondroitin-6-sulfate (Spicer et al., 1967). Since the first two histochemical criteria indicated that sulfated glycosaminoglycans were not a major component of the calcification zone, these results with testicular hyaluronidase suggested that the glycosaminoglycan of the calcification zone was similar to hyaluronic acid. However, as shown in Figure 11 the alcianophilia was not eliminated by incubation with bacterial hyaluronidase which digests only hyaluronic acid (Spicer et al., 1967). In fact, the skin sections exposed to bacterial hyaluronidase showed stronger alcianophilia than the controls. These results with bacterial hyaluronidase are subject to question because of this increased alcianophilia of the calcification zone, but cannot be discarded since the umbilical cord sections were less alcianophilic after exposure to bacterial hyaluronidase.

Discussion

The initial formation of granular calcium phosphate deposits in the tadpole skin appeared to be closely associated with differentiation of the dermis since prior to this time granular deposits were not observed (Figure 2). As the developing dermis separated from the epithelial cells, granular deposits which

were von Kossa and alcian blue positive were seen (Figure 3). The mineral granules, at the periphery of the newly formed dermis, separated progressively from the basal epithelial cells forming a zone which apparently developed into the stratum spongiosum (Figure 4). The sequence of events observed in the differentiation of R. catesbeiana dermis closely parallels that reported for R. pipiens (Kemp; 1959, 1961). The basement lamella, a group of collagenous fibers subjacent to the epidermis and embedded in a viscous ground substance, was reported to become detached from the basal epidermal cells forming a space called the stratum spongiosum. The basement lamella, having completely separated from the epidermis, was then called the dermal lamella which developed into the stratum compactum. No report of mineral granules was made in these studies by Kemp.

In contrast to Guardabassi and Sacerdote (1949) who reported few differences between the calcification zones of larval and adult R. esculenta, maturation of R. catesbeiana appeared to influence markedly the thickness and fibrous content of the calcification zone. Based on the limited observations of this study, the thickness of the calcification zone appears to change from values near 5 microns in ventral skins of the very young premetamorphic tadpole (Figure 4) to 70, 110, and 34 microns in skins from the prometamorphic tadpole, newly metamorphosed frog, and adult frog, respectively. Also, the calcification zone in dorsal skin from the early prometamorphic tadpole was approximately

one-half the thickness of the ventral zone but in the adult frog, these two zones were of similar thickness. The demineralized tissue suggested that although the calcification zones from larval and adult animals were morphologically similar, the adult skin had thicker and more densely packed fiber bundles in the calcification zone.

In contrast to that of the large adult, the calcification zones of prometamorphic and newly metamorphosed animals showed numerous convolutions on their peripheral border which appeared to penetrate the full thickness of the calcification zone (Figure 5) and through which vascular channels passed (Figures 7b, 8b, 11b). In all stages, granular calcium phosphate deposits were more densely packed in both the peripheral border of the zone and adjacent to the vascular channels penetrating the stratum compactum (Figures 6a and b). Guardabassi and Sacerdote (1951) also observed dense granular concentrations at the superficial border of the compactum zone in Rana esculenta skin which appeared to be closely related to the vascular channels penetrating the stratum sompactum. The greater availability of electrolytes at this border of the calcification zone and adjacent to the traversing vascular channels possibly explains this characteristic distribution of the mineral granules. Other calcification systems show similar characteristics. The hypermineralized perilacunar matrix in bone and the hypermineralized peritubular matrix in dentin are adjacent to the nutrient channels

of their respective tissues (Major, 1964). Furthermore, glycosaminoglycans are present throughout the calcification zone (Figure 7b) and some studies have shown that the physical nature of the glycosaminoglycans limits the diffusion of ions (Glimcher, 1960), thus possibly accounting for the decreased concentration of mineral granules in the central and medial areas of the calcification zone.

Numerous cell nuclei were observed scattered throughout the calcification zone of prometamorphic and newly metamorphosed animals (Figures 7a and b), but not that of mature adults. In adult skin the calcification zone was so heavily laden with mineral deposits that the granular appearance was replaced by a continuous, non-granular, von Kossa positive area (Figure 6c) which was quite distinct from that of the prometamorphic and newly metamorphosed frog.

The matrix of the calcification zone was moderately PAS-positive (Figure 8) and thus similar to that of other mineralizing systems; the matrix of cartilage, bone, and dentin are all strongly to moderately PAS-positive while the ground substance from other connective tissue is only negligibly colored (Mowry, 1968). Connective tissue acid mucopolysaccharides exhibit weak or no reactivity to PAS staining (Spicer et al., 1967), but the neutral connective tissue mucosubstances stain red to magenta. Mowry (1968) does not think that sialic acid containing substances, such as sialomucoproteins, explain the coloration of cartilage and bone

matrix since no distinctive histological distribution of sialomucoprotein has been established. Therefore, the matrix associated with the granular calcium phosphate deposits appears to contain neutral mucosubstances.

Skin stained with alcian blue-PAS showed a purple granular stain in the calcification zone which strongly suggested that a major component of the matrix was glycosaminoglycans (Figure 10a). This is consistent with the observations of Guardabassi and Sacerdote (1951) and Imamura (1965) who reported that the calcification zone showed metachromasia with toluidine blue. The following histochemical data indicated that the glycosaminoglycan component of the calcification zone matrix is similar to hyaluronic acid:

1) The alcian blue-glycosaminoglycan complex was stable only in the absence of $MgCl_2$ (Figure 9) suggesting the presence of either hyaluronic acid or a weakly acidic sulfomucin;
2) The alcianophilia of the calcification zone was eliminated when stained at pH 1.0 indicating the presence of non-sulfated glycosaminoglycans or weakly acidic, masked sulfated mucosubstances (Figure 10);
3) Finally, elimination of the alcianophilia with testicular hyaluronidase suggests the presence of hyaluronic acid since the data mentioned above rule

out the highly sulfated glycosaminoglycans such as the chondroitin sulfates which are also digested by this enzyme (Figure 11).

As shown in Figure 9, the major alcian blue positive area was coincident with the calcification zone. Chemical analysis of the glycosaminoglycans of dorsal R. catesbeiana tadpole skin by Lipson et al. (1964) showed that at least 96% of the total glycosaminoglycan was chemically identical to hyaluronic acid. Also, Imamura et al. (1965) reported that the ^{45}Ca accumulating zone in R. nigromaculata did not bind $^{35}SO_4$, and that its alcianophilia was reversable with mild methylation, thus, suggesting the presence of non-sulfated mucopolysaccharides. Therefore, most of the data from this and other studies suggest that hyaluronic acid is the major component of the matrix of the calcification zone.

However, the evidence indicating the presence of hyaluronic acid in the calcification zone is not entirely conclusive. Imamura et al. (1965) reported that testicular hyaluronidase did not destroy the metachromatic staining which is in contrast to the observation made in this study and by Lipson (1964) who reported that 96% of the total glycosaminoglycan in R. catesbeiana dorsal skin was susceptible to testicular glyaluroidase digestion. Secondly, in this study, bacterial hyaluronidase, which specifically digests hyaluronic acid, did not destroy the alcianophilia of the calcification zone (Figure 11) but did reduce the alcianophilia in the umbilical cord. The fact that the alcianophilia actually increased significantly in

the enzyme treated skins but decreased in the umbilical cord sections treated with the enzyme is inconsistent with the effect of testicular hyaluronidase but may be related to a binding effect by the calcification zone. Additional observations with other preparations of bacterial hyaluronidase as well as demineralized tissue sections should be made in future experiments.

CHAPTER 2

THE EFFECT OF MATURATION ON THE SKIN MINERAL DEPOSITS IN *RANA CATESBEIANA*

Introduction

The skin mineral in anurans has been suggested to act as a reservoir for skeletal ossification (Taylor et al., 1966). If such a function exists, then the mineral content of skin should change during skeletal maturation. The histological studies of the previous chapter suggested that the calcification zone changed considerably during the maturation of *R. catesbeiana*. From these studies, it appeared that the thickness of the calcified zone declined approximately 50% during maturation from the early prometamorphic tadpole to the mature adult frog. The thickness of the calcified zone appeared to be greater just after metamorphosis than during the prometamorphic stage.

In order to identify more clearly the changes in mineral content with maturation, the experiment described herein was designed to measure the chemical composition of the skin calcification system at several maturation stages.

Materials and Methods

R. catesbeiana were obtained, maintained, and sacrificed as previously described (Chapter 1). Dorsal and ventral body skin samples of 1.54 cm^2 were removed from early prometamorphic, early climactic, and late climactic tadpoles and from yearling and large adult frogs. In addition, ventral skin samples of varying sizes were taken from premetamorphic tadpoles and newly metamorphosed frogs collected from ponds in Alabama. The premetamorphic tadpoles were divisible into two groups on the basis of their ventral skin appearance. The first and apparently youngest group had transparent ventral skin; the second group had slightly opaque ventral skin. The number of animals in each group and their approximate sizes are shown in Table I.

All samples were taken from the caudal aspect of the body skin and were scraped lightly on the serosal surface to remove loosely bound mucinous material. The skins were weighed after drying at 105° C for 24 hours. Mineral weight was obtained after ashing at 600° C for 24 hours and the organic residue in each sample was calculated from the difference in dry and ash weights.

For chemical analysis, the ashed samples were dissolved in 1.0 ml of 6 N HCl, heated to just below boiling, and then diluted volumetrically with distilled water to 10.0 ml. Triplicate aliquots from each sample were analyzed for calcium (Bachra et al., 1958) and for phosphate (Bonting et al., 1961).

TABLE I. THE NUMBER AND DESCRIPTIVE CHARACTERISTICS OF THE RANA CATESBEIANA USED IN THE STUDY ON THE EFFECT OF MATURATION ON THE SKIN MINERAL DEPOSITS.

MATURATION STAGE	n	BODY LENGTH (cm^2)	TAIL LENGTH (cm^2)	HIND-LEG LENGTH (cm^2)
PREMETAMORPHIC (TRANSPARENT)	5	(<5.0 - Total length)		0
PREMETAMORPHIC (OPAQUE)	9	(<5.0 - Total length)		0
EARLY PROMETAMORPHIC	10	∿ 4.0	∿ 6.5	<1.0
EARLY CLIMACTIC	16	3.9 ± 0.2[3]	6.6 ± 0.7	4.7 ± 0.3
LATE CLIMACTIC	10	4.0 ± 0.2	0.7 ± 0.4	5.6 ± 0.4
NEWLY METAMORPHOSED FROG[1]	10	<5.0	0	___
YEARLING FROG[1,4]	10	∿ 10.0	0	___
ADULT FROG[1,2]	10	∿ 16.0	0	___

[1]Head-body length
[2]"Jumbo" bullfrogs ∿ 450 gms
[3]Mean ± standard deviation
[4]According to Dickerson (1906), yearling bullfrogs have a head-body length of ∿ 4 inches.

Results

In order to describe more accurately any changes in the granular layer of R. catesbeiana skin, the data are expressed in terms of both skin area and skin dry weight. This is because dry weight reflects the amount of both mineral and organic matter and real alterations in the mineral content might not be apparent from data expressed as the calcium "concentration" of skin if the organic weight of the skin was changing simultaneously and disproportionally.

As shown in Figures 12 and 13, the variability of the parameters measured fluctuated considerably with the maturational stage. The coefficient of variability was greatest during metamorphic climax (the period of most rapid morphological and functional change) and least variable at early prometamorphosis and in adult frogs (periods characterized by slow and steady growth). Due to this high variability, statistical analysis of the data was based on the Kruskall-Wallis one-way Analysis of Variance (a non-parametric method) followed by Duncan's Multiple Range Test (Steele and Torrie, 1960). The statistical results are expressed by ranking the sample means in increasing order and then underlining the groups of means which show no significant difference at $p<0.05$.

However, upon examination of the statistical results, two disadvantages of this type of analysis should be remembered. First, according to Steele and Torrie (1960) the validity of the Duncan's

Figure 12. The Effect of Maturation on the Coefficient of Variability of the Parameters Used to Study the Ventral Skin Mineral System of Rana catesbeiana.

Maturation Stages: EP = Early Prometamorphosis; EC = Early Climax; LC = Late Climax; NF = Newly Metamorphosed Frog; YF = Yearling Frog; AF = Adult Frog.

$$\text{Coefficient of Variability} = \frac{\text{Standard deviation}}{\text{mean}} \times 100\%$$

PARAMETERS

__ _ __ ORGANIC WEIGHT - mg/cm^2 SKIN

_____ CALCIUM - $\mu M/cm^2$ SKIN

------- PHOSPHATE - $\mu M/cm^2$ SKIN

. . . . . CALCIUM - mM/gm DRY SKIN

.-.-.-. PHOSPHATE - mM/gm DRY SKIN

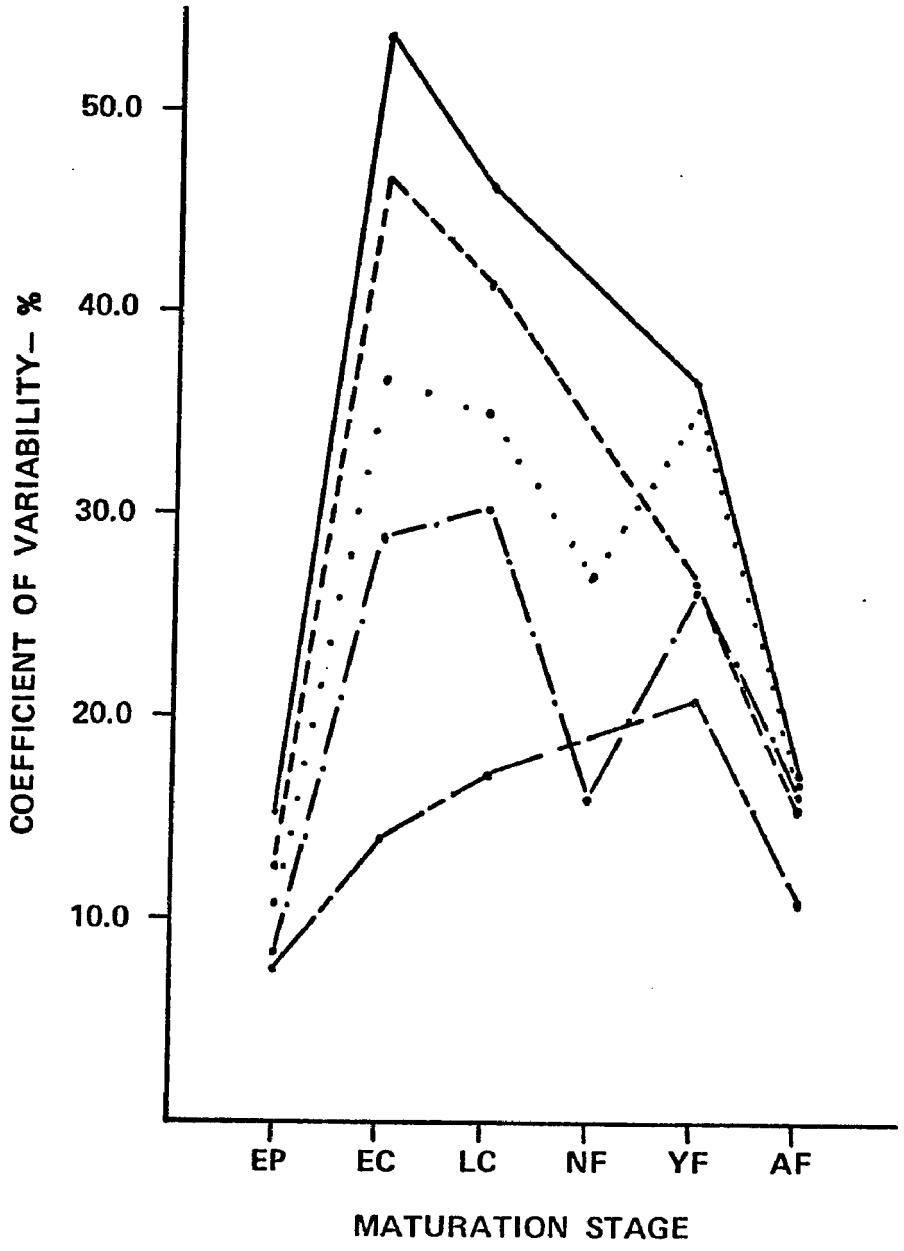

50.0
40.0
30.0
20.0
10.0
COEFFICIENT OF VARIABILITY– %
EP
EC
LC
NF
YF
AF
MATURATION STAGE

Figure 13. The Effect of Maturation on the Coefficient of Variability of the Parameters Used to Study the Dorsal Skin Mineral System of Rana catesbeiana.

Maturation Stages: EP = Early Prometamorphosis; EC = Early Climax; LC = Late Climax; NF = Newly Metamorphosed Frog; YF = Yearling Frog; AF = Adult Frog.

$$\text{Coefficient of Variability} = \frac{\text{Standard deviation}}{\text{mean}} \times 100\%$$

PARAMETERS

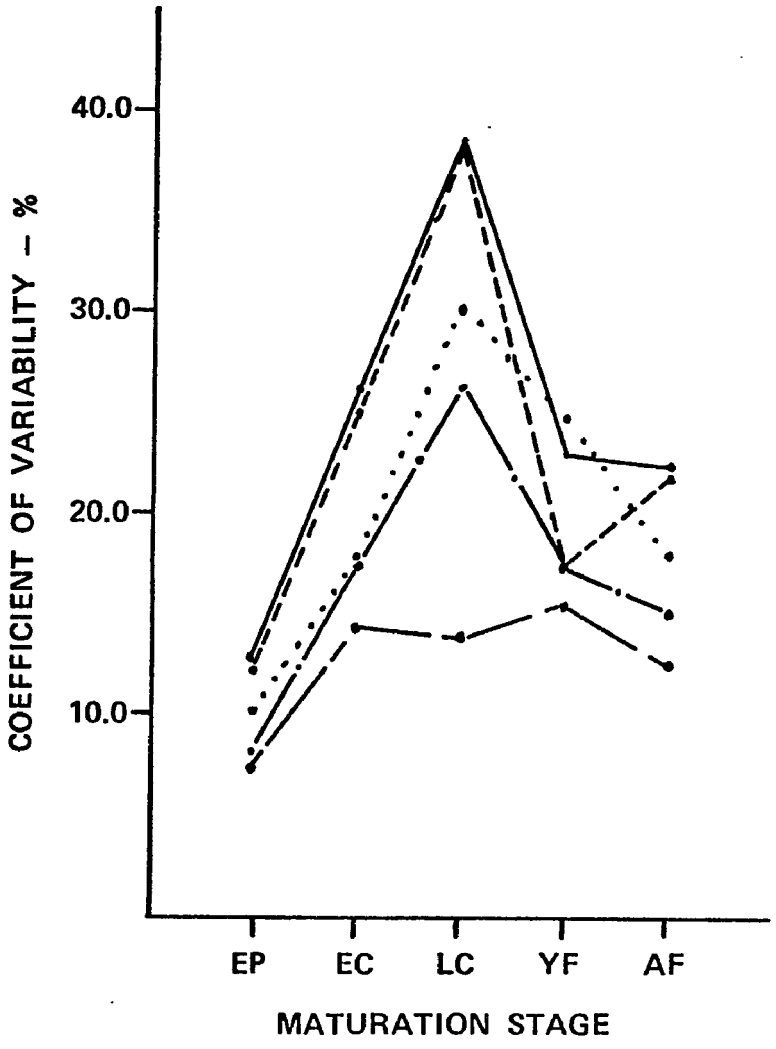
_ _ _ ORGANIC WEIGHT - mg/cm^2 SKIN

_____ CALCIUM - $\mu M/cm^2$ SKIN

------- PHOSPHATE - $\mu M/cm^2$ SKIN

..... CALCIUM - mM/gm DRY SKIN

.-.-.-. PHOSPHATE - mM/gm DRY SKIN


40.0
30.0
20.0
10.0
COEFFICIENT OF VARIABILITY – %
EP
EC
LC
YF
AF
MATURATION STAGE

Multiple Range Test following the Analysis of Variance in which the sample means show unequal variances has not been proven. Secondly, the Kruskall-Wallis test loses information if the tadpole population being sampled is normally distributed causing a Type II error (accepting a null hypothesis when it is false) to be made more frequently.

The organic residue (mg/cm^2) was unchanged through the yearling frog stage in ventral skin and through the late climactic stage in dorsal skin (Figure 14). However, the organic content of ventral skins from large adult frogs was about 4 fold higher than in tadpole skins. After a slight increase in the organic content of dorsal skin from yearling frogs, that from adult frogs was almost 6 fold higher than in tadpoles. At all larval stages, the ventral skin contained a greater amount of organic residue than the dorsal skin. However, in adult frogs the organic residue appeared to be greater in dorsal skins although this difference was not statistically verified.

The amounts of both calcium and phosphate ($\mu M/cm^2$) in ventral skin from all of the larval and older adult frogs were statistically equivalent. However, both of these elements were considerably reduced in ventral skin from yearling frogs (Figures 15 and 16). In dorsal skin, the content of calcium and phosphate did not significantly change during the tadpole stages. However, following late climax the calcium and phosphate were reduced in dorsal skin of yearling frogs. In adult frogs, the calcium and

Figure 14. The Effect of Maturation on the Organic Weight (mg/cm^2 Skin) of Ventral and Dorsal Skin from Rana catesbeiana.

MATURATION STAGE	n[1]	ORGANIC WEIGHT (mg/cm^{2}SKIN) VENTRAL	DORSAL
EARLY PROMETAMORPHIC (EP)	10	5.3 ± 0.4[2]	4.0 ± 0.5
EARLY CLIMACTIC (EC)	16	5.0 ± 0.7	3.5 ± 0.5
LATE CLIMACTIC (LC)	10	6.4 ± 1.1	4.3 ± 0.6
YEARLING FROG (YF)	10	4.8 ± 1.0	5.8 ± 0.9
ADULT FROG (AF)	10	20.2 ± 2.2	28.6 ± 3.6

[1]Number of animals
[2]Mean ± standard deviation

Duncan's Multiple Range Test. Each horizontal line denotes groups of means which show no significant differences ($\alpha = 0.05$).

SKIN (D=DORSAL, V=VENTRAL)	D	D	D	V	V	V	D	V	V	D
MATURATION STAGE	EC	EP	LC	YF	EC	EP	YF	LC	AF	AF

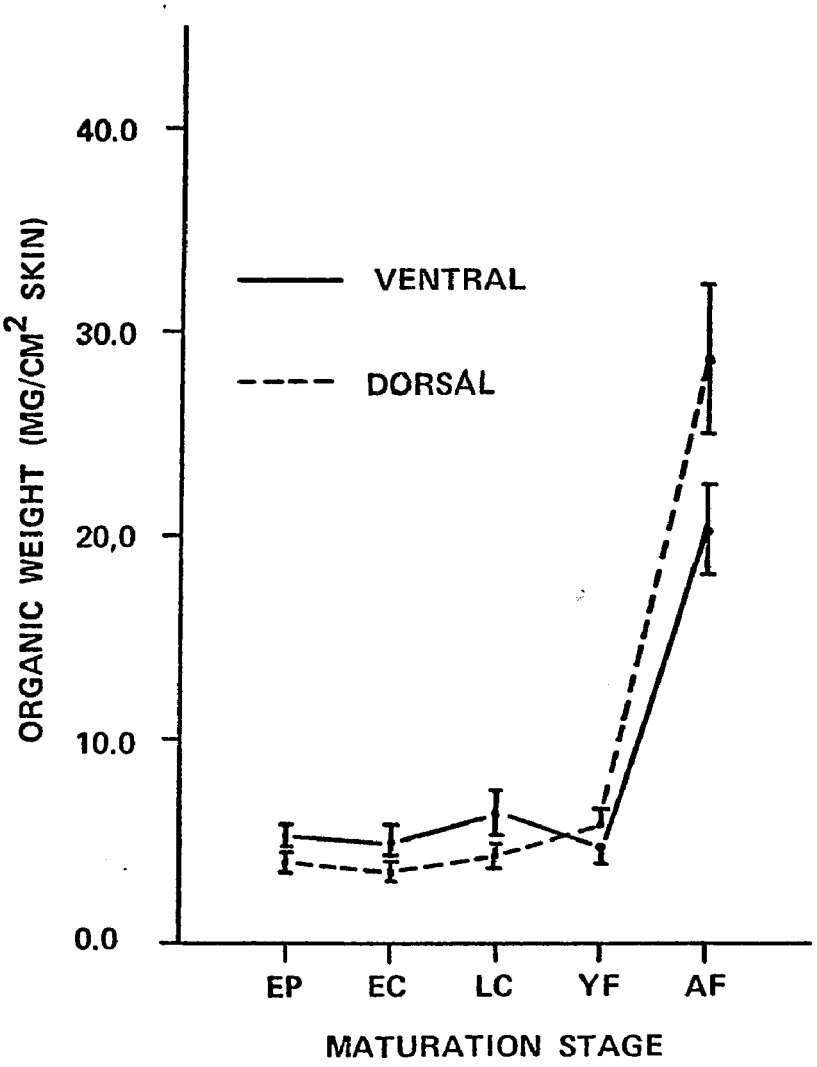

40.0
30.0
20,0
10.0
0.0
ORGANIC WEIGHT (MG/CM2 SKIN)
VENTRAL
DORSAL
EP
EC
LC
YF
AF
MATURATION STAGE

Figure 15. The Effect of Maturation on the Calcium ($\mu M/cm^2$ Skin) in Ventral and Dorsal Skin from Rana catesbeiana.

MATURATION STAGE	n[1]	CALCIUM ($\mu M/cm^2$ SKIN) VENTRAL	DORSAL
EARLY PROMETAMORPHIC (EP)	10	19.0 ± 2.9[2]	8.7 ± 1.1
EARLY CLIMACTIC (EC)	16	13.8 ± 7.4	7.3 ± 1.9
LATE CLIMACTIC (LC)	10	26.9 ±12.4	10.2 ± 3.9
YEARLING FROG (YF)	10	4.4 ± 1.6	5.2 ± 1.2
ADULT FROG (AF)	10	19.3 ± 3.8	29.3 ± 6.6

[1]Number of animals
[2]Mean ± standard deviation

Duncan's Multiple Range Test. Each horizontal line denotes groups of means which show no significant difference ($\alpha = 0.05$).

SKIN (D=DORSAL, V=VENTRAL	V	D	D	D	D	V	V	V	V	D
MATURATION STAGE	YF	YF	EC	EP	LC	EC	EP	AF	LC	AF

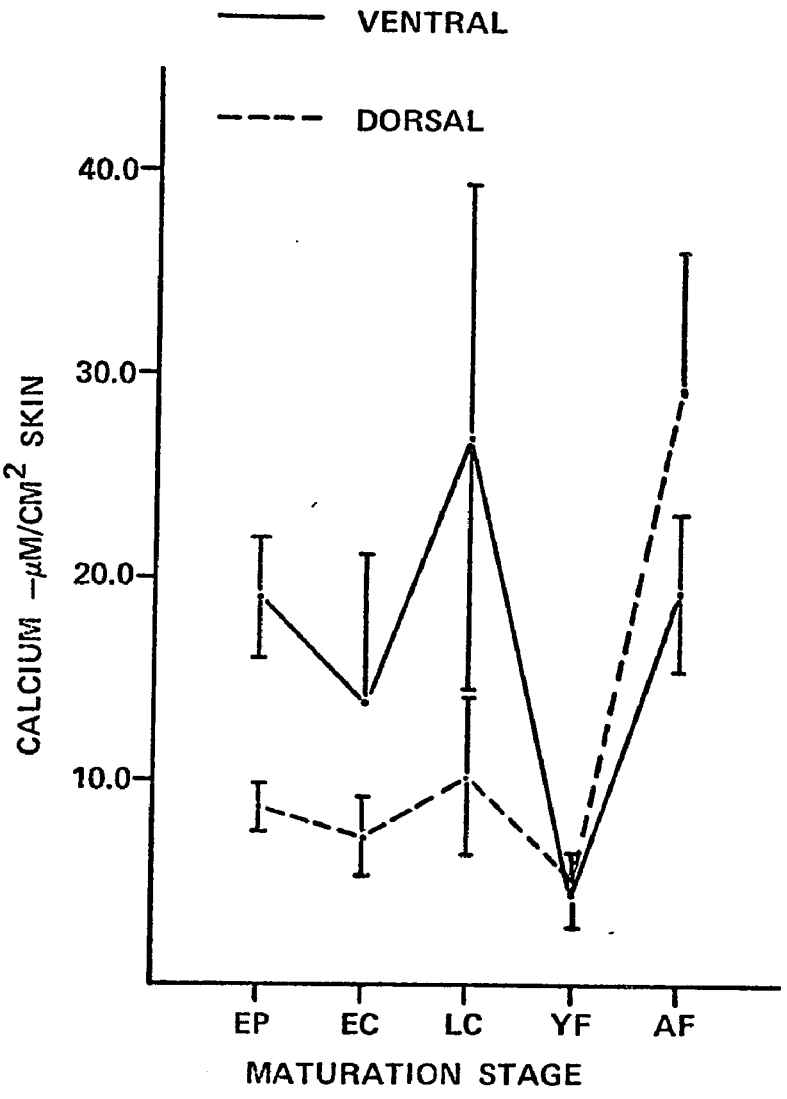

VENTRAL
DORSAL
40.0
30.0
20.0
10.0
CALCIUM –μM/CM2 SKIN
EP
EC
LC
YF
AF
MATURATION STAGE

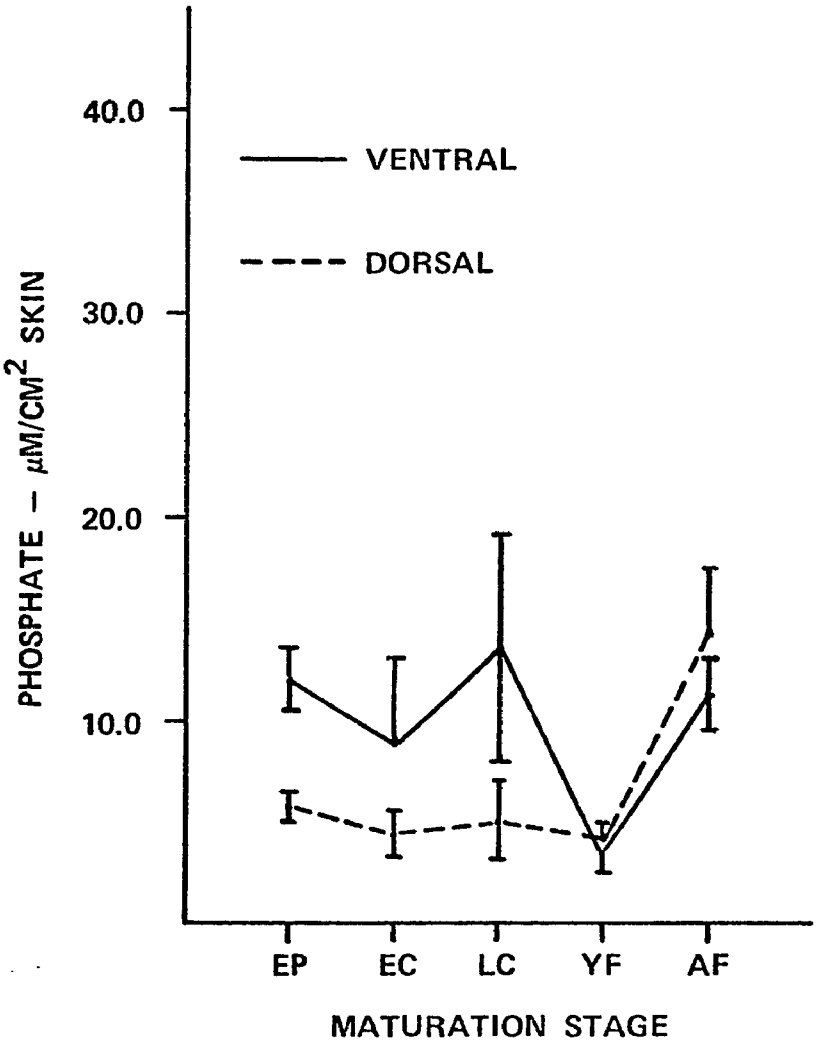
Figure 16. The Effect of Maturation on the Phosphate ($\mu M/cm^2$ Skin) in Ventral and Dorsal Skin from Rana catesbeiana.

MATURATION STAGE	n[1]	PHOSPHATE ($\mu M/cm^2$ SKIN) VENTRAL	DORSAL
EARLY PROMETAMORPHIC (EP)	10	11.9 ± 1.5[2]	5.7 ± 0.7
EARLY CLIMACTIC (EC)	16	8.8 ± 4.1	4.4 ± 1.1
LATE CLIMACTIC (LC)	10	13.5 ± 5.6	5.0 ± 1.9
YEARLING FROG (YF)	10	3.4 ± 0.9	4.1 ± 0.8
ADULT FROG (AF)	10	11.2 ± 1.7	14.3 ± 3.1

[1]Number of animals
[2]Mean ± standard deviation

Duncan's Multiple Range Test. Each horizontal line denotes groups of means which show no significant difference ($\alpha = 0.05$).

SKIN (D=DORSAL, V=VENTRAL)	V	D	D	D	D	V	V	V	V	D
MATURATION STAGE	YF	YF	EC	LC	EP	EC	AF	EP	LC	AF


40.0
30.0
20.0
10.0
VENTRAL
DORSAL
PHOSPHATE - μM/CM2 SKIN
EP
EC
LC
YF
AF
MATURATION STAGE

and phosphate were considerably increased above that observed in larval animals. The dorsal skin from tadpoles contained approximately 50% less calcium and phosphate than that of ventral skin. However, in adult frogs, the dorsal skin contained higher mean values for calcium and phosphate than ventral skin although they were not shown to be significantly different by the statistical test employed.

The calcium and phosphate "concentration" (mM/gm dry skin) in ventral skin increased until early prometamorphosis, remained unchanged through late climax, and finally, decreased approximately 3 fold in yearling and adult frogs (Figures 17 and 18). This "concentration" in dorsal skin also was unchanged from early prometamorphosis until late climax but was significantly reduced in yearling and adult frogs. The "concentration" of calcium and phosphate in dorsal skin at early prometamorphosis was about 33% less than that of ventral skin but was equal in dorsal and ventral frog skin.

Discussion

Although Taylor et al. (1966) noted a tendency for the skin phosphate "concentration" of R. catesbeiana tadpoles to be frequently higher than observed in adults, this difference was not statistically significant because of the great variability of their data. Guardabassi and Sacerdote (1949) reported that the calcified layer of larval and adult R. esculenta skin was similar. In contrast,

Figure 17. The Effect of Maturation on the Calcium "Concentration" (mM/gm dry skin) of Ventral and Dorsal Skin from Rana catesbeiana.

MATURATION STAGE	n[1]	CALCIUM (mM/gm DRY SKIN) VENTRAL	DORSAL
PREMETAMORPHIC "TRANSPARENT" (P-T)	5	0.20 ± 0.09[2]	____
PREMETAMORPHIC "SLIGHTLY OPAQUE" (P-O)	9	0.45 ± 0.18	____
EARLY PROMETAMORPHIC (EP)	10	2.56 ± 0.28	1.69 ± 0.17
EARLY CLIMACTIC (EC)	16	2.02 ± 0.74	1.73 ± 0.31
LATE CLIMACTIC (LC)	10	3.03 ± 1.06	1.92 ± 0.58
NEWLY METAMORPHOSED FROG (NF)	14	1.66 ± 0.43	____
YEARLING FROG (YF)	10	0.82 ± 0.33	0.81 ± 0.20
ADULT FROG (AF)	10	0.88 ± 0.15	0.94 ± 0.17

[1]Number of animals
[2]Mean ± standard deviation

Duncan's Multiple Range Test. Each horizontal line denotes groups of means which show no significant difference ($\alpha = 0.05$).

SKIN (D=DORSAL, V=VENTRAL)	V	V	V	D	V	D	V	D	D	D	V	V	V
MATURATION STAGE	P-T	PO	YF	YF	AF	AF	NF	EP	EC	LC	EC	EP	LC

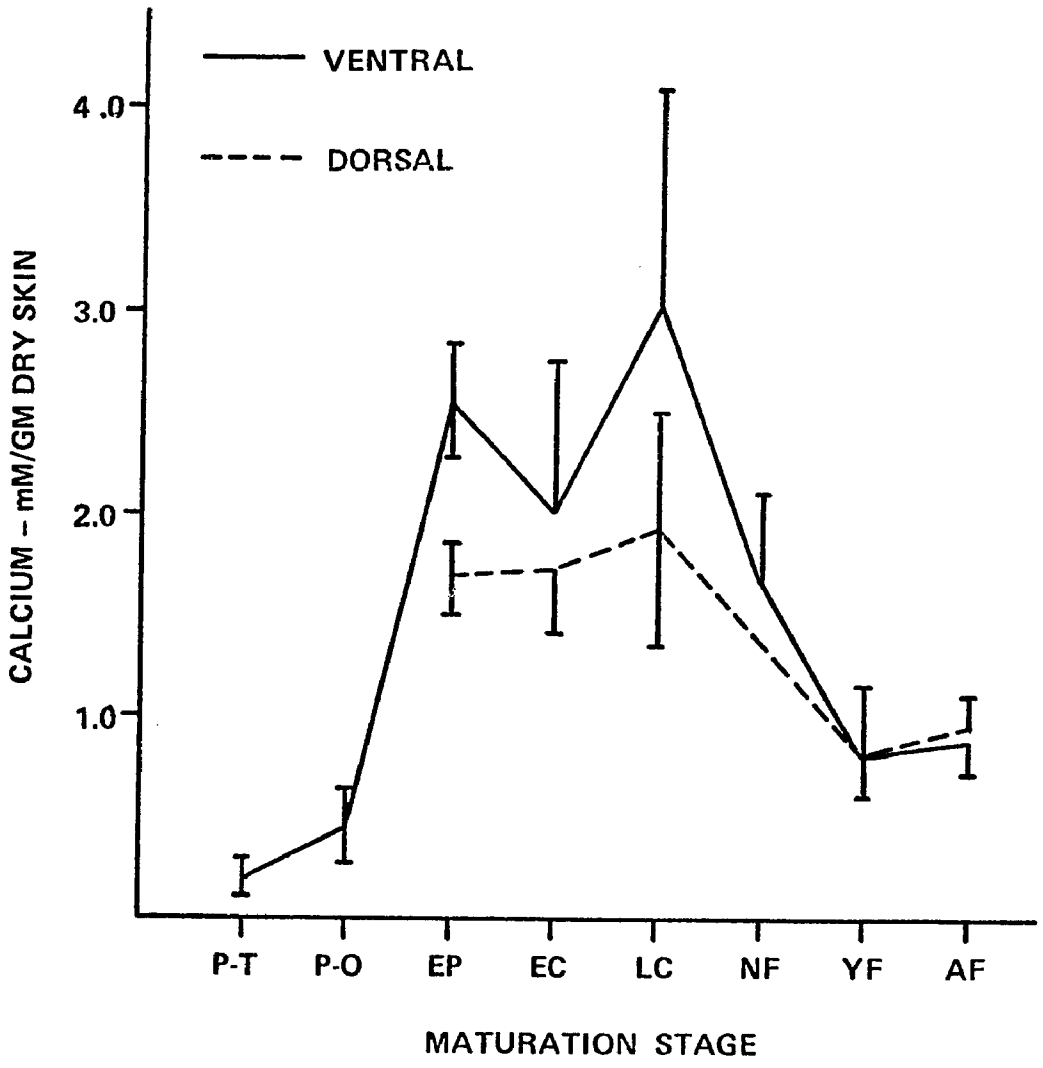

VENTRAL
DORSAL
4.0
3.0
2.0
1.0
CALCIUM - mM/GM DRY SKIN
P-T
P-O
EP
EC
LC
NF
YF
AF
MATURATION STAGE

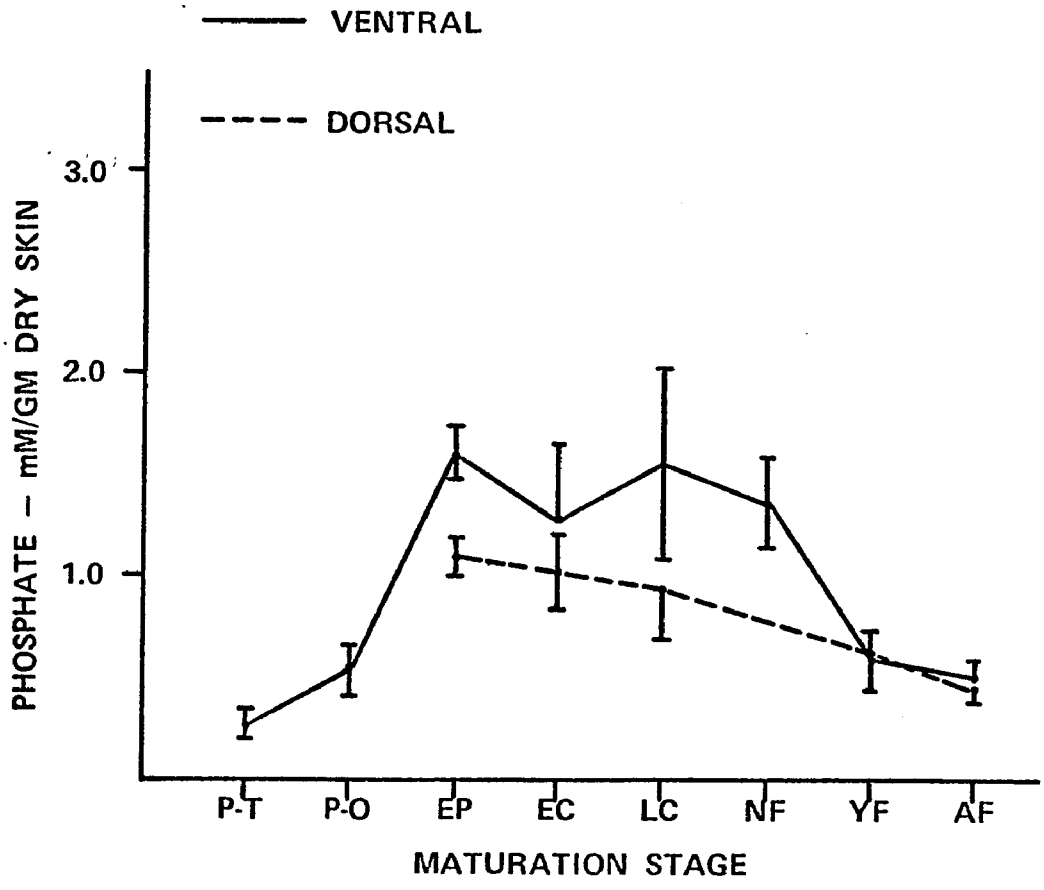
Figure 18. The Effect of Maturation on the Phosphate "Concentration" (mM/gm dry skin) of Ventral and Dorsal Skin from Rana catesbeiana.

MATURATION STAGE	n[1]	PHOSPHATE (mM/gm DRY SKIN) VENTRAL	DORSAL
PREMETAMORPHIC "TRANSPARENT" (P-T)	5	0.27 ± 0.07	____
PREMETAMORPHIC "SLIGHTLY OPAQUE" (P-O)	9	0.54 ± 0.12	____
EARLY PROMETAMORPHIC (EP)	10	1.61 ± 0.13	1.11 ± 0.09
EARLY CLIMACTIC (EC)	16	1.29 ± 0.37	1.03 ± 0.18
LATE CLIMACTIC (LC)	10	1.56 ± 0.47	0.95 ± 0.25
NEWLY METAMORPHOSED FROG (NF)	14	1.37 ± 0.22	____
YEARLING FROG (YF)	10	0.61 ± 0.16	0.63 ± 0.11
ADULT FROG (AF)	10	0.51 ± 0.08	0.46 ± 0.07

[1]Number of animals
[2]Mean ± standard deviation

Duncan's Multiple Range Test. Each horizontal line denotes groups of means which show no significant difference ($\alpha = 0.05$).

SKIN (D=DORSAL, V=VENTRAL)	V	D	V	V	V	D	D	D	D	V	V	V	V
MATURATION STAGE	P-T	AF	AF	PO	YF	YF	LC	EC	EP	EC	NF	LC	EP


VENTRAL
DORSAL
PHOSPHATE – mM/GM DRY SKIN
3.0
2.0
1.0
P-T
P-O
EP
EC
LC
NF
YF
AF
MATURATION STAGE

the results of this investigation indicate that significant changes in both the content ($\mu M/cm^2$) and "concentration" (mM/gm dry skin) of calcium and phosphate occur during the maturation of R. catesbeiana. Both the calcium and phosphate concentrations are considerably reduced following metamorphosis but this is quite misleading when the total amounts of calcium and phosphate in skin are considered. Actually, when expressed as amount per unit area, skins of early prometamorphic tadpoles and adult frogs have similar amounts of calcium as well as phosphate while the content of these elements in dorsal skin from adult frogs is increased about 3 fold above that of larval animals. Also, as the R. catesbeiana tadpole develops into a large frog the body skin surface area is substantially increased. Therefore, because the average content of calcium and phosphate in dorsal and ventral skins of adult frogs is higher than early prometamorphic tadpoles and because the adult frog has a much greater surface area of body skin, then total content of calcium and phosphate in adult frog skin should be much greater than in tadpoles. However, the skin calcium and phosphate/body mass should be reduced as frogs grow since the ratio of the skin surface area/body mass is inversely related to body size.

The adult frog skin appeared to have a greater ability to calcify than tadpole skin. Although the calcification zone in adult ventral skin appeared to be only one-half the thickness of that in tadpole skin (Chapter 1), the adult and early prometamorphic

skins had equal amounts of calcium as well as phosphate per unit area. The calcification zone of adult skin also contained much thicker fibrous tufts which might further reduce the space for mineral deposits. The explanation for this more densely packed mineral zone was not apparent.

The amount of granular calcium phosphate deposits was observed to be considerably reduced in yearling frogs. The fact that ventral organic weight/cm^2 was the same in yearling frogs as in tadpoles suggested that the reductions in mineral content were not correlated with a uniform decrease in skin thickness but appeared to represent a physiological depletion of the calcium phosphate deposits. Although not supported by the non-parametric statistical analysis, the data possibly suggested that a reduction in skin mineral may have also occurred at early climax. Support for a decreased skin content of mineral at early climax came from the observation that the mineral weight (mg)/cm^2 of ventral skin fell from 2.1 ± 0.2(S.D.) at early prometamorphosis to 1.4 ± 0.6 at early climax. It is interesting to speculate that if this study had sampled tadpoles only at early prometamorphosis and early climax, the evaluation of the differences in the two sample means, using Li's (1968) modification of the Student's t-test for sample means with unequal variances, would show a significant reduction in calcium and phosphate at early climax.

The following observations support the hypothesis that the skin mineral serves as a physiological reservoir of calcium and phosphate. The possible reduction in skin mineral at early

climax occurred at the end of an interval during which the hind-leg grew from less than 1 cm in length at early prometamorphosis to approximately 5 cm at early climax. Also, the endolymphatic sacs in R. temporaria which contain $CaCO_3$, are not thought to provide a reservoir for hind-limb ossification; but act as a calcium reservoir system which, in fact, has been reported to continue accumulating calcium during this period of major hind-limb growth (Pilkington and Simkiss, 1966). Therefore, ossification of the growing hind-limb before early climax may be dependent on calcium and phosphate from the environment, a physiological reservoir, or both. Secondly, newly metamorphosed frogs with snout to cloacal lengths of approximately 4 cm grew to about 10 cm (2.5 fold increase) during the first year following metamorphosis. At approximately one year following metamorphic climax, skin calcium and phosphate were considerably reduced. When the need for calcium and phosphate during this period including major skeletal growth, new feeding habits, and finally, hibernation (Dickerson, 1906) is considered, then the skin calcification system is strongly suggested to represent a potential reservoir system.

Although this study showed reductions in the skin mineral which were associated with stages of maturation that demand calcium and phosphate for ossification of the skeleton, further investigations utilizing animals from a homogeneous and carefully controlled population are needed to prove that the skin mineral is necessary

for skeletal calcification. R. pipiens, perhaps, would represent such a population since they can be produced and raised under genetically controlled laboratory conditions.

CHAPTER 3

THE CALCIUM PHOSPHATE RESERVOIR IN THE BODY SKIN OF *RANA CATESBEIANA*

Introduction

As reported by Taylor et al. (1966) and observed in Chapter 2 of this dissertation, the larval *R. catesbeiana* contains high amounts of calcium and phosphate in its dorsal and ventral skin. The reduced amounts of skin mineral observed in yearling frogs (and possibly in early climactic tadpoles) suggest that the skin mineral may represent a dynamic reservoir for calcium and phosphate metabolism. Based on this hypothesis, an experiment was designed to determine the percentage of the tadpoles' total calcium and phosphate which is found in the body skin.

Materials and Methods

Ten early prometamorphic tadpoles were pithed and placed in separate petri dishes. After determining their wet weight, total length, body length (from snout to beginning of tail), and hind-leg length, their total body skins were removed and placed in firing crucibles. The tadpole carcasses were then placed in separate firing crucibles to which the washings from each respective petri dish were added. After drying 24 hours at 105° C, the individual skin and

body samples were ashed at 600° C for 24 hours and dissolved in HCl as previously described (Chapter 2). Aliquots of each sample were then analyzed for calcium (Bachra et al., 1958), and phosphate (Bonting et al., 1961).

Results

As shown in Table II, the body skin of early prometamorphic tadpoles contained 29.5 ± 4.2% and 26.3 ± 2.4% of the animals' total calcium and phosphate, respectively.

The data collected in this experiment (Table II) also provided an estimate of the relationship between the descriptive characteristics of early prometamorphic tadpoles and the content of calcium and phosphate in their body skin. The body length and total length showed the highest correlation to the skin calcium and phosphate while the animals' wet weight and HLL/BL ratio showed the lowest (Table III).

Discussion

During ontogenetic development, mammals, birds, reptiles, and amphibians all utilize a reservoir system to provide the minerals necessary for skeletal ossification (Simkiss, 1967). In mammals, the fetal skeleton is calcified by minerals transported from the maternal system via the placenta. The eggshell of birds and some reptiles provides calcium for initial skeletal ossification. The skeletal development of amphibians follows a period of

TABLE II. TOTAL CALCIUM AND PHOSPHATE IN THE BODY SKIN OF EARLY PROMETAMORPHIC TADPOLES.

ANIMAL	WET WEIGHT (gm)	TOTAL LENGTH (cm)	BODY LENGTH (cm)	HIND-LEG LENGTH (cm)	HLL[1] / BL	TOTAL CALCIUM (mM) SKIN	TOTAL CALCIUM (mM) BODY	TOTAL PHOSPHATE (mM) SKIN	TOTAL PHOSPHATE (mM) BODY	% CALCIUM IN SKIN	% PHOSPHATE IN SKIN
1	10.5	10.2	3.9	1.3	0.34	0.20	0.36	0.14	0.36	35.7	27.9
2	10.0	10.2	3.9	1.0	0.25	0.14	-	0.11	-	-	-
3	9.0	10.8	4.1	1.4	0.33	0.21	0.43	0.15	0.36	32.8	30.0
4	9.0	10.0	3.9	0.8	0.22	0.17	0.41	0.13	0.34	29.3	27.5
5	9.5	9.6	3.9	0.7	0.17	0.17	0.35	0.14	0.36	32.7	28.1
6	9.0	10.1	3.8	1.0	0.26	0.14	0.51	0.12	0.34	21.5	25.3
7	8.5	9.5	3.9	1.1	0.28	0.13	0.34	0.09	0.29	27.6	24.7
8	8.0	9.7	3.7	1.3	0.35	0.15	0.34	0.12	0.34	30.6	26.7
9	9.8	10.4	4.0	1.6	0.40	0.15	0.42	0.12	0.39	26.3	22.7
10	11.2	10.6	4.2	1.5	0.36	0.20	0.48	0.14	0.46	29.4	23.7
$\bar{x}$	9.4	10.1	3.9	1.2	0.30	0.17	0.40	0.13	0.36	29.5	26.3
s	1.0	0.4	0.1	0.3	0.07	0.03	0.06	0.02	0.05	4.2	2.4
C.V.%[2]	10.6	4.2	3.6	26.7	23.3	17.6	15.0	15.4	13.9	14.2	9.1

[1]Hind-leg length/body length.
[2]Coefficient of Variability ($\frac{\text{standard deviation}}{\text{mean}} \times 100\%$)

TABLE III. CORRELATION COEFFICIENTS BETWEEN THE DESCRIPTIVE CHARACTERISTICS OF EARLY PROMETAMORPHIC TADPOLES AND THEIR BODY SKIN CONTENT OF CALCIUM AND PHOSPHATE.

Correlation Coefficients Were Based on Data Shown in Table II.

	CORRELATION COEFFICIENTS	
	BODY SKIN CALCIUM	BODY SKIN PHOSPHATE
ANIMAL WEIGHT	+0.50	+0.41
HLL/BL	+ 0.24	+0.07
BODY LENGTH	+0.63*	+0.59
TOTAL LENGTH	+0.63*	+0.73*

* $p<0.05$

larval growth in which reservoirs of calcium and phosphate are accumulated. The calcium carbonate deposits in the endolymphatic sacs of Bufo bufo (Guardabassi, 1960), and Rana temporaria (Pilkington and Simkiss, 1966) represent one such reservoir and have been reported to be utilized in skeletal ossification. The body skin of tadpoles was observed in this experiment to represent an additional potential reservoir which contained a major fraction of the animals' total calcium and phosphate.

The correlations between skin content of calcium and the tadpoles' wet weight, body length, or the ratio of hind-leg length to body length are of interest because reliable indices for skin calcium are needed in order for paired sampling of tadpoles. The data collected in this experiment suggest that neither wet weight, nor ratio of hind-leg length to body length are meaningful indices to select animals with similar skin calcium. Although body length and total length were better indices of total skin calcium and phosphate, the correlation coefficients suggest that about 35 to 53% of the variability of body length or total length explains the variability observed in skin calcium and phosphate.

CHAPTER 4

CHEMICAL AND PHYSICAL CHARACTERISTICS OF THE CALCIUM PHOSPHATE DEPOSITS IN *RANA CATESBEIANA* SKIN

Introduction

The calcium phosphate granules in *R. catesbeiana* skin were reported to be 0.1 to 1.5 microns in diameter and to consist of randomly arranged, needle-like particles measuring 10 to 20 Å in thickness and up to 500 Å in length (Taylor et al., 1966). Since the mean molar Ca/P ratio of the skin was 1.5, Taylor et al. suggested that the principal calcium phosphate component was $Ca_3(PO_4)_2$.

These granular deposits are morphologically similar to other biologically formed granular calcium phosphate deposits. Pautard (1960) reported that in *Spirostomum ambiguum*, a protozoa, calcium phosphate is precipitated in the form of dense round particles about 1.0 micron in diameter which have peripheral needle-like projections and give an x-ray diffraction pattern resembling hydroxyapatite (HA). These granules showed a close resemblance to the foci or islands of calcification observed in fetal ox femurs during endochondral ossification. The fundamental units of the granules in the unicellular animals resembled beaded threads averaging 215 Å x 64 Å in size and were comparable to the fundamental units observed in dentin.

Since the granular calcium phosphate deposits of R. catesbeiana skin closely resemble other foci of calcification and have not been previously analyzed, experiments were designed to determine the chemical composition and x-ray diffraction pattern of the isolated mineral from the skin of R. catesbeiana.

The chemical and physical nature of bone mineral is a major concern in calcification research. Recent investigations have shown bone mineral to consist of two major components -- an amorphous calcium phosphate and crystalline hydroxyapatite (Termine, 1967). The amorphous or non-crystalline solids are defined as being devoid of long range or periodic order but possessing short range or local order. These solids are generally considered to possess a higher energy state than their crystalline derivatives. The amorphous calcium phosphate (Ca/P = 1.5) was shown to decrease from 67% of the total mineral phase in the femur of 3-day old rats to 37% in that of 80-day old rats. The amorphous phase has also been shown to be present in dentin, cartilage, and epiphyseal, trabecular, haversian, and non-haversian compact bone tissues. The non-crystalline mineral was proposed to be the first mineral deposited which gradually is transformed into crystals approximating the composition and crystal form of hydroxyapatite (Ca/P = 1.67). Also, the amorphous calcium phosphate is thought to represent a labile pool of calcium phosphate functioning in mineral metabolism.

Other investigators have proposed that the initial crystalline form of calcium phosphate is octacalcium phosphate (OCP), which exists in layers alternating with layers of hydroxyapatite (Brown, 1966). OCP (Ca/P = 1.33) is postulated to form and then "hydrolyze" to hydroxyapatite (HA) as written:

$$8Ca^{++} + 2H^{+} + 6\ PO_4^{---} + 5H_2O = Ca_8H_2(PO_4)_6 \cdot 5H_2O\ [OCP]$$

$$Ca_8H_2(PO_4)_6 \cdot 5H_2O[OCP] + 2\ Ca^{++} = Ca_{10}(PO_4)_6(OH)_2[HA] + 4H^{+} + 3H_2O$$

According to Brown, the evidence that OCP has at least a transient role in bone and tooth formation is based on the following:

1. The platy or blade-like shape of OCP crystals is observed in bone and dentin.
2. Low Ca/P ratios have been found in the early stages of mineralization.
3. OCP heated above 200° C is partially converted to pyrophosphate. Mineralized tissues, but not pure HA, are partially converted to pyrophosphate when heated. The amount of pyrophosphate is greatest in tissues in early stages of mineralization and decreases with age and with time after formation of the mineral.
4. During <u>in vitro</u> calcification of cartilage in phosphate-carbonate solutions, Ca/P ratios of exactly 1.33 were observed.

5. Based on solubility data, calf and child bone were observed to contain a solid phase with a Ca/P ratio of about 8/6 (1.33) whereas adult bone gave a ratio of 10/6 (1.67).
6. Fetal enamel showed an x-ray diffraction pattern with two diffuse lines which corresponded to OCP.
7. The occurrence of OCP and its higher solubility would help explain the higher concentration of serum phosphate in young animals.

However, Urist and Dowell (1967) have disputed the existence of OCP in calcifying systems on the basis that (1) OCP is not present in sufficient quantities to be detected in the presence of apatite by x-ray diffraction and (2) when the several forms of calcium phosphate were implanted into the anterior chamber of the rat eye, OCP was not observed to hydrolyze to HA. However, in their implantation studies, the large size of the platy crystals may have prevented such "hydrolysis". From their study, a protein-$CaHPO_4$ complex was proposed by Urist and Dowell as a possible initial mineral phase in bone. This was based on observations that the zone of provision calcification in epiphyseal cartilage from rabbit long bones contained a mineral phase having a Ca/P ratio of 1.48 ± 0.8 and giving a broad, ill-defined x-ray diffraction pattern which suggested the presence of poorly crystalline apatite with no positive evidence of OCP. It was suggested that the low Ca/P ratio observed in the provisional calcification zone was caused by a mixture of apatite and

the protein-$CaHPO_4$ complex. In response to the argument that $CaHPO_4$ cannot exist at the pH of body fluids, $CaHOP_4 \cdot 2H_2O$ was shown to be associated with degenerating cartilage without "hydrolyzing" immediately into apatite (McCarty et al., 1966). Also, protein-$CaHPO_4$ was reported to exist at physiologic pH in lower vertebrates with a "physiologic hypercalcemia" (Urist, 1962).

Another proposed intermediate precursor to HA was suggested by Dallemagne (1964) to be hydrated tricalcium phosphate - (TCPH) containing $9Ca^{++}$ ions for $6PO_4^{---}$ groups and written as:

$$Ca_9(PO_4)_6(O_3PO\text{-}H\text{-}OPO_3)$$

Dallemagne suggested that the low Ca/P ratio of bone mineral (1.5) and the high yield of pyrophosphate produced when bone mineral is subject to ignition at 325^{o} C could be explained by an admixture of HA and TCPH.

McLean and Urist (1968) suggested a model system of bone mineral maturation which incorporated the amorphous calcium phosphate, OCP, TCPH, and HA into one system. This model began with the initial deposition of amorphous calcium phosphate which is redissolved and then deposited in the initial crystalline form. This initial crystalline form is either OCP or something of similar composition and crystalline structure and having a low Ca/P ratio of 1.33. The crystal maturation proceeded as a continuum that progressed through a series of changes in which calcium was visualized as being continuously and randomly added to the mass of mineral. The next stage after OCP, was

suggested to be Dallemagne's hydrated tricalcium phosphate having a Ca/P ratio of 1.5. Further maturation continually adds calcium until HA is formed.

While the granular calcium phosphate deposits in R. catesbeiana skin resemble the foci of calcification in other newly calcifying systems, these deposits are unique in that they do not appear to coalesce as observed in other systems. Therefore, these deposits provide an excellent model system in which to study the chemical and physical characteristics of the immature form of calcium phosphate depositions.

Material and Methods

An initial study was designed to compare the calcium, phosphate, and magnesium content of total body skin with that of the isolated mineral granules from R. catesbeiana skin. The body skins from ten early prometamorphic tadpoles were scraped on their serosal surface to remove loosely bound mucinous substances, washed briefly in distilled water, drained on filter paper, and weighed. After drying at 105° C for 24 hours, weighing, and then powdering in a Wiley mill, the sample was divided into two portions and weighed again after drying. The first portion was ashed at 600° C for 24 hours, weighed after drying at 105° C, and finally dissolved in HCl as described earlier (Chapter 2). Triplicate aliquots of each skin sample were analyzed for calcium (Bachra et al., 1958), phosphate

(Bonting et al., 1961), and magnesium (Perkin-Elmer atomic absorption spectrophotometer, Model 290B).

The second portion of the pooled skin sample was refluxed with anhydrous ethylenediamine (Termine and Posner, 1967) for 12 hours in order to solubilize the organic components of the skin. After centrifugation the supernatant and mineral precipitate were collected separately. The mineral was washed once with absolute alcohol, centrifuged, and the washings added to the previous supernatant. The separate mineral and supernatant fractions were then dried, ashed, and dissolved in HCl after which calcium, phosphate, magnesium, and sodium (Instrumentation Laboratory flame photometer, Model 143) analyses were performed on the supernatant and mineral samples.

A second study was designed to determine the amount of calcium and phosphate as well as the x-ray diffraction patterns of the following pooled mineral samples from R. catesbeiana:

1. the body skin mineral from three large adult frogs;
2. the body skin mineral from ten early prometamorphic tadpoles (total length, 10.4 ± 6.0 (SD) cm; hind-legs length <0.5 cm);
3. the bone mineral from ten pairs of femurs from late prometamorphic tadpoles (total length, 10.5 ± 4.0 cm; hind-leg length, 4.1 ± 0.7 cm);
4. the bone mineral from three pairs of femurs from the above three adult frogs.

As each skin sample was removed, it was immediately frozen in a lyophilization jar bathed in dry ice and acetone. Each femur was cleaned of the muscular and marrow tissue and then frozen in a similar manner.

Following lyophilization, each sample was refluxed twice with 150 ml of anhydrous ethylenediamine in order to dissolve the organic residue in the samples. After centrifugation, the isolated mineral samples were washed once with acetone and dried in a vacuum descicator for 48 hours.

After each sample was ground and mixed in a mortar and pestle, the x-ray diffraction patterns of the powdered samples were obtained by Dr. Charles Bugg, Institute for Dental Research, University of Alabama in Birmingham with a Debye-Scherrer Powder Camera utilizing nickel-filtered copper radiation (45KV, 20ma).

The resulting diffraction patterns were compared with a pattern developed from powdered tooth enamel which was used as a standard for biological apatite. A major portion of each sample was then placed in firing crucibles, weighed after drying at 105^{o} C for 12 hours, ashed at 600^{o} C for 24 hours, again weighed after drying at 105^{o} C for 12 hours, dissolved in HCl as previously described (Chapter 2) and analyzed in triplicate for calcium (Bachra et al., 1958) and phosphate (Bonting et al., 1961).

Results

The skin from the bodies of early prometamorphic tadpoles contained 87.7% water, 9.8% organic matter and 2.5% mineral. As shown in Table IV, the isolated skin mineral granules contained 99% of the total calcium and phosphate of the body skin but only 82% of the total magensium. The skin mineral from the early prometamorphic tadpoles was characterized by a Ca/P (molar) ratio of 1.33. The major constituents of the isolated mineral were calcium and phosphate but sodium and magnesium were present in trace amounts; the sum of calcium, phosphate, sodium, and magnesium accounted for 94% of the total mineral (Table V).

As shown in Table VI, the phosphate content (mg/gm mineral) of skin and bone was similar in both larval and adult bullfrogs. However, the calcium content of the skin mineral was considerably lower than that of bone mineral in both frogs and late prometamorphic tadpoles. Again, skin mineral was characterized as having a low Ca/P ratio. Interestingly, calcium content of bone from late prometamorphic tadpoles was found to be somewhat less than that of adult bone. Since each type of mineral represented a pooled sample, no basis existed for statistical evaluation of the differences in calcium composition.

The x-ray diffraction patterns showed the crystalline nature of skin mineral to be similar to that of bone from the tadpole and adult frog (Figure 19). Although the x-ray reflections of the bone and skin samples were often ill-defined, the patterns of each

TABLE IV. COMPARISON OF THE MINERAL COMPOSITION IN THE BODY SKIN AND ISOLATED SKIN MINERAL OF R. CATESBEIANA TADPOLES.

	BODY SKIN	ISOLATED SKIN MINERAL
	mM/gm DRY SKIN	
CALCIUM	1.72	1.69
PHOSPHATE	1.34	1.28
MAGNESIUM	0.11	0.09
Ca/P (MOLAR)	1.28	1.33

TABLE V. COMPOSITION OF THE ISOLATED SKIN MINERAL FROM R. CATESBEIANA TADPOLES.

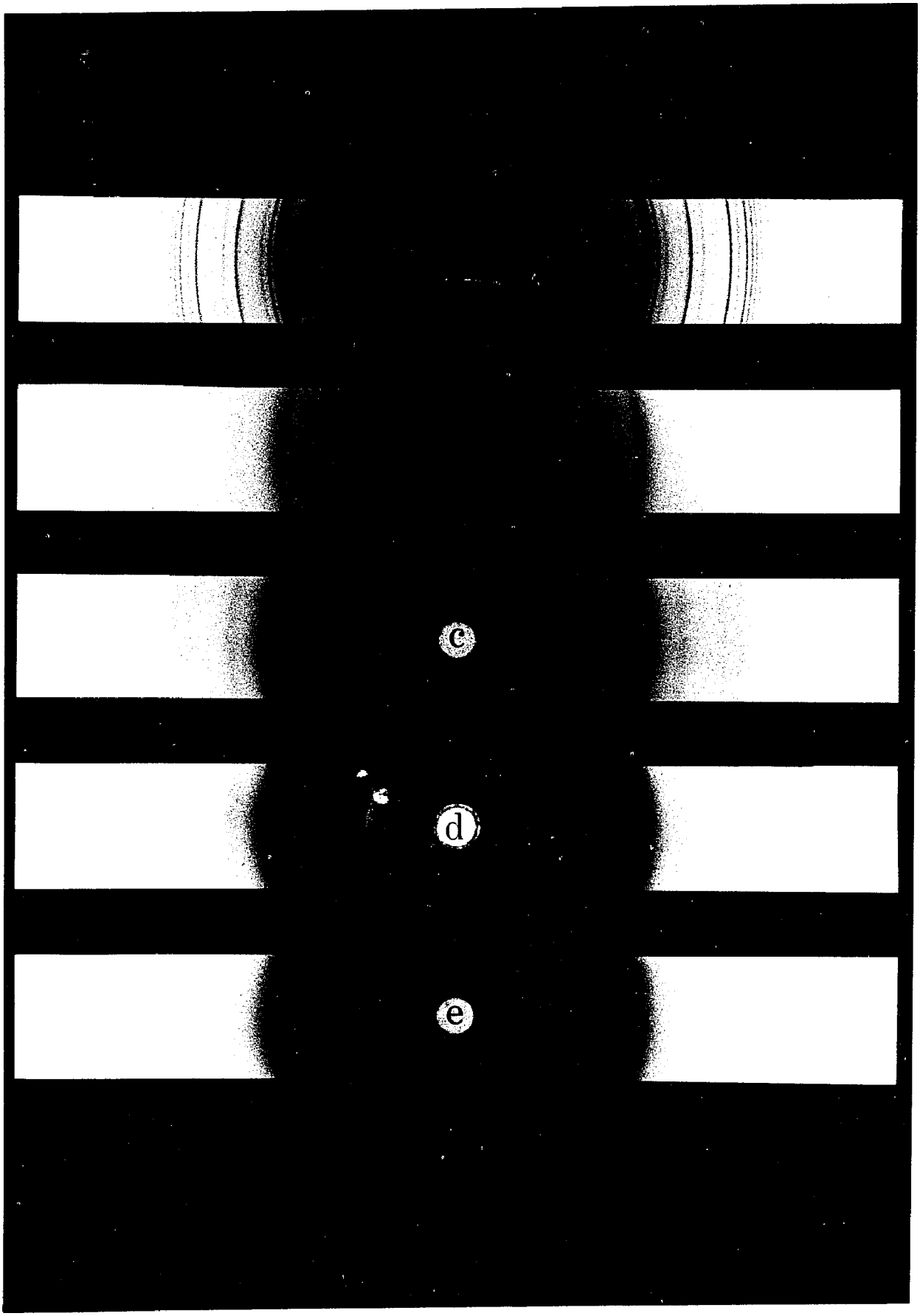
	mg/gm MINERAL
CALCIUM	326.7
PHOSPHATE	586.2
MAGNESIUM	10.8
SODIUM	16.0

TABLE VI. THE CALCIUM AND PHOSPHATE CONTENT OF THE BONE AND ISOLATED SKIN MINERAL OF R. CATESBEIANA.

	BONE MINERAL		ISOLATED SKIN MINERAL	
	FROG	TADPOLE	TADPOLE	FROG
CALCIUM - mg/gm MINERAL	381	357	321	304
PHOSPHATE - mg/gm MINERAL	563	584	562	554
Ca/P - MOLAR	1.59	1.44	1.35	1.30

Figure 19. X-ray Diffraction Patterns of Rana catesbeiana Bone and Skin Mineral.

(a) Tooth Enamel, (b) Bone Mineral from Frogs, (c) Bone Mineral from Tadpoles, (d) Skin Mineral from Tadpoles, and (e) Skin Mineral from Frogs: Debye-Scherrer Powder Camera, Cu radiation (45 KV, 20 ma), Ni filtered.


c
d
e

sample closely corresponded to the pattern for enamel apatite.

Discussion

The skin mineral in R. catesbeiana was characterized by a low molar Ca/P ratio of approximately 1.33 and contained about 32% calcium and 57% phosphate. These findings do not support previously reported observations that strongly suggest that $Ca_3(PO_4)_2$ was the principal calcium phosphate in the skin (Taylor et al., 1966) since tricalcium phosphate contains 38.75% calcium and 61.25% phosphate and has a calcium to phosphate ratio of 1.5.

Since the skin mineral had a diffraction pattern similar to biological apatite but its calcium composition was considerably lower than apatite, then at least two calcium phosphate phases may exist in the granular skin mineral. It is unlikely that this mineral with a low Ca/P ratio was spontaneously converted to hydroxyapatite during the isolation process since a non-aquous procedure was utilized. Therefore, the question again arises concerning the exact composition of the granular mineral. Brown's (1966) argument concerning a coexistence of octacalcium phosphate and hydroxyapatite is difficult to apply to the skin mineral since the Ca/P ratio of skin mineral corresponds almost exactly with that of 100% octacalcium phosphate. The model suggested by Brown should require at least 20% hydroxyapatite which would increase the Ca/P ratio to 1.40. Finally, it is unlikely that a sample of mineral (Ca/P - 1.33) containing a large percentage of octacalcium phosphate and a small percentage

of apatite could show an apatite diffraction pattern that was comparable to adult frog bone mineral and to dental enamel.

The only known calcium phosphate precipitate which could effectively lower the calcium to phosphate molar ratio to near 1.3 would be $CaHPO_4 \cdot 2H_2O$. Urist (1967) proposed that early calcifications were composed of a mixture of $CaHPO_4$ and hydroxyapatite -- a combination which could explain the low calcium to phosphate ratios of newly calcifying tissues. Since the skin mineral has a calcium to phosphate ratio of approximately 1.33 and the mineral is not likely to be composed of more than 80% octacalcium phosphate and less than 20% hydroxyapatite, then the presence of $CaHPO_4 \cdot 2H_2O$ in the skin mineral seems possible.

The percentage of phosphate in hydroxyapatite, octacalcium phosphate, and secondary calcium phosphate ranged from 55.4% to 58.0% which makes evaluation of the mineral components difficult when based on phosphate content. However, the percentages of calcium are 23.3%, 32.6%, and 39.9% in secondary calcium phosphate, octacalcium phosphate, and hydroxyapatite, respectively. The percentage of calcium in the skin mineral is almost identical to that of octacalcium phosphate but since the mineral apparently contains a major apatitic component, a mixture with octacalcium phosphate should increase the percentage of calcium in the skin mineral above 32%. Again, when assuming a mixture of calcium phosphates is present in skin, secondary calcium phosphate may be in part responsible for the low average value of 32% calcium observed in skin mineral.

The amorphous calcium phosphate described by Termine (1967) with a calcium to phosphate ratio of 1.5 cannot be rationalized as a major mineral fraction responsible for the low Ca/P ratio of skin mineral. However, this certainly does not rule out its presence in the granular skin mineral.

As shown morphologically (Taylor et al., 1966) and by the chemical composition and x-ray diffraction patterns of this study, the granular calcium phosphate deposits in R. catesbeiana skin closely resemble other foci of calcification. As previously discussed, the morphological characteristics of these skin deposits closely correspond to other granular deposits reported in newly calcifying unicellular animals, ox femurs, and dentin. Newly calcified tissues are often characterized by low Ca/P ratios as was observed for the skin mineral. However, in spite of these low ratios, x-ray diffraction analysis usually suggests the presence of a poorly crystalline apatite as was observed in the skin mineral. Therefore, because of the similarities between granular mineral deposits in skin, bone, and teeth, and because the skin granular deposits do not coalesce to form solid layers of hard tissue, this study indicates that the calcium phosphate in R. catesbeiana skin may represent a valuable model system in which to study the initial form of calcium phosphate in newly calcifying tissues.

CHAPTER 5

THE FUNCTION OF GLYCOSAMINOGLYCANS IN THE CALCIFICATION SYSTEM OF *RANA CATESBEIANA* SKIN

Introduction

The reactive properties of glycosaminoglycans have been reported to represent a mechanism which influences biological calcification (Bowness, 1966). Since the granular calcium phosphate deposits in the skin of *R. catesbeiana* are closely associated with glycosaminoglycans (Chapter 1), an investigation was set up to study the *in vitro* uptake of ^{45}Ca by *R. catesbeiana* skin and to determine how this uptake is influenced by the skin glycosaminoglycans.

For ease of presentation, these experiments are divided into two groups and discussed separately. The first group concerns the *in vitro* uptake of ^{45}Ca by *R. catesbeiana* skin while the second relates to the influence of glycosaminoglycans on this ^{45}Ca uptake.

A. *In Vitro* Uptake of ^{45}Ca by Ventral Skin from *R. catesbeiana*.

Introduction

Before testing any specific hypothesis concerning the mechanisms involved in the calcification of *R. catesbeiana* skin, the general characteristics of ^{45}Ca uptake by larval ventral skin were investigated. Studies were designed to determine the following:

1. the rate of ^{45}Ca uptake;
2. the specific location of ^{45}Ca binding;
3. the influence of larval maturation stages on ^{45}Ca uptake;
4. the effect of cellular and tissue alterations on ^{45}Ca uptake.

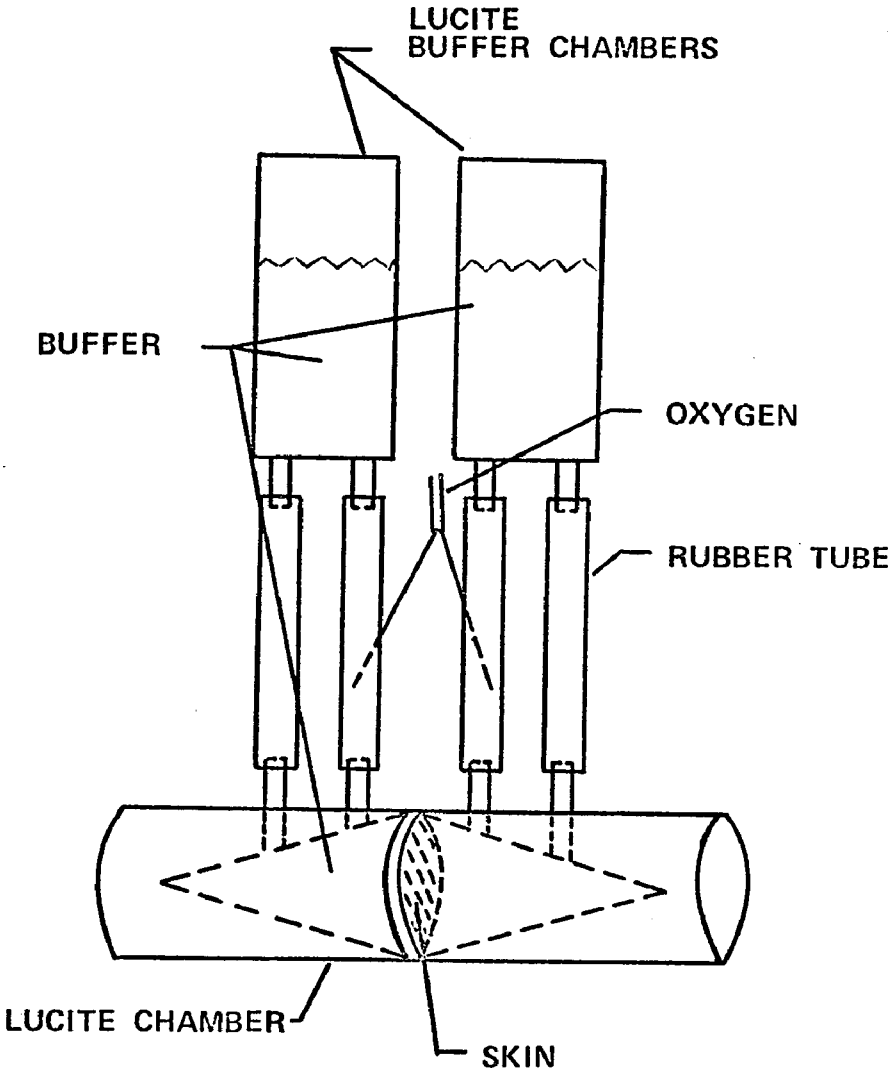
Methods and Results

Ventral skin from R. catesbeiana tadpoles was mounted between two Lucite chambers (Figure 20) and bathed on both the serosal and mucosal surface with either an amphibian Ringer's solution buffered at pH 7.3 with 5.0 mM sodium phosphate or a solution containing 95 mM sodium chloride, 1.0 mM calcium chloride, and 1.0 mM sodium phosphate and buffered at pH 7.3 with 50 mM tris-(hydroxymethyl)-aminomethane (tris). In order to circulate and aerate the buffer solutions, 100% oxygen was bubbled into each chamber half. Radiocalcium chloride, 0.25 μCi dissolved in 0.2 ml of buffer, was added to the serosal solution while 0.2 ml of unlabeled buffer solution was added to the mucosal chamber.

After the skins had been exposed to the isotope for a specific time interval, the chambers were drained, and rinsed with distilled water in order to remove any excess buffer solution from the skin. The skins were then removed, dried, ashed and put into solution as previously described (Chapter 2). Radioactivity of each sample was measured with a liquid scintillation counter (Packard,

Figure 20. The Lucite Chamber System Utilized in the ^{45}Ca Uptake Studies.

Ventral skin was mounted between the two lower chambers and bathed on both the serosal and mucosal surfaces with a tris-buffered NaCl solution or amphibian Ringer's phosphate buffer. Circulation through the chambers was provided by bubbling 100% oxygen into the chambers as shown.


LUCITE
BUFFER CHAMBERS
BUFFER
OXYGEN
RUBBER TUBE
LUCITE CHAMBER
SKIN

Tri Carb-3320). Radiocalcium uptake was expressed as a percentage of the total ^{45}Ca counts which had been initially added to the chamber.

To observe the movement of ^{45}Ca within the chamber system, early prometamorphic tadpoles were randomly divided into 4 groups containing 12 tadpoles (Table VII). The ventral skins from these 4 groups were exposed to ^{45}Ca in the tris buffer system for 4, 8, 12, or 16 hours after which each skin was trimmed of excess skin not exposed to the serosal buffer. The skins as well as the serosal and mucosal buffer solutions were then analyzed for total calcium (Bachra et al., 1958) and ^{45}Ca activity.

The percent ^{45}Ca in the serosal buffer decreased concomitantly with an increase in the percent ^{45}Ca of the skin and mucosal buffer (Figure 21). After 16 hours, the ^{45}Ca was found distributed as follows: serosal fluid, 52%; mucosal fluid, 7%; and skin, 37%.

The specific activity of the serosal solution appeared to decrease exponentially from 0 to 16 hours (Figure 22). This decrease was apparently caused by the loss of skin calcium into the buffer solutions since, at 16 hours, the total calcium in the serosal and mucosal buffer solution had increased 72% and 30%, respectively (Figure 23). The specific activity (cpm/μg calcium) of the mucosal solution increased progressively from 0 to 16 hours. The data showing a linear increase in specific activity of the skin are not shown since a consistent technical error in the analysis of total skin

TABLE VII. DESCRIPTIVE CHARACTERISTICS OF THE R. CATESBEIANA TADPOLES USED IN THE STUDY OF THE RATE OF IN VITRO ^{45}Ca UPTAKE.

ANIMAL GROUPS (HOURS)	n	BODY LENGTH	TOTAL LENGTH	HIND-LEG LENGTH
4	12	4.3 ± 0.4[1]	11.4 ± 1.2	0.29 ± 0.14
8	12	4.2 ± 0.5	10.8 ± 1.4	0.22 ± 0.10
12	12	4.1 ± 0.5	10.6 ± 1.3	0.17 ± 0.12
16	12	4.1 ± 0.2	10.6 ± 0.7	0.27 ± 0.18

[1]cm, mean ± standard deviation

Figure 21. Changes in the Percent ^{45}Ca Activity in the Serosal Buffer, Skin, and Mucosal Buffer During the *In Vitro* Study of ^{45}Ca Uptake by *Rana catesbeiana* Skin.

Each point represents the mean of 12 observations; the vertical bars represent ± 1 standard deviation.

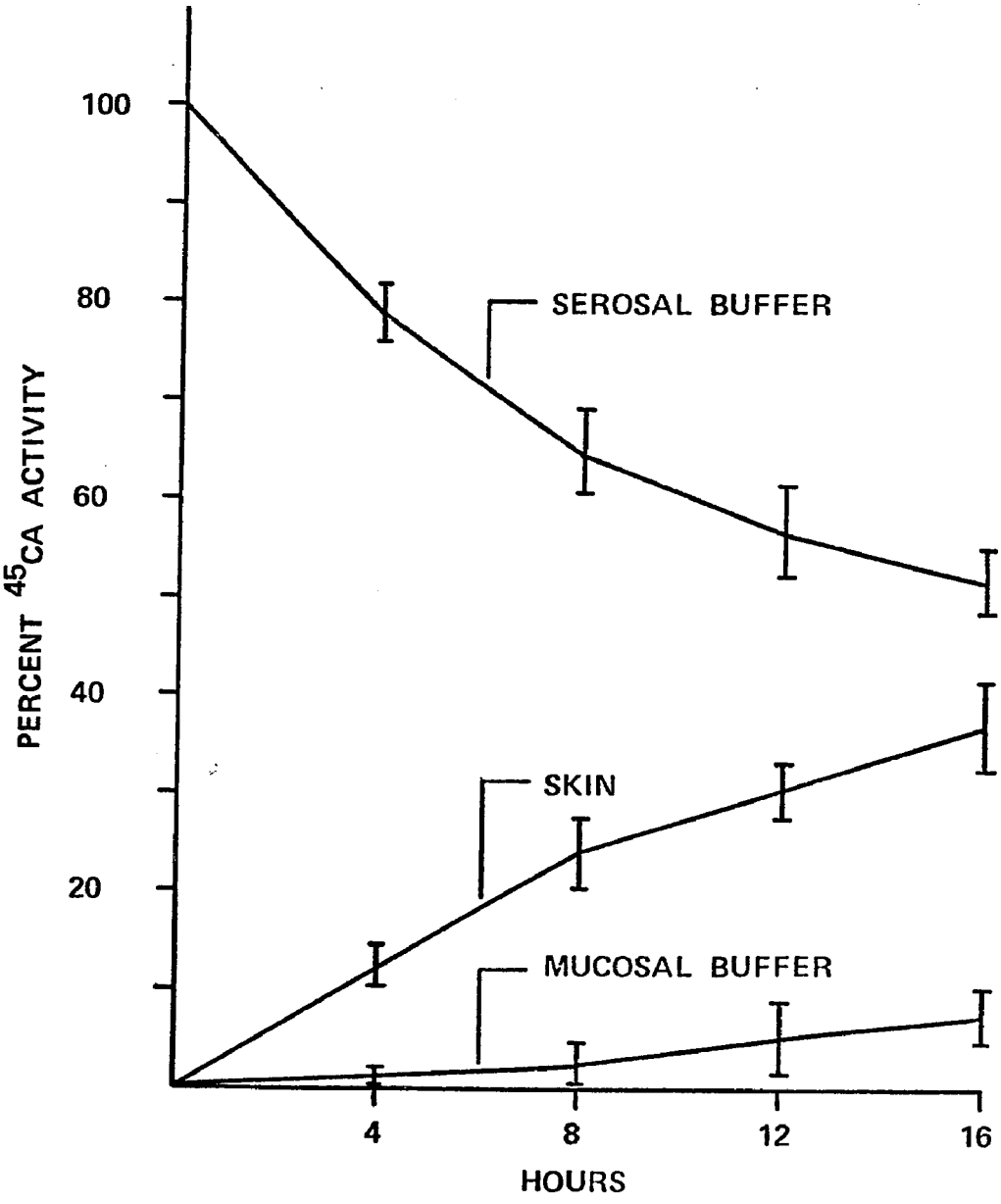

100
80
60
40
20
PERCENT ^{45}CA ACTIVITY
SEROSAL BUFFER
SKIN
MUCOSAL BUFFER
4
8
12
16
HOURS

Figure 22. Changes in the Specific Activity (CPM/µg Ca) of the Serosal Buffer and Mucosal Buffer during the *In Vitro* Study of ^{45}Ca Uptake of *Rana catesbeiana* Skin.

Each point represents the mean of 12 observations; the vertical bars represent ± 1 standard deviation.

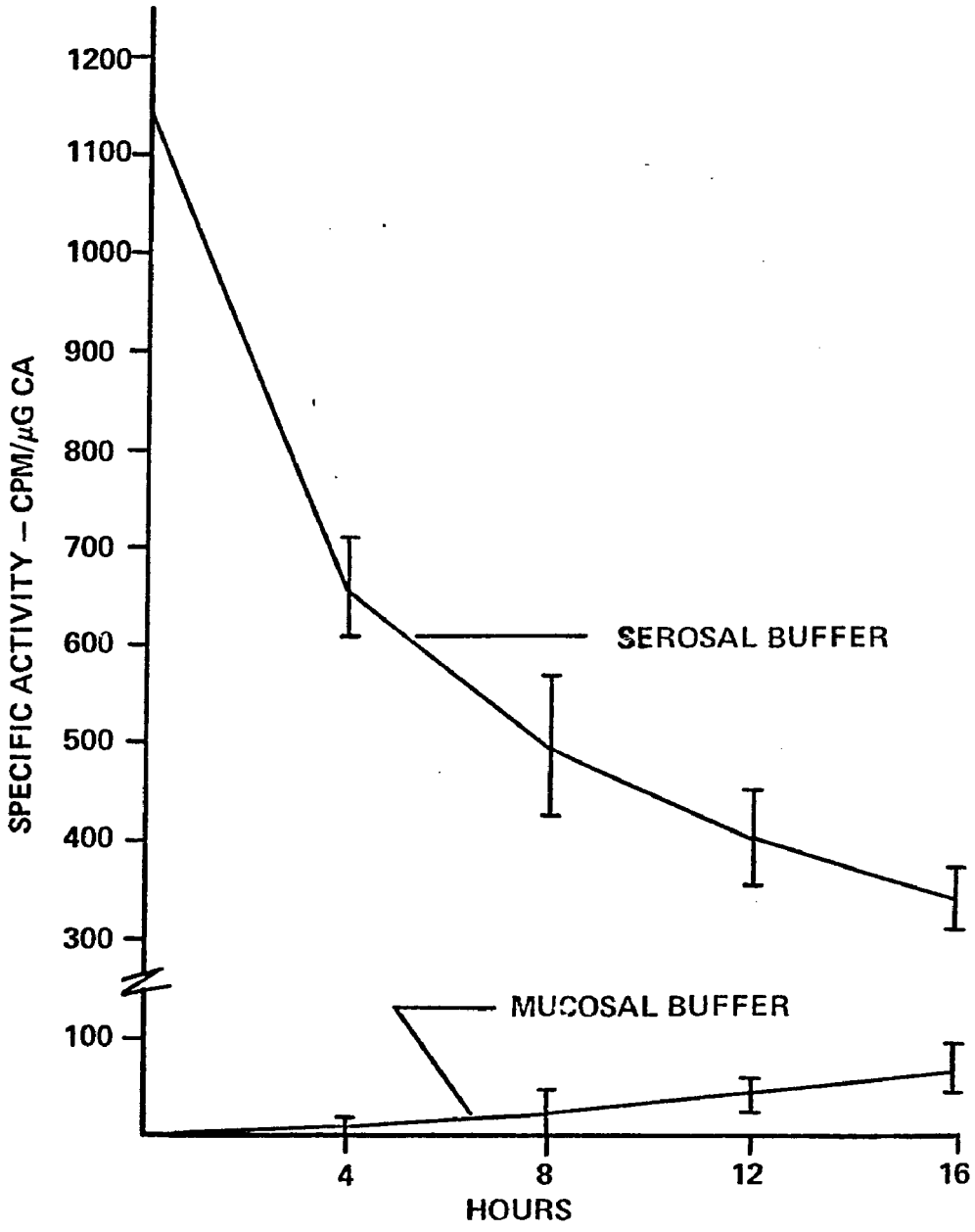
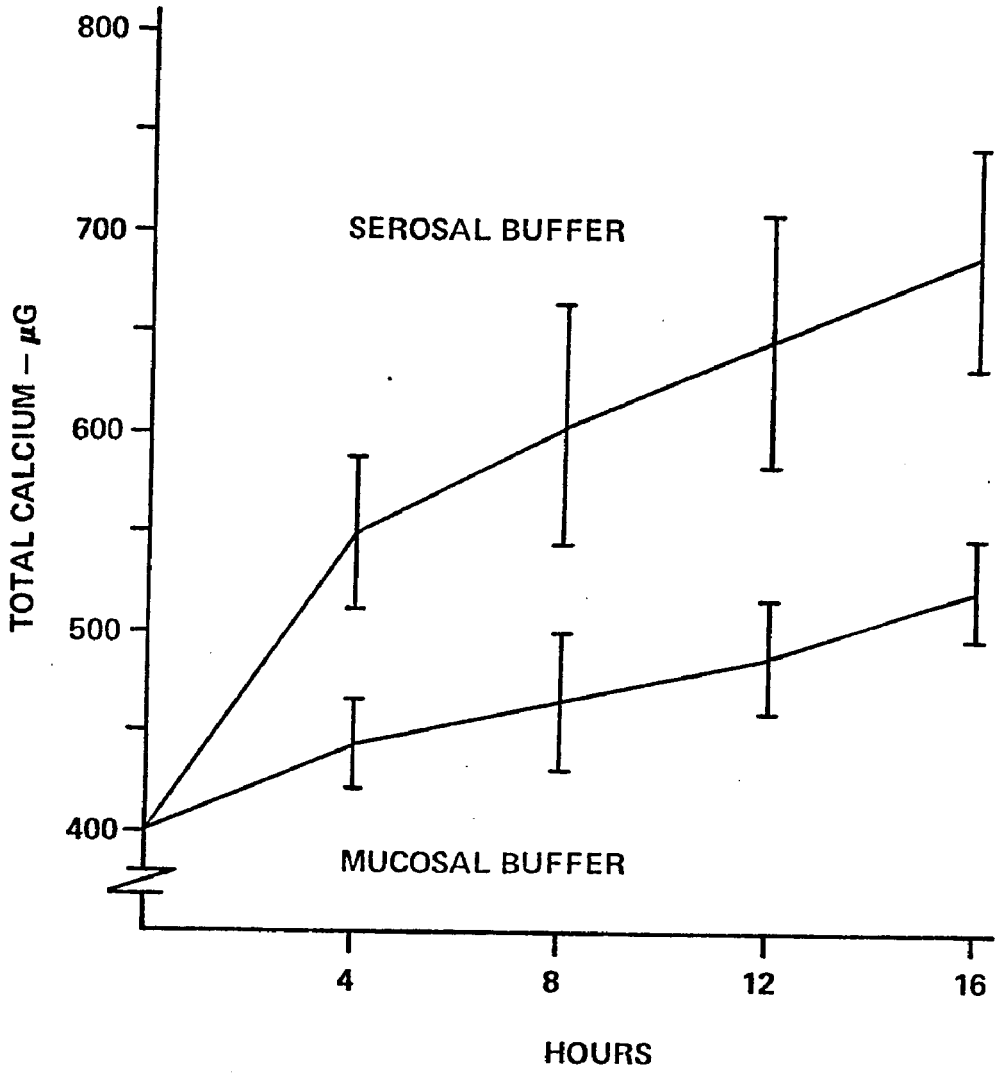

1200
1100
1000
900
800
700
600
500
400
300
100
SPECIFIC ACTIVITY – CPM/μG CA
SEROSAL BUFFER
MUCOSAL BUFFER
4
8
12
16
HOURS

Figure 23. Changes in the Total Calcium (μg) of the Serosal Buffer and Mucosal Buffer during the In Vitro Study of ^{45}Ca Uptake by Rana catesbeiana Skin.

Each point represents the mean of 12 observations; the vertical bars represent ± 1 standard deviation.


800
700
600
500
400
TOTAL CALCIUM – µG
SEROSAL BUFFER
MUCOSAL BUFFER
4
8
12
16
HOURS

calcium was suspected, but could not be verified.

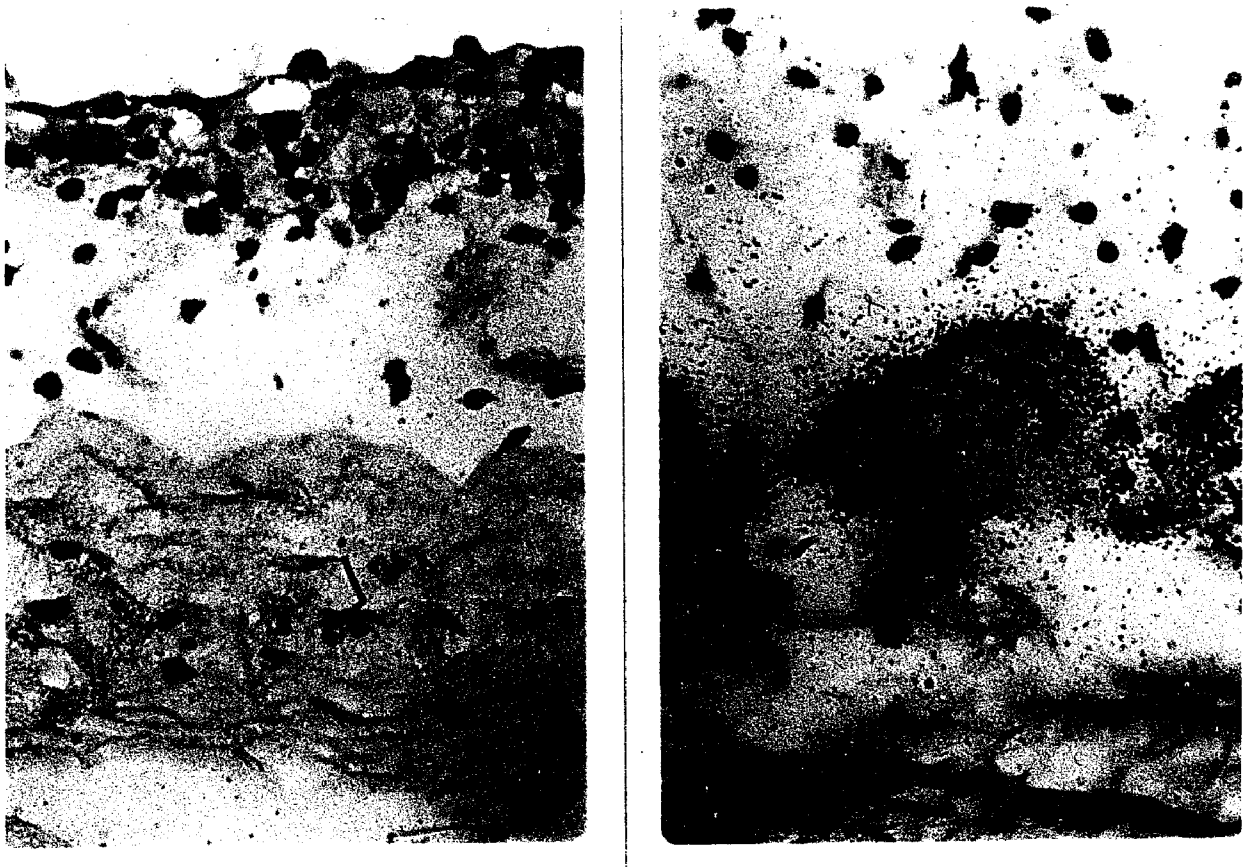
The specific location of ^{45}Ca binding was determined by autoradiography. Ventral skin from an early prometamorphic tadpole was bathed on both surfaces with the tris solution and then exposed for 4 hours to ^{45}Ca. Next, the skin was fixed in 10% neutral buffered formalin for 24 hours and prepared for histological sectioning. Tissue sections (approximately 5 microns in thickness) were mounted on glass slides and coated in absolute darkness with NTB-3 Nuclear Track Emulsion (Kodak). After 82 days of incubation in a refrigerator at 4^{o} C, the emulsion was developed after which the tissue sections were stained with hematoxylin and eosin. A control skin exposed only to buffer solution was treated identically.

As shown in Figure 24, ^{45} Ca label was specifically concentrated in an area that corresponds to the location of the calcification zone. Also, as expected from the uptake studies, in which ^{45}Ca was detected in both the skin and the mucosal buffer, scattered ^{45}Ca label was seen in all areas of the skin. The emulsion in the control skin was noticeably free of background exposure.

The next experiment measured ^{45}Ca uptake in ventral skins from tadpoles at three maturation stages. Ventral skins from R. catesbeiana at early prometamorphosis, late prometamorphosis and late climax were exposed as previously described to ^{45}Ca in amphibian Ringer's solution for 6 hours. Utilizing these early and late prometamorphic tadpoles as controls, the effect of loss of cellular viability on ^{45}Ca uptake and the effect of tissue fixation on ^{45}Ca

Figure 24. The In Vitro Binding of ^{45}Ca in the Calcification Zone of Tadpole Skin.

(a) Control Autoradiogram; (b) ^{45}Ca-Autoradiogram: The ventral skin of an early prometamorphic tadpole was bathed for 4 hours on both surfaces with a tris buffer containing ^{45}Ca in the serosal solution. The control skin was treated identically with the exception that no ^{45}Ca was placed in the serosal solution. The autoradiogram of the skin exposed to ^{45}Ca (b) showed the calcification zone to specifically bind ^{45}Ca.



a b

uptake were tested. Ventral skins from late prometamorphic tadpoles were frozen for 2 hours in order to destroy cellular activity (Coriell et al., 1964), thawed, and placed in the above chamber system for 6 hours. Finally, ventral skins from early prometamorphic animals were soaked for 24 hours in a fixative containing 9 parts absolute alcohol and 1 part concentrated formaldehyde and then exposed as above to ^{45}Ca.

Ventral skins from early and late prometamorphic tadpoles bound similar amounts of ^{45}Ca (Table VIII); however, late climactic skins showed a 35% decrease. Loss of cellular viability caused no significant change in ^{45}Ca uptake, but fixation of the skins sufficiently altered the tissue so that uptake increased 54%.

Discussion

The *in vitro* skin chamber system provided a good model to observe the uptake of ^{45}Ca in skin since the uptake increased almost linearly through the eighth hour (Figure 21) and the skin calcification zone specifically bound the ^{45}Ca (Figure 24). After eight hours the rate of ^{45}Ca uptake in skin fell concomitantly with an increase of calcium accumulation in the mucosal buffer.

Calculations on the total quantity of calcium exchanged in the skin would be difficult because a mathematical model with unverified assumptions is required. Interestingly, 52% and 41% of the

TABLE VIII. THE PERCENT ^{45}Ca UPTAKE IN 6 HOURS BY VENTRAL SKIN FROM TADPOLES.

SKINS	n[1]	PERCENT ^{45}Ca UPTAKE
EARLY PROMETAMORPHIC	6	11.4 ± 1.7[2]
LATE PROMETAMORPHIC	4	11.4 ± 0.4
LATE CLIMACTIC	6	7.4 ± 1.1
FROZEN-THAWED, LATE PROMETAMORPHIC	4	12.7 ± 1.4
ALCOHOL-FORMALDEHYDE FIXATION, EARLY PROMETAMORPHIC	6	17.6 ± 5.3

[1]Number of animals
[2]Mean ± standard deviation

total increase in the calcium of the serosal and mucosal solution, respectively, had occurred by 4 hours.

The ionic composition of the buffer appeared to influence the uptake of ^{45}Ca since ventral skins bathed in amphibian Ringer's solution bound less ^{45}Ca at 6 hours than skins bathed in the tris buffer. However, this should be expected since the amphibian Ringer's solution with a higher ionic strength would decrease the activity of calcium. While the unphysiological characteristics of the tris buffer system might not support cellular activity as long as Ringer's solution, any loss of cellular viability should not have affected uptake since no difference in uptake was observed in skins which had been previously frozen.

Ethyl alcohol and formalin have been reported to shrink tissue and bind terminal amino residues, respectively (Urist and Adams, 1966). The effect of fixation with alcohol-formaldehyde was to increase considerably the uptake of ^{45}Ca (Table VIII) suggesting that the native organic matrix in skin may modulate the uptake of calcium by the calcification zone.

The ventral skins from late climactic tadpoles bound considerably less ^{45}Ca than skins at early and late prometamorphosis (Table VIII). The significance of this decrease in ^{45}Ca uptake is not apparent. However, the decreased ability of the skin to exchange ^{45}Ca may be related to some protective mechanism which inhibits calcium from being incorporated in the skin during a period in which the skeleton is rapidly developing.

B. The Influence of the Glycosaminoglycans on ^{45}Ca Uptake in *R. catesbeiana* Skin.

Introduction

The granular calcium phosphate deposits in the skin of *R. catesbeiana* are closely associated with an amorphous matrix (Taylor et al., 1966) which was observed to contain a glycosaminoglycan with histochemical properties similar to hyaluronic acid (Chapter 1). The function of the glycosaminoglycans in calcification systems has received considerable attention resulting in the contradictory reports of facilitation and inhibition of calcifying tissues. Since the role of glycosaminoglycans remains unsettled, the objective of this study was to determine how the glycosaminoglycans of *R. catesbeiana* skin influence the *in vitro* incorporation of ^{45}Ca into the calcification zone.

The facilitative role of glycosaminoglycans has been based on numerous reports. Pfaundler (1904)[1] observed that cartilage, which contains a high concentration of glycosaminoglycans, selectively bound calcium from a $CaCl_2$ solution but this was disputed by Wells (1906) who reported that the calcium loss from the $CaCl_2$ solution was due to dilution of the solution with the cartilage water. However, Rubin and Howard (1950) observed a close association in cartilage between its metachromatic staining (denoting glycosaminoglycans) and subsequent mineral deposition. Boyd and Neuman (1951) found

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[1]Cited by Schubert and Pras (1968).

that the cations bound by cartilage were equivalent to the sulfate of the tissue providing evidence that cation binding power is due mainly to chondroitin sulfate. Since hyaline cartilage failed to calcify, chondroitin sulfate from mineralizing tissues was suggested to be structurally different. Howard (1951) observed metachromatic staining prior to calcification in the transitional zone of cartilage and concluded that mucopolysaccharide is a basic component of most calcification systems.

Miller (1952) noted that metachromatic dyes inhibited cartilage calcification in vitro, a phenomenon which was reversed by calcium, phosphate, and Orange G. On the basis of these observations, he suggested that chondroitin sulfate was essential to calcification. Also, Sobel (1960) prevented calcification of cartilage by blocking chondroitin sulfate with toluidine blue, thus supporting the importance of chondroitin sulfates to mineralizing systems. Chondroitin sulfate, together with collagen, was thought to initiate deposition of calcium phosphate by formation of nucleation centers. This process was thought to be related to a rapid turnover rate of mucopolysaccharide which was observed in calcifying bone and cartilage. Autoradiographic studies showed a high sulfate-35 uptake at the forming edges of bone and in the calcifying regions of cartilage. In non-calcifying cartilage, the rate of synthesis was low when compared to calcifying cartilage although the actual amounts present in the two types of cartilage were similar.

Acid mucopolysaccharides were proposed by Berger and Eger

(1965) to be responsible for initial calcium binding at calcification sites. Thereafter, a depletion of the acid mucopolysaccharides releasing calcium and an increase in concentration of neutral mucopolysaccharides which bound phosphate was suggested. The calcium released from the degraded acid mucopolysaccharides was suggested to react with the phosphate to initiate the deposition of inorganic mineral.

In a study of mucopolysaccharides in cutaneous calcifications of humans, Johnson et al. (1964) observed that sulfated acid mucopolysaccharide was associated with calcified sites in basal cell carcinoma and adnexal carcinoma. In metastatic and dystrophic calcinosis cutis, epidermoid cysts, and pseudoxanthoma elasticum, the calcium deposits were associated with a non-sulfated acid mucopolysaccharide which contained hyaluronic acid in part. These authors suggested that mucopolysaccharide elaboration was an intermediate step in the pathogenesis of calcification and functioned because of the ability of mucopolysaccharide to bind iron initially which was subsequently replaced by calcium.

Other reports in the literature support the concept that mucopolysaccharides inhibit the calcification of tissues. Logan (1935) noted that chondroitin sulfate was lost during calcification of cartilage and speculated that this loss probably favored calcification. Hass (1943) concluded from his experiments that calcification of cartilage depended on the loss of chondroitin sulfate levels in the intercellular matrix. Sylven (1947) wrote that normal calcification was characterized by degeneration and digestion of cartilage and then replacement by bone. Gersh and Catchpole (1949) suggested

that calcification was due to depolymerization of the cartilage intercellular matrix.

Glimcher (1960) disagreed with the concept that ground substance components, alone or in combination with collagen, are important in initiating calcification. The amount of mucopolysaccharide in non-calcifying hyaline cartilage was found to be higher than in calcifying cartilage. The author stated that the types of mucopolysaccharide in calcifying tissues were unimportant because all the various acid mucopolysaccharides found in adult and growing bone were also present in non-calcifying tissues. Also, the physical state of the mucopolysaccharides was thought to limit the diffusion of ions into a calcifying zone. Finally, he felt the cation binding properties of mucopolysaccharides could selectively bind calcium, thus inhibiting nucleation within or on the collagen fibers.

Dulec (1960)[1] observed calcium, phosphate, and uronic acid concentrations in epiphyseal cartilage at several stages of calcification. Also, he measured the alkaline phosphate, ATPase, pyrophosphatase, phosphorylase, and other enzymes related to hexosamine formation. During the first stages of ossification, a decrease in uronic acid and an increase in phosphate occurred. In ossifying cartilage, less calcium was bound than in hyaline cartilage. Also, a decrease in chondroitin sulfate and an increase in phosphate transferring enzymes was observed. This author proposed that the breakdown

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[1]Cited by Bowness (1960).

and disappearance of chondroitin sulfate resulted in increased calcium activity facilitating calcification.

Detailed observations on the distribution of sulfate in endochondrial calcification systems were made by Weatherell and Weidman (1963) who reported that sulfate density was essentially the same in areas where calcification began as in non-calcifying hyaline cartilage. However, at the visible calcification front where cartilage was being replaced by osseous tissue, sulfate concentrations fell. Their evidence indicated that sulfated mucopolysaccharides disappeared shortly before calcification began.

Dunstone (1962) concluded from his studies that acid mucopolysaccharides bound cations in a rather non-specific way by electrostatic forces and such binding would not be expected to provide sites for crystal growth.

In order to determine how glycosaminoglycans influence the calcification system in R. catesbeiana skin, two experimental criteria were tested. First, ^{45}Ca uptake was observed in the presence and absence of toluidine blue. Toluidine blue or dimethyltoluthionine chloride, is a cationic dye which is used to stain connective tissue polysaccarides. Its metachromatic staining reaction is due to the formation of salts with polyanions in which the metachromatic dye is the counterion. Such salts have been isolated and found to contain equivalent amounts of dye and chromotrope (Schubert and Hamerman, 1968).

The rationale of this experiment was based on the assumption that toluidine blue would neutralize the major reactive properties of glycosaminoglycans, thereby, eliminating the effect of skin glycosaminoglycans on ^{45}Ca uptake. If the ^{45}Ca uptake was increased by toluidine blue, the skin glycosaminoglycans would be suggested to inhibit uptake. However, a decrease in ^{45}Ca uptake by toluidine blue would suggest a facilitative role for glycosaminoglycans.

Secondly, the correlation between ^{45}Ca uptake and the skin content of uronic acid (an indicator for glycosaminoglycans) was tested. If glycosaminoglycan inhibited uptake of ^{45}Ca, then a negative correlation might exist which should become less negative in skins treated with toluidine blue. However, a positive correlation which should become less positive after treatment would be predicted if glycosaminoglycans facilitated uptake of calcium into the calcification zone.

Materials and Methods

Twenty-four prometamorphic tadpoles were paired according to body lengths which were shown to be a reasonably good index of body skin calcium content (Chapter 3). Ventral skins from each pair were exposed to 0.25 μCi ^{45}Ca for 6 hours as previously described. One skin selected randomly from each pair was exposed to 0.005% toluidine blue in the serosal isotope solution. At 6 hours, each skin was rinsed with distilled water in order to remove excess buffer and circular areas of 2.6 cm^2 were removed from the central portion

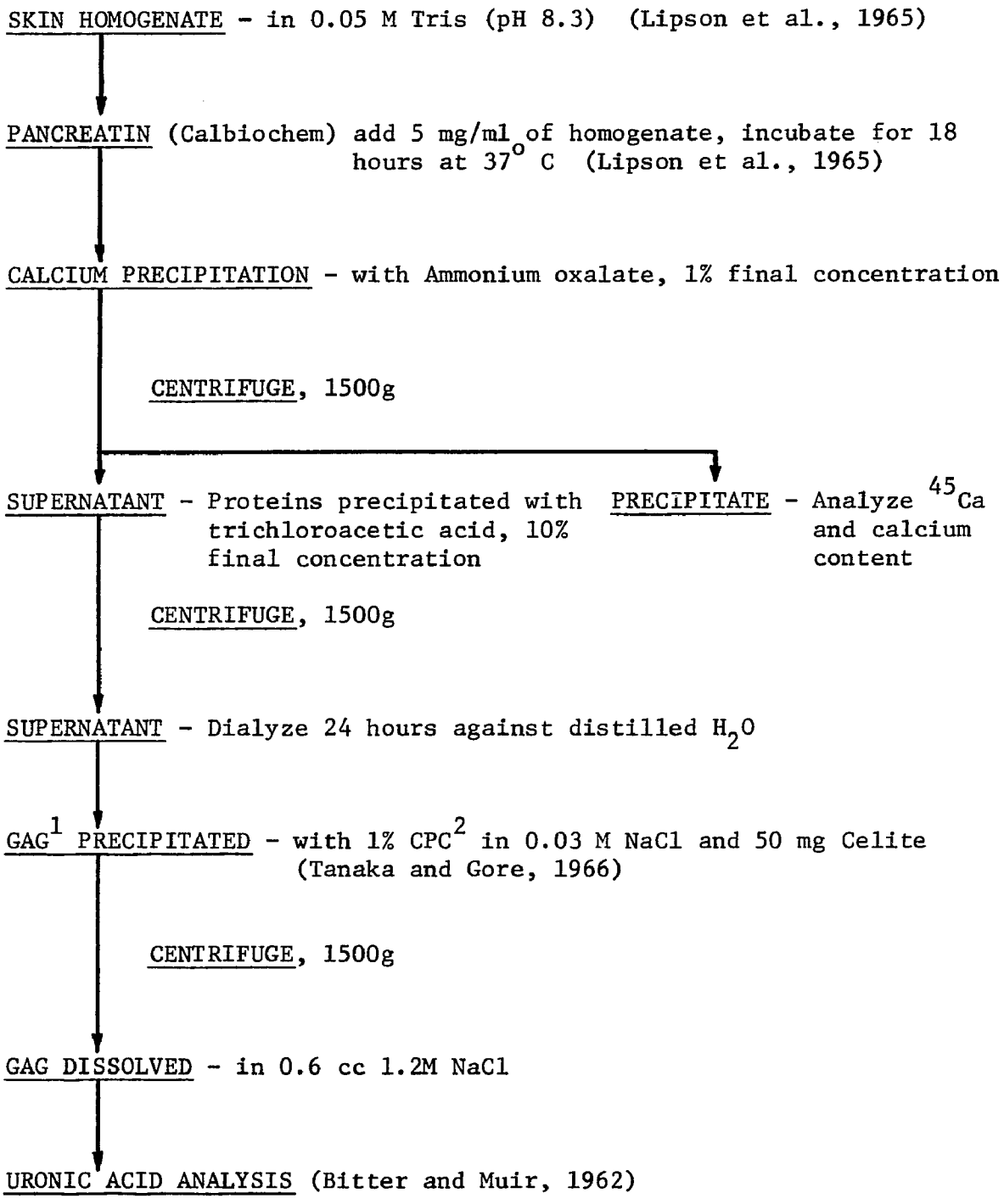
of each skin. After obtaining the wet weight, each standard area of skin was frozen until the analysis for calcium, ^{45}Ca, and uronic acid content could be conducted.

The twenty-four skin samples (12 controls, 12 treated with toluidine blue) were analyzed for calcium, ^{45}Ca, and uronic acid as depicted in Figure 25. Ammonium oxalate at a final concentration of 1%, while precipitating 98.5% of the calcium in the homogenate, did not interfere with the uronic acid analysis. Since only one aliquot for uronic acid per 2.6 cm^2 skin was possible, the variability of the uronic acid analysis had to be minimal. Testing eight replicate samples, the coefficient of variability was 8.3% for the uronic acid analysis.

Results

As shown in Table IX, control and experimental animal groups which were selected by pairing animals with similar body lengths had similar total lengths, wet weights and hind-leg lengths. Exposure of the toluidine blue did not significantly affect the water content of the skins and although there was considerable variability in the skin content of uronic acid and calcium, the mean values were not significantly different (Table X). In the presence of the above similarities between the control and experimental groups, analysis by the Student's t-test (for paired samples) indicated that ^{45}Ca uptake was significantly higher ($p<0.05$) in the toluidine blue-treated skins.

Figure 25. Outline of the Analysis of the Calcium, ^{45}Ca, and Uronic Acid in Rana catesbeiana Skin.



[1]Glycosaminoglycan
[2]Cetylpyridinium chloride

TABLE IX. DESCRIPTIVE CHARACTERISTICS OF THE EARLY PROMETAMORPHIC TADPOLES USED TO DETERMINE THE FUNCTION OF GLYCOSAMINOGLYCANS ON ^{45}Ca UPTAKE.

ANIMAL PAIR	BODY LENGTH (cm)		TOTAL LENGTH (cm)		WET WEIGHT (gm)		HIND-LEG LENGTH (cm)	
	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL
1	3.87	3.87	9.42	9.90	9.2	10.0	0.69	0.40
2	3.80	3.75	9.50	9.22	8.5	6.7	0.40	0.55
3	3.61	3.67	9.55	9.13	8.2	8.3	0.45	0.58
4	3.57	3.55	8.75	8.72	7.8	8.3	0.60	0.38
5	3.52	3.52	8.90	9.19	7.4	7.5	0.79	0.55
6	3.50	3.50	9.20	8.63	6.7	6.5	0.47	0.65
7	3.41	3.44	8.64	8.88	6.5	7.0	0.30	0.45
8	3.40	3.37	8.22	8.74	6.0	6.7	0.32	0.68
9	3.33	3.36	8.60	8.58	6.1	7.0	0.52	0.38
10	3.30	3.28	8.82	8.24	7.2	5.8	0.38	0.34
11	3.22	3.25	8.45	8.09	5.8	6.0	0.60	0.73
12	3.21	3.04	7.90	8.17	5.2	5.3	0.30	0.41
$\bar{x}$	3.48	3.47	8.83	8.79	7.1	7.1	0.49	0.51
$\bar{d}$ (Exp.-Cont.)	-0.01		-0.04		+0.04		+0.02	
S.E.($\bar{d}$)	±0.03		±0.11		±0.24		±0.06	

TABLE X. THE WET WEIGHT, SKIN CONTENT OF URONIC ACID AND CALCIUM, AND ^{45}Ca UPTAKE IN VENTRAL SKINS OF THE TADPOLES DESCRIBED IN TABLE IX.

Experimental skins treated with toluidine blue.

ANIMAL PAIR	WET WEIGHT (gm) 2.6 cm^2 SKIN		TOTAL URONIC ACID (µg) 2.6 cm^2 SKIN		TOTAL CALCIUM (mg) 2.6 cm^2 SKIN		^{45}Ca UPTAKE (%)	
	CONTROL	EXPERIMENTAL[1]	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL
1	0.1131	0.1065	9.37	10.51	2.60	3.31	13.05	16.31
2	0.0939	0.0867	4.73	9.99	1.53	2.43	10.67	9.44
3	0.0829	0.0850	9.45	11.42	2.27	2.20	11.21	11.86
4	0.0883	0.0824	7.75	4.73	2.27	2.25	11.33	11.03
5	0.0648	0.0790	8.72	2.82	1.76	2.89	7.71	12.04
6	0.0849	0.0896	7.91	11.35	1.97	2.40	9.79	13.34
7	0.0917	0.0897	7.75	6.42	2.44	2.55	9.89	13.73
8	0.0814	0.0868	9.85	10.41	2.26	1.92	8.65	13.00
9	0.0755	0.0761	10.51	8.34	1.55	1.88	9.57	10.12
10	0.0979	0.0694	8.82	6.39	2.11	1.75	12.17	14.28
11	0.0730	0.0863	4.15	10.48	2.37	1.92	13.80	13.45
12	0.0582	0.0631	6.29	6.65	2.35	1.70	11.86	10.23
$\bar{x}$	0.0838	0.0834	7.94	8.29	2.12	2.26	10.81	12.40
$\bar{d}$ (Exp.-Cont.)	-0.0004		+0.35		+0.14		+1.59*	
S.E.($\bar{d}$)	±0.0032		±1.03		±0.16		±0.64	

*$p<0.05$

The correlation coefficient between ^{45}Ca uptake and the skin content of uronic acid was -0.44 ($p = 0.148$) in the control group and +0.20 ($p = 0.534$) in the experimental group (Table XI). The probability that these coefficients were changed by the toluidine blue-treatment was only 0.85. The correlation between ^{45}Ca uptake and the skin content of calcium was +0.53 ($p = 0.072$) and +0.42 ($p = 0.169$) in the control and experimental groups, respectively. The lowest correlation was observed between skin content of calcium and uronic acid.

When a multiple regression for the control group was run with ^{45}Ca as the dependent variable and calcium, uronic acid, and wet skin weight as the independent variables, the multiple correlation coefficient was 0.744 ($p = 0.078$). A similar analysis for the experimental group produced a coefficient of 0.579 ($p = 0.327$).

In both regressions, a test of the sequential and partial sum of squares added little to the analysis:

1. For the control group, the sequential and partial sum of squares for calcium had a borderline significance ($p = 0.052$ and $p = 0.095$, respectively). The sequential and partial sum of squares for uronic acid also had a borderline significance ($p = 0.100$ and $p = 0.073$, respectively);
2. For the experimental group, none of the sequential or partial sums of squares was significant ($p = 0.179$).

TABLE XI. CORRELATION COEFFICIENTS BETWEEN THE ^{45}Ca UPTAKE, URONIC ACID, AND CALCIUM IN VENTRAL SKIN FROM RANA CATESBEIANA.

Correlation Coefficients are based on the data in Table X.

	CORRELATION COEFFICIENTS	
	CONTROL	EXPERIMENTAL
^{45}Ca UPTAKE vs URONIC ACID	-0.44	+0.20
^{45}Ca UPTAKE vs CALCIUM	+0.53	+0.42
URONIC ACID vs CALCIUM	-0.01	-0.04

(No significance at $p<0.05$)

Discussion

Based on the assumption that toluidine blue neutralized the reactive groups of glycosaminoglycans, the increase in ^{45}Ca uptake in toluidine blue-treated skins suggests that the glycosaminoglycans inhibit uptake in control skins. Also, the data suggest that a strong correlation does not exist between ^{45}Ca uptake and the skin content of uronic acid. Possibly, the content of glycosaminoglycan in areas other than the calcification zone is too great to allow correlations which reflect the true relationship between the glycosaminoglycans of the calcification zone and ^{45}Ca uptake. This was suggested by the very low correlation between the content of uronic acid and calcium in skin (Table XI). Therefore, while glycosaminoglycans appear to represent an inhibitory mechanism to calcium uptake, the overall importance of this mechanism will remain unclear until studies specifically determine the role of the glycosaminoglycans in the calcification zone.

However, a single inhibitory role of glycosaminoglycans is difficult to rationalize especially when other observations are reviewed. Both the initial formation of the granular deposits and the changes in calcium and phosphate content during maturation are associated with a matrix containing glycosaminoglycans (Chapter 1). Therefore, the glycosaminoglycans are intimately associated with systems both increasing and decreasing in the amount of calcium phosphate deposits. This apparent paradox may possibly be explained

on the basis of Bowness' (1966) proposal that the mucopolysaccharides may actually perform both functions of facilitation and inhibition of calcifying sites. Two compartments were proposed to exist; first, the chondroitin sulfate and fibers which are related to nucleation and, secondly, the chondroition sulfate in the "true" ground substance which is related to inhibition of calcification. In areas adjacent to active endochondral calcification (and possibly other calcifying tissues) a rapid synthesis and high concentration of sulfated mucopolysaccharide combined with non-collagenous protein was observed. These intercellular protein-polysaccharides tend to overlap and form watery domains which will accumulate calcium in preference to magnesium, sodium and potassium, will contain a lower concentration of inorganic phosphate than average for the tissue, and will retard distribution of water and dissolved solutes. Calcium activity is decreased by interaction with chondroitin sulfates and largely separated from the phosphate compartment. Therefore, calcium phosphate does not precipitate. Precipitation of calcium and phosphate was proposed to depend on one of two possible mechanisms which may or may not operate together. Interaction of collagen fibers (binding the phosphate groups) and the soluble protein-polysaccharide could promote a precipitation reaction. Also, catabolism of protein-polysaccharides by enzymes could destroy macromolecular domains and decrease the effect of phosphate exclusion and calcium binding, thus facilitating precipitation.

Although the above model system has not been proven conclusively, the function of the glycosaminoglycans in the calcification zone of R. catesbeiana demands further consideration of Bowness' proposal. Tadpole skin has been reported to contain hyaluronidase (Lipson et al., 1964) which may differentially influence the state of polymerization of the glycosaminoglycans during maturation, thereby affecting the calcification system in the skin. Therefore, studies determining turnover of the glycosaminoglycan during maturation would be of considerable interest.

Finally, the mechanisms which limit the size of the calcium phosphate deposits and which prevent granular coalescence represent another consideration for glycosaminoglycan function. Early calcifying systems with small granular deposits are also associated with a matrix rich in glycosaminoglycans (Bernard and Pease, 1969). However, as these granules grow and unite to form bone the quantity of glycosaminoglycans is reduced. In the skin calcification system, the granular mineral deposits, which closely resemble the initial foci of calcification in bone and dentin, are continually associated with glycosaminoglycans which may limit their growth and prevent coalescence.

CHAPTER 6

THE CONCENTRATION OF CALCIUM AND PHOSPHATE IN *RANA CATESBEIANA* PLASMA

Introduction

Neuman and Neuman (1958) reported that the serum phosphate concentration in newborn children was about 2 fold higher than that of adults. However, serum calcium showed little variation with age and metabolic state. This elevation of plasma phosphate is thought to be important for skeletal development (Travis and Neuman, 1964; MacGregor and Brown, 1965). Since changes in plasma phosphate may occur during maturation in *R. catesbeiana* and, if so, may affect the skin calcification system, an experiment was designed to measure the plasma calcium and phosphate levels at three developmental stages.

Materials and Methods

Whole blood was collected in heparinized microhematocrit tubes from the aortas of 10 early prometamorphic and 10 late climactic tadpoles. Following centrifugation, each plasma sample was measured volumetrically and then ashed at 600° C. After reconstitution of each sample in HCl as described in Chapter 2, the calcium and inorganic phosphate were measured.

Since the individual plasma samples from late climactic tadpoles were too small to allow both calcium and phosphate determinations, whole blood was collected as described above from 20 tadpoles at late climax. The plasma was pooled and analyzed for calcium and inorganic phosphate as described above.

Finally, whole blood was collected in heparinized syringes from 5 large adult frogs (420 - 470 gms). After centrifugation, the plasma samples were analyzed directly for calcium according to Bachra et al. (1958). The proteins in the plasma samples were precipitated with 10% trichloroacetic acid and the supernatant was analyzed for phosphate (Bonting et al., 1961).

Although the plasma from adult frogs was analyzed differently from that of tadpoles this procedural change should not have affected the accuracy of the plasma calcium and phosphate determinations (Bachra et al., 1958; Taylor et al., 1966).

All plasma samples were collected during the summer months.

Results

As shown in Table XII, the concentration of plasma calcium (approximately 2.0 mM/l) did not appear to vary with the maturation stage of R. catesbeiana. Also, as measured at early prometamorphosis and in adult frogs, the variability of plasma calcium was low. In contrast, both the concentration and variability of plasma phosphate appeared to be influenced considerably by maturation. Relative to the values measured in plasma from early prometamorphic tadpoles, the

TABLE XII. THE CONCENTRATION OF CALCIUM AND PHOSPHATE IN *R. CATESBEIANA* PLASMA.

ANIMAL	n[1]	CALCIUM mM/liter	PHOSPHATE mM/liter
EARLY PROMETAMORPHIC TADPOLES	10	1.9 ± 0.1[2]	2.9 ± 0.7
LATE CLIMACTIC TADPOLES	10		5.3 ± 1.3
LATE CLIMACTIC TADPOLES	(pooled)	2.1	5.0
ADULT FROGS	5	2.0 ± 0.1	1.5 ± 0.2

[1]Number of animals
[2]Mean ± standard deviation

plasma phosphate was 85% higher at late climax, and 50% lower in adult frogs. The variability of plasma phosphate in tadpoles averaged about 23.5% compared to 14.4% in adult frogs.

Discussion

While Waggener (1903) observed that the concentration of calcium in the blood of R. catesbeiana frogs was approximately 2.9 mM/1, Spector (1956) reported the plasma concentration of calcium to be only 1.6 mM/1. The measurements described herein show the plasma calcium of both larval and adult bullfrogs to be approximately 2 mM/1 with little, if any, variation during maturation. However, reductions in the plasma calcium concentration have been reported to occur simultaneously with seasonal cytolysis and regeneration of the amphibian parathyroid (Simkiss, 1967). In R. catesbeiana, vacuolation and breakdown of these cells starts in February and regeneration is completed in May. Therefore, apparent differences in the reported values of plasma calcium in R. catesbeiana may be related, in part, to seasonal variation.

The functional significance of the skin mineral in adult frogs may be related to the regulation of plasma calcium during the seasonal cytolysis of amphibian parathyroid glands. Although complete parathyroidectomy of adult R. catesbeiana results in death (Waggener, 1930), parathyroidectomy of R. ridibunda caused no ill effects (Studitsky, 1945)[1]. A study designed to correlate the skin

[1]Cited by Simkiss (1967).

calcium content with seasonal parathyroid cytolysis and with the effects of parathyroidectomy would be of considerable interest.

As observed in other immature vertebrates, larval R. catesbeiana showed a higher plasma phosphate than adults. Moreover, during late climax the plasma phosphate increased 85% above early prometamorphic concentrations. Since the content of calcium and phosphate in skin at late climax was not reduced but sometimes was slightly elevated (Chapter 2), the highly elevated plasma phosphate at late climax may be related to the dissolution of calcium phosphate deposits and organic phosphate in the resorbing tail (although unpublished, a thin layer of basophilic granules has been observed in the skin of the tadpole tail). This along with utilization of calcium from the endolymphatic sacs (Pilkington and Simkiss, 1966) would create a more favorable environment for skeletal ossification. Therefore, the calcium phosphate stored in the body skin of R. catesbeiana tadpoles may not be required during climax. However, after the reservoirs of calcium and phosphate in the tail and the endolymphatic sacs are depleted, the skin mineral may be required for continual calcification and growth of the skeleton at a time when the animals' feeding and life habits are changing.

The increased concentration of plasma phosphate at late climax without a change in plasma calcium also raises the question of how hypercalcification of the skin is prevented. Ventral skin from late climactic tadpoles bound 35% less ^{45}Ca than skins at early

prometamorphosis suggesting an inhibitory mechanism controlling calcium incorporation into the skin (Chapter 5). The increased uptake of ^{45}Ca by toluidine blue-treated skin suggests that the skin glycosaminoglycans may represent one such inhibitory mechanism. Interestingly, Bowness (1966) reported that glycosaminoglycans inhibited calcification by accumulating calcium and excluding phosphate. As shown in Chapter 2, the mean calcium content in ventral skin at late climax was increased 36% whereas phosphate was increased only 14% above values seen in early prometamorphosis. Also, the mean "concentration" of calcium in ventral skin at late climax increased 18% above that at early prometamorphosis in contrast to a slight decrease in phosphate "concentration". Although the above observations do not prove conclusively that an increased skin content of glycosaminoglycan at late climax prevents hypercalcification of the skin, this correlative evidence does permit a basis from which to study the metabolism and content of glycosaminoglycans during maturation.

SECTION III

CONCLUSIONS

1. The granular calcium phosphate deposits of R. catesbeiana skin appeared to develop at the periphery of the basement lamella when this latter structure began to differentiate in the stratum compactum.
2. The granular calcium phosphate deposits were associated with an unsulfated glycosaminoglycan which was digested by testicular hyaluronidase but not bacterial hyaluronidase. Despite the latter, the data suggest that the glycosaminoglycan is hyaluronic acid.
3. Although the thickness of the ventral calcification zone in adult frog skin appeared to be one-half the thickness of that in skin from early prometamorphic tadpoles, equal amounts of calcium as well as phosphate per unit area were observed in skin from early prometamorphic tadpoles and adult frogs.
4. Although the thickness of the dorsal calcification zone in skin from the early prometamorphic tadpole was similar to that from the adult frog, the adult dorsal skin contained about 3 times more calcium and phosphate.
5. Based on the observations summarized in 3 and 4, the adult skin had an increased ability to form or a decreased ability to mobilize the calcium phosphate deposits.
6. The dorsal and ventral calcium phosphate deposits were reduced considerably in yearling frogs without a concomitant decrease

in organic skin weight. This observation supports indirectly the proposal that the skin calcification system functions as a reservoir for calcium and phosphate.

7. The body skin of early prometamorphic tadpoles, which represented about 9% of the animal's total wet weight, contained approximately 26% and 29% of the tadpoles' total calcium and phosphate, respectively.
8. The isolated body skin mineral from tadpoles and frogs had a low Ca/P molar ratio of 1.33 but produced an apatitic x-ray diffraction pattern similar to that of bone mineral and tooth enamel.
9. The calcification zone in ventral skin specifically bound ^{45}Ca *in vitro*.
10. Destruction of cellular viability by freezing and thawing the skin did not significantly influence the *in vitro* uptake (and presumably binding) of ^{45}Ca. However, alteration of the matrix by fixation in alcohol-formalin resulted in an increase in ^{45}Ca uptake.
11. Ventral skin from late climactic tadpoles bound about 35% less ^{45}Ca than early prometamorphic skin suggesting that a protective mechanism exists which inhibits calcium uptake during a period of development when the plasma phosphate was considerably elevated and plasma calcium was unchanged or slightly elevated.
12. The *in vitro* uptake of ^{45}Ca was increased by toluidine blue which suggested that the skin glycosaminoglycans may function to inhibit the incorporation of calcium into the calcification

zone.

13. Since the granular calcium phosphate deposits closely resemble (morphologically, chemically, and physically) other newly calcifying deposits but do not coalesce to form sheets of mature bone, the skin calcification system represents a unique model from which the nature of the initial form of calcium phosphate precipitated *in vivo* can be studied.

DISCUSSION

As previously mentioned in the introduction, the basic understanding of mineralizing systems will continue to be strengthened through studies of various calcifying tissues by revealing commonly shared principles or basic mechanisms. The results of this investigation indicated that the granular calcium phosphate deposits in R. catesbeiana skin share several characteristic properties with newly mineralizing bone.

For example, Bernard and Pease (1969) and Bernard (1969) reported that in mouse bone the nucleation sites of hydroxyapatite crystals were osteoblastic "extrusions" or "buds" which were observed in the osteoid (principally composed of collagen and glycosaminoglycan). These buds were observed among the newly formed fibrils and possessed, in part, polysaccharide cores. While migrating through the osteoid and away from the osteoblast, the progressive growth of well-defined hydroxyapatite crystals within these osteoblastic buds formed "bone nodules" which grew from about 0.15 μ to 0.75 μ in diameter. As these bone nodules grew, the density of the polysaccharide decreased in the core of the granules but increased at its periphery. After completing migration through the osteoid, the bone nodules coalesced forming seams of bone.

Also, Anderson et al. (1970) observed and isolated "matrix vesicles" in the epiphyseal plates of mice. These vesicles

were observed in the longitudinal septa (rich in glycosaminoglycans) and were most numerous in the longitudinal septa of the hypertrophic zone. The earliest detectable deposits of apatite were seen in these matrix vesicles as electron-dense, needle-like shapes estimated to be 40 Å wide and approximately 1000 Å long. These vesicles which contained the newly formed hydroxyapatite also possessed significant alkaline phosphatase and ATPase activity, but low acid phosphatase activity.

The osteoblastic buds and matrix vesicles which grow into calcium phosphate granules of limited size exist in an environment rich in glycosaminoglycan -- a condition shared by the granules of R. catesbeiana skin. Hence, this common observation may have important implications concerning the role of glycosaminoglycans as facilitators or inhibitors of calcification.

Although the mineral granules of bone and R. catesbeiana skin are both closely associated with glycosaminoglycans, the specific glycosaminoglycan types are dissimilar. Chondroitin sulfate is generally associated with the matrix of calcifying bone (McLean and Urist, 1968) whereas a component histochemically similar to hyaluronic acid was observed in the skin calcification zone of R. catesbeiana. However, since both chondroitin sulfate and hyaluronic acid are found also in non-calcifying tissues, the specificity of these glycosaminoglycans does not appear to be the major factor determining whether or not a tissue will calcify.

Morphologically and chemically, the inorganic calcium phosphate deposits of newly calcifying tissues are quite similar to those of bullfrog skin. As reported by Taylor et al. (1966), the skin granules were 0.1 to 1.0 μ in diameter which closely corresponded to the bone nodules (0.15 μ to 0.75 μ) described by Bernard and Pease. The low Ca/P molar ratio and the apatitic x-ray diffraction pattern of the skin granules are also characteristic of other newly calcifying tissues (Brown, 1966; Urist and Dowell, 1967).

Another characteristic commonly shared by the skin and bone granules is that neither is closely associated with newly-forming collagen (Taylor et al., 1966; Bernard and Pease, 1969). The results of this dissertation support the observation of Taylor et al. (1966) by noting that the areas containing the most densely packed granules were relatively free of fibrils. Therefore, these common observations do not support the hypothesis that collagen is the site of bone-crystal nucleation (Glimcher, 1960).

The apparent non-specificity of glycosaminoglycans in calcification systems as well as the observation that calcification loci are cellular derivatives and are not closely associated with collagen fibers give support to Pautard's (1966) cellular-nucleation hypothesis. An electron microscope study designed to observe whether the skin granules are also derivatives of cellular calcification loci would be of considerable interest. Perhaps such a study

would support further Pautard's proposal that "mineralization is basically an intracellular event" in which "the cell creates the the image of the mineral".

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