A CHEMICAL, RADIOGRAPHIC AND HISTOLOGICAL ANALYSIS OF SKELETAL GROWTH IN THE NORMAL RHESUS (Macaca mulatta) FETUS

by

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A THESIS

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

A. Introductory Remarks

The investigation herein described represents an analysis of certain aspects of the dynamics of skeletal growth, differentiation and internal metabolism in the Rhesus monkey (Macaca mulatta) observed during critical periods of fetal life. Data presented in this study are analyzed in the light of current concepts of bone structure and metabolism which are briefly reviewed in this initial section.

For most of man's history bones were thought of as essentially little more than "biologic struts." The ancient Biblical story of the bones being revived and reassembled to form living men (Ezekiel 37) heralds a connotation long since held by many scientists, that bone was essentially a dead supporting structure for the vertebrate body. Bone was a structure that one could cut, shape, and treat as inanimate building material. Most recent monographs refer to Frey, a histologist of the last century, for his summary on what was, until recently, thought to be the function of bone: "Owing to their hardness and solidarity, the bones are peculiarly well adapted for the mechanical construction of the body. . . . They serve to protect internal organs, and form systems of levers." But Frey goes on to say "the bones

take part also, to a great extent, in the chemical occurrences of the organism, owing to the lively interchange of matter going on in them."(11) Such a statement is, in fact, the modern outlook on bone.

Investigators in the last half century have elucidated and defined the living participation of the skeleton in many vital processes. New biochemical, morphological, and biophysical techniques have allowed better identification of cellular and subcellular constituents. Knowledge has advanced in many areas with each new experimental parameter tending to precipitate accelerated use of others. A decided impetus to investigate the skeleton has been given by bone-seeking nuclides.

B. Some Current Concepts of Bone Metabolism

1. Bone Solubility

It is impossible to isolate one facet of the metabolism of the skeleton from another. The structure of bone, its formation, reactivity, and response to physiologic regulators are all interdependent events occurring in relation to the total body metabolism. Fundamental to ideas regarding the participation of the skeleton in body processes is the relationship of bone to its surrounding fluids. Only recently has a unified concept of the solubility of bone mineral in the body rested on a framework of

substantial experimental data.[50] This concept developed by Neuman and Neuman is formulated in terms of activities of ions, rather than ion concentrations. If such correction factors are not used in the case of $CaHPO_4 \cdot 2H_2O$, its solubility at physiological ionic strength is stated to be in error by over 1200 per cent.[50] The activity product, a Ca^{++} x a $HPO_4^{=}$, of normal serum at physiological conditions has been calculated to be about 1×10^{-7}.[69] Neuman and Neuman have shown that this mean value is both undersaturated and supersaturated at one and the same time. Such a paradox is resolved in terms of two different mineral solids. The solid phase initially precipitating is secondary calcium phosphate, a solid undersaturated with respect to serum. The only solid phase stable at physiological pH is hydroxyapatite, a solid supersaturated with respect to serum.[36] Bone mineral does not exhibit a fixed solubility product and is greatly influenced in its solubility by the presence of other ions in the body fluids.[36] Serum is thus postulated to be supersaturated in bone and in areas of forming bone, but undersaturated in areas of resorption of bone.[46] Such variable solubility is probably dependent on local changes of carbonate and hydrogen ion and citrate concentration. Cellular mechanisms in bone are thought to be responsible for the maintenance of these variable

differences in ion concentration and are likely under control of the parathyroid hormone and other unknowns. (The role of parathyroid hormone will be discussed in a following subsection.)

2. Bone Structure

Roentgen-ray diffraction studies, as early as 1926, showed that the crystals of bone were similar to apatite minerals. The subject has since been constantly investigated and there have been various divergent views as to ionic relationships in the bone crystal; the problem remains unresolved.[15,18] The apatite lattice is not a compound; rather, it is an arrangement in space of the various ions contained in the mineral.[43] The theoretical molar ratio of calcium/phosphorus should be 1.67; the composition of most hydroxyapatite preparations does not agree with the above prediction, though they give the same x-ray diffraction pattern. It is mainly this divergence between predicted and observed composition that has resulted in several names and formulas for bone mineral. Most workers use $Ca_{10}(PO_4)_6(OH)_2$ as the general formula for the hydroxyapatite unit cell.[50]

3. Surface Exchange

Exchange of ionic elements on the bone crystal surface is an important cause for the variable composition of

bone mineral.(50) The crystalline surface is large; values range from ten to more than two hundred square meters per gram wet ashed bone.(50) X-ray diffraction and electron microscopic studies have shown the size of the crystals to be 200 by 30-70 Angstrom units.(60) The apatite bone crystals are thus small and have a large surface area. In addition, strong asymmetric electric fields exist at the crystal surface, probably accounting for the fact that these crystals can bind a large volume of water (1.9 times their own volume); therefore, bound ion layers at the surface acquire an insulating hydration shell.(50)

The exchange of ions between the surrounding solution and hydration shell is extremely rapid. Exchange between ions in the hydration shell and the crystal surface is less rapid, being dependent on the ion in question and the rate at which the surface ion diffuses out from its lattice position. Though the calcium and phosphate ions are of primary importance, a wide variety of heterions are able to substitute for calcium or phosphate on the crystal surface.(50) This permits the hydroxyapatite crystals to contain variable ratios of ions and to "mirror," to a certain extent, the composition of fluids with which they are in contact.(49) Intracrystalline exchange also occurs; this process is slow and depends to some extent on lattice

defects.(47) Strontium^{90} can partake in intracrystalline exchange.(12)

Qualitative features of ion exchange and fixation have been elucidated using radioactive isotopes. Quantitation has not been possible because of the many simultaneous events occurring at varying rates from crystal to crystal.(50) Some idea of the complexity of these skeletal exchange mechanisms and their modifying influences may be obtained from a listing of phenomena that are known to influence an ion entering or leaving the mineral phase. The list presented is modified from the literature.(50)

I. Physiochemical mechanisms:
   a. New crystal formation: the formation of new crystals in organic matrix.
   b. Recrystallization of recently deposited crystals.
   c. Surface exchange: exchange for an unlabeled ion on a pre-existing crystal surface.
   d. Intracrystalline exchange: diffusion of an exchanged surface ion into the crystal interior.
   e. Crystal growth: continuing growth of crystals in established calcified structures.

II. Modifying influences:
   a. Matrix formation: synthesis of new endochondral

cartilage and osteoid in growth and remodeling.

b. Resorption of previously calcified bone and cartilage in the growth and remodeling of bone.

c. Maturity-maturation: age of the structural element influences the rates of diffusion and recrystallization, the extent of intracrystalline exchange and growth.

d. Nutritional and endocrine influences, including parathyroid hormone, vitamin D, diet, etc.

e. Motion and trauma.

4. The Skeleton as an Important Physiologic Reservoir

Radioisotope studies have helped to identify the importance of bone as a major electrolyte reservoir for the body and its particular importance in disorders of hydrogen ion concentration.(50) Besides calcium and phosphate, the skeleton's role in buffer processes includes provision of H_3O^+, Na^+, Mg^{++}, $CO_3^=$ and citrate. The extent to which ions of the bone mineral are able to exchange with body fluid constituents has been studied largely with sodium^{22}. The availability of bone sodium is relatively reduced as age and maturation progress. Some 40% of the sodium in bone is available in the adult rat.(25) The availability of skeletal ions is probably also influenced by many other factors, including parathyroid hormone.

The effect of maturation of the skeleton poses a most difficult problem for the radiation biologist, for maturation is largely responsible in making "available" osteons "unavailable" as new crystal growth progresses. For example, over 90% of a calcium^{45} intraperitoneal injection is retained in the skeleton of a normal immature rat.(58) After a period of time this radioisotope resides almost wholly in the stable mineral fraction of the skeleton and is unavailable to the body fluids.(63) Bone seeking isotopes thus incorporated remain inescapably fixed.

5. Physiological Regulators

a. Parathyroid Hormone

Parathyroid hormone appears to have a unique and direct effect on the skeleton.(43) Through its action on the skeleton, parathyroid hormone has a significant role in regulating calcium ion concentration in the blood. Experimental aberrations and clinical disease of this gland produce certain well-known effects in animals and man:(50)

(a) Removal of the parathyroid glands causes a sharp drop in calcium concentration in serum from a usual value of 10 mg. % to 6 to 7 mg. %; such values are frequently accompanied by tetany. Thereafter, skeletal mineral and other

unknown factors maintain the serum calcium at this level.

(b) An excess of the parathyroid hormone leads to an increase in the calcium ion concentration of the blood and may induce profound morphological and chemical changes in the skeleton.

Conflicting concepts as to the primary mode of action of parathyroid hormone have traditionally been bifurcated between a primary bone effect and a primary kidney effect. Although the precise chemical nature of the active principle is still unknown, experimental evidence now seems to give both sites a primary role. The biochemical events by which the hormone mediates its effects are unknown, though it appears to reside in certain pathways of intermediary metabolism. Recent work has pointed to a direct effect of the hormone on the cell processes of bone itself, since part of the skeleton not bathed by the body fluids is liberated when the hormone is administered exogenously. Such a direct effect on the bone cell may be closely related to the production of citrate. Great increases in citrate output from bone have been observed almost immediately following the injection of parathyroid extract.(48) Sixty to eighty per cent of the body's citrate is found

in the skeleton.[17] Its source, however, has not been proven to be bone. Citrate is known to solubilize hydroxyapatite by surface exchange, to be an efficient chelator of calcium, to lower pH, and to be rapidly oxidized by the kidney and other tissues.[50] Clinically, serum citrate is high in hyperparathyroidism and low in hypoparathyroidism.[50] Dixon and Perkins have found that mature bone has greatly reduced amounts of isocitric dehydrogenase in relation to the condensing enzyme and aconitase.[17] Isocitric dehydrogenase is needed for efficient citrate utilization in the Krebs cycle. These findings have been interpreted to support the concept that parathyroid secretion induces bone cells to produce citrate which brings about an efficient bone-blood transfer, thus maintaining the supersaturated state of the serum and preventing spontaneous growth of the bone crystals.

b. Vitamin D

Much evidence now points to the existence of a direct effect of vitamin D on the skeleton, as well as its long-known effects on the gut absorption of calcium. Like parathyroid hormone, its mode of action is unknown; recent studies also suggest that its effect involves intermediary metabolism. For example, citrate levels

have been shown to be low in many organs in the rat during vitamin D deficiency, and to increase after administration of calciferol.(68) In prolonged vitamin D deficiency, cartilage is unable to oxidize pyruvic acid as substrate in Warburg studies.(75) (Such an effect could also be secondary to an occult hyperparathyroidism.) Zetterstrom and Ljunggren have shown that phosphorylated vitamin D_2 activates kidney motochondria, metabolizing glutamate as substrate.(82) Most current hypotheses suggest that vitamin D mobilizes calcium from bone by an alteration in carbohydrate metabolism that somehow also affects citrate. Thus, parathyroid hormone and vitamin D are thought to act synergistically on the skeleton, but at different points in the cycles of intermediary metabolism.(44) It has long been well known that the activity of these regulators is not identical. One cannot substitute for the other. Such a tentative scheme provides a valuable framework for future investigation in this field even though it leaves many questions unanswered.

6. Process of Calcification

The most critical problem of calcification is its initiation. Calcification has been studied in many ways for many years.(18) Most studies have been done in vitro on cartilage slices. Many techniques have been so unphysiological

that the applicability of data to *in vivo* conditions remains doubtful.(44) Robison's finding in 1923 of the enzyme alkaline phosphatase in areas of forming bone focused attention on the mechanics of the calcification process. He theorized that the enzyme simply liberates phosphate ions from organic combination to precipitate with calcium. This theory was found untenable and Robison himself later abandoned it. Despite this, one still finds this concept in the medical literature, even though "its ghost has been properly laid to rest" by current investigators.(50)

The concept that alkaline phosphatase has some close connection with calcification has been broadened to include the glycolytic cycle. Many of the enzymes and intermediates of the glycogen cycle have been shown to exist in calcifiable cartilage.(2) The identification of an intermediate in this cycle that would be the substrate for alkaline phosphatase has been unrewarding.(43)

It has also been claimed that alkaline phosphatase has to do with the property of calcifiability rather than calcification per se; i.e., it has to do with the production of a calcifiable protein matrix.

ATP is also thought to be intimately associated with calcification. This consideration has been strengthened by the demonstration of pyrophosphate as a constituent of normal

bone.[54] The role of ATP suggested is that of transferring pyrophosphate to some component of the organic matrix with a transference mechanism.[44] The glycolytic cycle would then function to syntheisze ATP.

The observation by the electron microscope of a close, well-oriented relationship of the bone mineral with its osteoid matrix has suggested a role for collagen as the initiator of the mineralization process. Crystals are seen to be oriented in the direction of the long axis of the fiber.[60] Crystals have been reported inside the collagen fibrils as well.[28] Several proposals have been offered which imply a template property in the collagen structure for nucleation and growth of the hydroxyapatite crystals.[44] Terms such as epitaxy and crystal seeding have been used to describe this concept. A recent study by Glimcher is of interest in this regard.[28] He dissolved the collagen fibers from rat tail tendon using neutral buffers and weak acids and subsequently reaggregated the macromolecules into native-like fibrils with typical axial repeat and intra-period find structure. He was able to induce the formation of apatite crystals in these reconstituted fibrils. He next prepared other reconstituted fibrils with different axial periods, and some fibrils with no periodicity. He could not induce mineralization in such preparations. Such a

finding indicates that mineralization is dependent on the grouping and configuration of the fibril and not on an intrinsic property of the tropocollagen macromolecules.

From his electron microscopic studies of *in vivo* calcification of embryonic bone, Glimcher claims that mineral salts are first deposited in a regular fashion along the fibrils. These areas he terms nucleation centers. Such centers have not been accurately identified with respect to the intraperiod fine structure of the collagen fibrils, but seem to correspond to certain of the band (electron dense) areas. Amino acids with long polar side chains are postulated to reside in these regions.(28)

In this regard, Solomons and Irving have been able to distinguish the collagen of soft and mineralized tissue by their respective content of Σ-amino groups.(67) Progressive demineralization of dentine and bone increased the content of Σ-amino groups of lysine and hydroxylysine in collagen. These amino acids may have an important chemical relation to mineral salt bonding.

The collagen of bone has been difficult to study, but has characteristics in common with those of other forms of connective tissue.(44) Why mineralization occurs only in selected locations, and why most of the collagen goes through life uncalcified are questions which are at present unanswered.

Ground substance with its content of polysaccharides containing hexosamines exists in bone. Its role is unknown, though it has been postulated to be critical both to the formation of mineral salts and to protect the collagen from mineralization.(66,28)

Some unanswered questions necessary for a more exact definition of the mineralizing system are as follows:(66)

(1) What is the chemical potential of mineral formation?

(2) What groups in the osteoid matrix participate in the capture of calcium and phosphate ions?

(3) What is the role of the mucopolysaccharides or mucoproteins?

(4) What is the degree of specificity and selectivity of the mineralizing mechanism?

(5) What are the circumstances that bring about mineralization at abnormal sites in the body?

This review of current concepts and problems of calcification could be greatly extended. It does reveal that from the physical to the physiological, bone is an active, complex participant in the total body processes of growth, development and homeostasis. An integrated application of many disciplines and techniques is undoubtedly the key to a more erudite elucidation of exact mechanisms and relationships.

C. Study Plan

The purpose of this study is to define normal skeletal growth and development in the Rhesus monkey (Macaca mulatta) fetus from 75 through 150 days conceptional age by chemical, radiographic and histological parameters.

Fetuses were removed from their mothers by cesarean section at conceptional ages of 75, 90, 105, 120 and 150 days. Analyses were performed on selected bone samples as well as the total skeleton.

D. Justification for the Animal Used

The present study is introductory to a long range investigation of skeletal metabolism in the immature Rhesus monkey (Macaca mulatta). Metabolic studies performed with this animal have been few, but lesions induced and data obtained from such studies have demonstrated a great similarity to the human. For example, studies in this laboratory have revealed a distinct morphological and metabolic lesion in the Rhesus monkey on pure vitamin D deficiency closely resembling its human counterpart.(55) In contrast, skeletal studies with vitamin D deficiency in the rat have had dubious applicability to man since the rat does not demonstrate gross ricketic features on a pure vitamin D-deficient diet, but requires a markedly distorted calcium to phosphorus ratio to effect such changes.(81,23) Moreover, thyroid ablation at birth in the

Rhesus monkey results in a picture of cretinism that mimics the clinical and pathological features of this disease in the human.(56) Additional advantages include the adequacy of tissue from a single specimen for detailed studies using a variety of techniques, the ease with which the monkey's diet is standardized and controlled, and the accuracy of determining age from the date of conception.(26) Such facts permit a persual of an integrated approach on a standardized preparation that is neither desirable with man or possible with smaller mammals.

A discoid placenta identical in structure to that of the human nourishes the Rhesus monkey fetus for a normal gestational period of 168 days.(19) The ages selected for study in this investigation cover the period from early fetal life to viability, and have been selected with care to elicit distinct changes in growth and differentiation using a relatively small number of animals.

E. Justification of Methods Used

Several parameters of estimating skeletal growth and differentiation utilized in this study have been applied in an attempt to define as completely as possible the growth dynamics of the fetal skeleton. The tibia has been selected for detailed regional analysis, being sectioned into nine separate regions as shown in Figure one. The tibia has

frequently been used in other studies as a representative long bone.[14,58]

The division of the tibia into separate regions was undertaken in an attempt to define and distinguish the composition and growth of morphologically distinct areas within a long bone. The morphology of metaphyseal bone formation in which delicate trabeculae are laid down on a core of calcified cartilage matrix is distinctly different from bone which forms under the periosteum and is rapidly developed into Haversian systems.[42] The bone formed by virtue of these two morphological patterns is analyzed chemically in a separate fashion in the present study. Another consideration involves different growth rates in different areas. The turnover of bone in the metaphysis is much more rapid than that in the shaft.[6] Growth of a long bone is a coordinated pattern of growth in different areas, each area at any one time proceeding at an intrinsic rate.

Thus, the division of the tibia into separate areas was based on morphological features of bone formation and differentiation, such that interpretations of chemical and growth data would have an anatomical basis.

II. METHODS AND MATERIALS

A. Adult Animals, Their Environment and Daily Care

The adult animals used in this study were disease-free and maintained on the standard regimen of the colony for a minimum of four months before entering the study. All animals were kept indoors in separate cages in a room maintained at 75° F. ± 2° the year around. Cages were cleaned daily. Each animal was weighed at monthly intervals.

The maternal diet consisted of three main items: (1) monkey biscuit,(59) (2) reconstituted milk formula, and (3) fresh banana. From this diet, each adult animal was offered daily approximately 23 gms. of protein (including all essential amino acids), 85 gms. of carbohydrate and 22 gms. of fat, with a complete source of vitamins and minerals. The daily calcium intake from this diet was calculated to be 1.3 gms. and the phosphorus intake 0.7 gms., with a calcium/phosphorus ratio of 1.9. The daily intake of vitamin D was approximately 400 USP units. A detailed breakdown of the constituents in each food item is listed in the appendix.

B. Breeding Procedure(76)

Ten days after the first day of a female animal's menstrual period she was exposed to a male breeder. Twenty-four hours later a vaginal smear was taken and examined for the presence of sperm. If none were present, another male

was used on the following day. Each animal was then immediately isolated from the male. Six weeks later the exposed animal underwent a rectal examination for palpation of the uterus. If enlarged, the animal was considered pregnant and the age of the fetus calculated from the day of conception. If the uterus were normal in size, the animal was subsequently re-exposed ten days after commencing her next menstrual period.

C. Calculation of Fetal Age

The age of each fetus was determined from the date of conception. This day of conception was termed day zero. Subsequent age was therefore expressed as the conceptional age in days.

D. Procedure for Handling and Processing Fetal Tissue

Each fetus was delivered from the mother by cesarean section under ether anesthesia. After removal from the uterus, the fetus was immediately weighed, autopsied, and appropriate parts were x-rayed. The left tibia was utilized for chemical analysis by freeing it of muscle, fascia, and surrounding fibrous periosteum. It was sectioned according to the procedure described below. From the x-ray the presence or absence of secondary centers was noted. If no ossification centers were present in the proximal or distal

tibial cartilage, this cartilage was cut in approximately equal parts: the upper, sections 1A and 7B and the lower, sections 1B and 7A (see Figure one). If ossification were present, the proximal and distal epiphyses were again cut in two sections; areas 1A and 7B now were cut to include the secondary centers of ossification while sections 1B and 7A represented the cartilage of the epiphyseal plates.

After both epiphyseal areas were removed, the remaining shaft, arbitrarily called the "diaphyseal length," was measured and sectioned as shown in Figure one. Sections 2, 3, 5 and 6 represented 15% of the total diaphyseal length while section 4 represented 40% of the total diaphyseal length. Certain sections conform generally to known anatomical regions of long bones. Sections 2 and 6 contained the primary and secondary spongiosa, while section 4 was representative of compact, cortical bone.

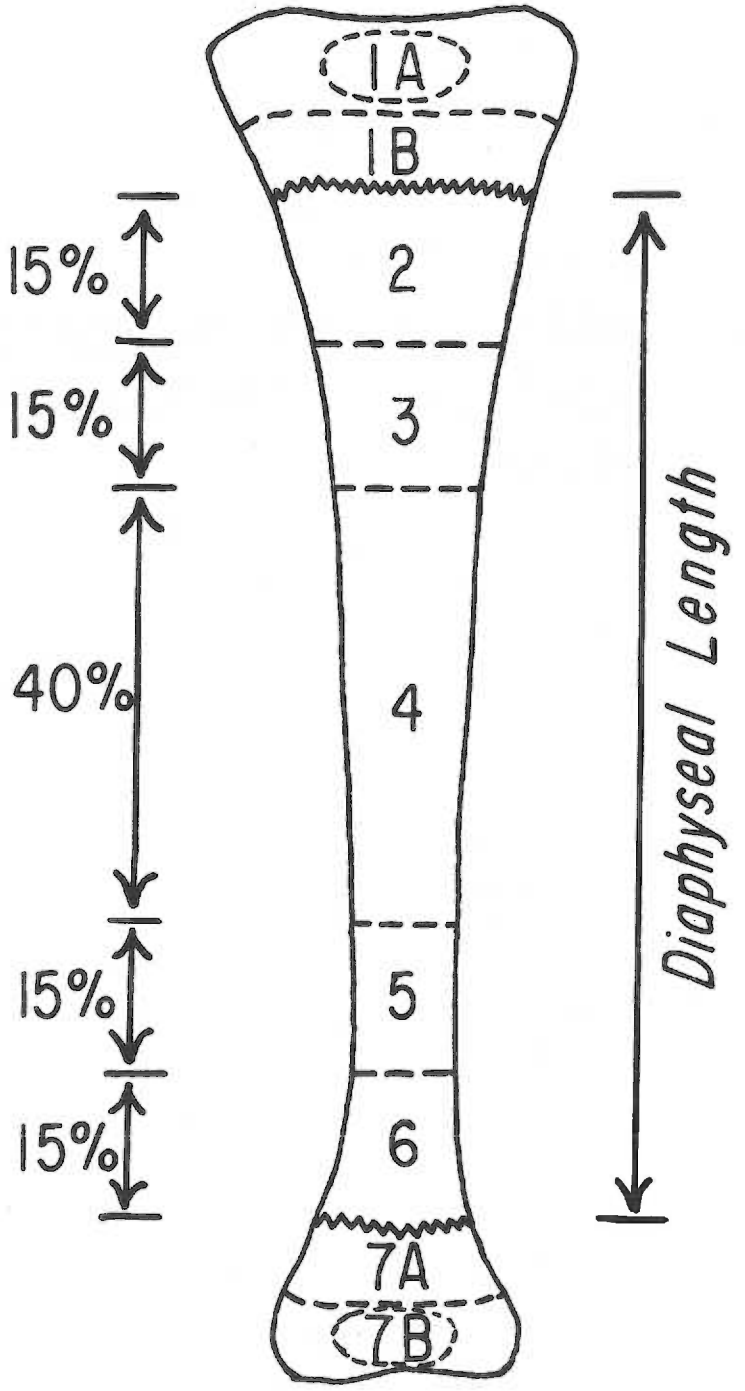
The cleaned, sectioned regions of the left tibia were processed as follows:

(a) They were crushed and placed in a 1:1 ether, alcohol solution for fat extraction, and were mechanically shaken for an hour in each of three changes of such a solution.

(b) The skeletal tissues were placed in weighed 10 ml. beakers, dried at 115-120° C. for six hours,

Figure One: Tibial section scheme for chemical studies. (Radiochemical data are not included in this study.)

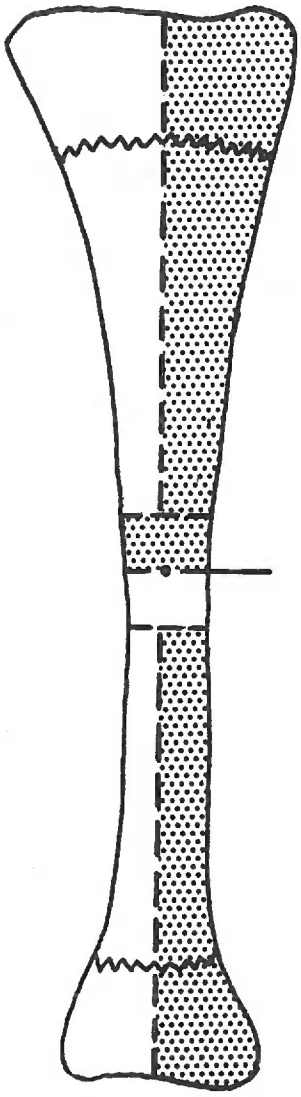
TIBIAL SECTION SCHEME
for chemical and radiochemical studies



The right tibia was removed for histological and histochemical studies. Excess soft tissues were removed, but the periosteum was left intact. After measuring its length and determining its midpoint, the right tibia was sectioned according to the scheme shown in Figure two. One of the paired, longitudinal sections was placed in chilled veronal buffered (pH 7) formalin and the other in chilled (4°C.) acetone. These sections were cut to demonstrate and visualize endochondral bone formation. Bone formation under the periosteum was studied by using cross sections from the midshaft as shown in Figure two. One section was placed in chilled, buffered formalin; the other, in chilled acetone, as before.

The remaining skeleton, after removal of the bones mentioned above, was freed of excess tissue, fat extracted, dried under the heat lamps, and finally ashed for 12 hours at 400° C. and for 48 hours at 800° C. The ashed bone was dissolved in approximately 40 ml. of concentrated nitric acid and diluted to 100 ml. or 250 ml. total volume with demineralized water. This solution was then used in the determination of total skeletal calcium. Several workers have shown that the calcium of soft tissues makes no significant contribution to the total body calcium.[26,53] Hence total calcium determined in this way is termed skeletal calcium. The

TIBIAL SECTION SCHEME
for morphological studies



A-In chilled acetone
for histochemistry

B-In veronal buffered formalin
(pH 7.2) for histology

mid-point of tibial
diaphyseal length

Figure Two: Tibial section scheme for histological and histochemical studies

calcium content of the left tibia (x 2) plus the left femoral calcium content were added to the above value to obtain total skeletal calcium.

E. Histological and Histochemical Procedure

Sections to be stained with hexmatoxlyn and eosin were removed from their fixative solutions and placed in a 70% alcohol, 5% nitric acid solution for demineralization.(40) When complete, the specimens were washed in water for 24 hours and neutralized in versene. After again washing for 24 hours they were placed for four hours time each in 60%, 70%, 80%, 90% alcohol and absolute alcohol, respectively. Next, they were immersed in two changes of alcohol ether (12 hours) and then into paraffin.

The paraffin blocks were mounted, sectioned at 10 mμ, and stained.

Specimens for alkaline phosphatase staining were left at 4° C. for at least three days. They were then demineralized and stained according to a modification of the Gomori technique.(37)

F. Roentgenological Procedure

1. Positioning of the specimen

The specimen was positioned by taping the dismembered left lower limb to cardboard. Anterior-posterior and lateral views of this extremity were obtained.

2. Film

Kodak Blue Brand medical x-ray film, 11 x 14 inches, was used with a hi-speed screen casette. In addition, 11 x 14 inch no-screen medical x-ray film was used.

3. Machine

A Westinghouse Autoflex unit was used in taking all films.

4. Exposure

Two target distances were used. A distance of 40 inches was used for films intended to record bony configuration and secondary ossification centers. Machine settings at this distance were: kilovolts, 40; milliamps, 50; time, 1/2 second, with no-screen film being used. A distance of 72 inches was used to obtain films for long bone measurements. Settings at this distance were: kilovolts, 60; milliamps, 50; time, 1/20 second, with screen film.

5. Measurements

The linear measurements of tibia and femur were obtained directly from the 72 inch roentgenograms with the aid of a sensitive calipers (John Bull, British Indicators, Ltd.). The tibial and femoral measurements via x-ray were identical with measurements obtained at autopsy, thereby demonstrating the validity of the roentgen technique. Diaphyseal length of the tibia and femur was the parameter

used to record linear growth. Diaphyseal length is defined in Figure one.

G. Microchemical Procedures

Bone calcium was determined from a modification of the method for determining serum calcium as described in the Beckman application data sheet DU-9-B.(7) This method employed the Beckman Model DU Spectrophotometer with flame and photomultiplier attachments. The sample was precipitated as calcium oxalate to eliminate interfering substances.

Phosphorus in bone was determined by a modification of the method of Taussky and Shorr.(72)

Nitrogen was determined by a modification of the micro-Kjeldahl method after Kabat and Mayer, and Kirk.(33, 34) The digestion rack and distilling apparatus were purchased from Microchemical Specialties, Berkeley, California.

An outline and analysis of each of these three chemical procedures can be found in the appendix.

III. RESULTS

In this section chemical data pertaining to the nineteen monkey fetuses analyzed at the five different conceptional ages are presented as a basis for the graphic presentation and correlative interpretation that will follow in the DISCUSSION.

The radiographic and histological material will be presented in the DISCUSSION and will be correlated with chemical findings. Table one records the body weight and certain chemical and linear growth measurements on each Rhesus monkey (Macaca mulatta) fetus.

Table two presents the mean and standard deviation of the chemical determinations and selected molar ratios for each age group on the tibial diaphysis. The tibial diaphysis is defined in Figure one. It includes regions 2 through 6 and excludes the cartilage ends and secondary centers. Calcium, phosphorus and nitrogen are expressed in terms of extracted, or fat-free, dry weight to minimize discrepancies between animals and areas considered. Such an expression is superior to a wet weight basis for comparison.(20) The molar ratios of calcium/phosphorus and calcium/nitrogen are also given in Table two. Calcium and phosphorus are, of course, the main mineral elements in the hydroxyapatite structure of the bone crystal.(18) The calcium/nitrogen ratio has been

Table One: Fetal growth data

TABLE ONE

Animal No.	Conceptional Age (days)	Body Weight (gms.)	Total Skeletal Calcium (mg.)	Tibial Diaphyseal Length (mm.)	Total Tibial Calcium (mg.)	Femoral Diaphyseal Length (mm.)	Total Femoral Calcium (mg.)
PP-59-8f	75	41.8	118	12.0	2.44	124	3.3
PP-59-9f	75	31.3	90.1	10.6	1.57	110	3.2
Y-960	75	—	108	9.1	—	100	—
Average		36.5	105	10.6	2.01	111	3.25
PP-59-6f	90	94.6	435	19.9	11.3	214	16.1
PP-59-11f	90	91.2	380	17.9	8.5	194	10.83
PP-58-4f	90	94.2	427	18.5	10.3	196	14.8
PP-58-1f	90	—	414	18.9	8.2	200	11.6
Y-989	90	—	358	17.1	9.0	196	—
Average		93.3	403	18.5	9.5	200	13.3
PP-58-6f	105	154	816	24.0	18.8	264	26.1
PP-58-7f	105	171	1016	25.4	22.7	269	27.4
PP-59-1f	105	146	928	23.0	21.6	250	32.1
PP-59-2f	105	127	828	22.2	16.4	242	24.9
PP-59-10f	105	164	1019	25.3	23.9	277	40.9
Average		152	921	24.0	20.7	260	30.3
PP-59-5f	120	243	1908	31.4	47.8	324	71.6
PP-58-8f	120	236	2024	30.6	49.3	330	73.3
PP-58-3f	120	214	1739	30.0	43.1	321	61.5
PP-58-2f	120	219	1721	33.5	46.1	349	57.5
PP-58-5f	122	287	2374	34.0	71.4	364	95.1
Average		240	1953	31.9	51.5	338	66.0
PP-59-3f	150	400	4413	40.5	108.8	434	151
PP-59-4f	150	470	4551	42.2	115.1	434	162.4
PP-59-7f	150	490	5193	42.9	147.3	438	198.9
Y-979	150	—	4670	40.3	153	429	—
Average		453	4707	41.3	131	434	171

Table Two: Chemical Anatomy of the Fetal Tibial Diaphysis

(includes sections 2 through 6--see Figure one)

TABLE TWO

Age (days)	mg. per gm. fat-free dry weight						molar ratio			
	Calcium		Phosphorus		Nitrogen		Ca/P		Ca/N	
	mean	± S.D.	mean	± S.D.	mean	± S.D.	mean	± S.D.	mean	± S.D.
75	165	17.3	90.2	1.42	59.6	1.42	1.44	.173	0.983	.10
90	197	25.5	95.0	7.20	62.3	5.29	1.60	.10	1.17	0.226
105	200	22.9	96.9	2.91	60.7	3.46	1.60	.148	1.17	0.205
120	207	6.33	98.5	5.54	59.6	4.58	1.64	.086	1.23	0.132
150	205	19.0	93.5	6.85	63.4	6.64	1.70	.071	1.14	0.214

shown to express the ratio of the change of combustible and non-combustible elements in growing bone.[58] It therefore relates the mineral phase of bone to its organic matrix. Table three presents P values for a comparison of the mean at 75 days with the means at subsequent ages for the elements and ratios presented in Table two. P values from .05 to 0.1 will be interpreted as "suggestive;" from .05 to .01, as "significant;" and greater than .01, as "highly significant."

The nine regions of the sectioned left tibia, as shown in Figure one, have been analyzed separately for calcium, phosphorus and nitrogen. Values are again expressed in terms of fat-free dry weight. Tables four, five, six, seven and eight give the results of these analyses, the calcium/phosphorus and calcium/nitrogen molar ratios for each fetus, and the average for each age group. Tables nine, ten and eleven present P values for the comparison of selected means. (Not all calculated P values have been given). Attention will be focused on areas 2, 6 and 4. Areas 2 and 6 contain the primary and secondary spongiosa of endochondral bone formation while area 4 contains cortical bone formed under the periosteum.

Table Three: Comparisons of means of the total diaphyseal tibial chemical constituents, as calculated on a fat-free dry weight basis, and of selected molar ratios.

TABLE THREE

	P Values			
	75 day vs.			
	90 day	105 day	120 day	150 day
Calcium	> .2	> .1	< .01	> .05, < .1
Phosphorus	> .4	> .02, < .05	< .1	> .5
Nitrogen	> .5	> .6	> .9	> .5
Molar ratio, Calcium:Phosphorus	> .1	> .2	> .05, < .1	> .05, > .1
Molar ratio, Calcium:Nitrogen	> .3	> .2	> .1, < .05	> .4

Table Four: Skeletal Parts: Calcium Content

(Calcium [mg.]/Fat-free Dry Weight [gm.])

TABLE FOUR

Conceptional Age	Fetus No.	Tibial Section								
		1A	1B	2	3	4	5	6	7A	7B
75 days	PP-59-8f	*	*	185	181	187	169	163	*	*
	PP-59-9f	*	*	152	156	170	127	132	*	*
	Average	*	*	169	169	179	148	148	*	*
90 days	PP-58-1f	*	*	169	186	177	173	179	*	*
	PP-58-4f	*	*	201	224	214	310	215	*	*
	PP-59-6f	*	*	197	201	180	190	186	*	*
	PP-59-11f	*	*	178	173	178	187	164	*	*
	Average	*	*	186	196	187	215	186	*	*
105 days	CP-58-6f	*	*	219	194	194	196	203	*	*
	PP-58-7f	*	*	209	191	195	199	213	*	*
	PP-59-1f	*	*	225	230	237	238	232	*	*
	PP-59-2f	*	*	198	180	176	169	177	*	*
	PP-59-10f	*	*	194	190	178	187	176	*	*
	Average	*	*	209	197	196	198	200	*	*
120 days	PP-58-2f	*	*	217	208	203	212	213	*	*
	PP-58-3f	*	*	206	210	213	222	235	*	*
	PP-58-8f	*	*	230	215	201	202	211	*	*
	PP-59-5f	*	*	210	209	202	195	209	*	*
	PP-58-5f#	*	*	199	196	212	198	190	*	*
	Average	*	*	212	208	206	206	212	*	*
150 days	PP-59-3f	60.7	19.5	193	202	207	183	197	12.3	54.7
	PP-59-4f	50.5	11.6	181	175	205	188	181	9.02	58.9
	PP-59-7f	31.7	17.2	236	219	234	211	217	32.2	60.4
	Average	47.6	16.1	203	199	215	194	198	17.8	58.0

*Less than 10 mg. calcium per gram fat-free dry weight of bone

#122 days

Table Five: Skeletal Parts: Phosphorus Content

(Phosphorus [mg.]/Fat-free Dry Weight [gm.])

TABLE FIVE

Conceptional Age	Fetus No.	Tibial Section								
		1A	1B	2	3	4	5	6	7A	7B
75 days	PP-59-8f	2.7	12.4	91.7	93.0	92.3	88.3	88.1	27.8	3.6
	PP-59-9f	2.8	14.8	92.6	90.5	89.4	89.7	82.8	22.9	—
	Average	2.75	13.6	92.2	91.8	90.8	89.9	85.5	25.4	3.6
90 days	PP-58-1f	4.0	13.7	89.4	89.6	92.9	89.3	91.7	38.7	2.8
	PP-58-4f	2.6	12.6	102	105	106	106	98.8	16.7	2.5
	PP-59-6f	2.7	12.5	97.7	98.9	96.6	93.9	92.3	13.3	3.1
	PP-59-11f	4.3	16.0	88.2	89.6	86.3	89.8	90.6	12.6	4.6
	Average	3.4	13.7	94.3	95.5	95.5	94.8	93.4	20.3	3.3
105 days	PP-58-6f	2.9	11.4	96.3	94.7	98.0	102	104	15.5	2.5
	PP-58-7f	1.9	20.7	99.1	91.1	91.2	95.7	96.7	22.6	2.9
	PP-59-1f	2.2	12.6	99.6	95.3	102.3	103.7	107.4	10.4	2.6
	PP-59-2f	3.7	19.3	96.3	95.7	94.2	95.9	98.3	18.4	2.7
	PP-59-10f	—	7.6	98.7	96.6	93.5	96.2	88.7	8.0	—
	Average	2.7	13.4	98.0	94.7	95.8	98.7	99.0	13.8	2.7
120 days	PP-58-2f	1.2	7.0	106	102	98.4	101	94.4	16.6	2.0
	PP-58-3f	1.3	8.6	103	105	106	106	111	10.3	2.2
	PP-58-8f	4.4	19.6	108	101	91	97	100		4.9
	PP-59-5f	1.1	11.2	89.2	92.0	92.4	90.2	91.1	12.8	1.7
	PP-58-5f*	0.7	11.2	98.9	98.7	98.7	96.7	98.7	18.1	2.0
	Average	1.7	11.5	101	99.7	97.3	98.2	99.0	14.5	2.6
150 days	PP-59-3f	32.1	9.8	92.3	97.7	98.6	90.7	95.9	11.8	27.0
	PP-59-4f	23.9	8.6	84.2	86.0	83.5	91.8	86.2	9.8	27.5
	PP-59-7f	14.1	13.1	102	95.5	103	95.5	94.6	20.5	27.6
	Average	23.4	10.5	92.8	93.1	95.0	92.7	92.3	14.0	27.4

*122 days

Table Six: Skeletal Parts: Nitrogen Content

(Nitrogen [mg.]/Fat-free Dry weight [gm.])

TABLE SIX

Conceptional Age	Fetus No.	Tibial Section								
		1A	1B	2	3	4	5	6	7A	7B
75 days	PP-59-8f	108	108	58.8	59.5	61.1	64.7	61.7	87.9	110
	PP-59-9f	112	111	58.7	59.9	56.4	53.5	60.0	—	121
	Average	110	110	58.8	59.7	58.8	64.1	60.9	87.9	116
90 days	PP-58-1f	196	96.6	57.2	71.1	68.6	73.3	70.3	125	113
	PP-58-4f	147	120	59.6	59.3	54.8	55.3	61.5	79.6	136
	PP-59-6f	119	102	55.2	53.0	60.6	61.4	57.4	91.8	—
	PP-59-11f	120	109	64.8	70.6	66.1	67.4	62.9	109	131
	Average	146	107	59.2	63.5	62.5	64.4	63.0	101	127
105 days	CP-58-6f	127	105	59.8	63.6	61.7	55.9	55.3	96.4	130
	PP-58-7f	128	103	54.0	67.7	66.9	64.0	58.9	105	130
	PP-59-1f	120	109	54.7	56.8	57.0	54.3	50.4	82.0	124
	PP-59-2f	127	103	59.7	61.6	66.6	63.9	67.4	103	99.0
	PP-59-10f	123	104	55.0	59.2	63.5	64.3	64.4	106	129
	Average	125	105	56.6	61.8	63.1	60.5	59.3	98.5	122
120 days	PP-58-2f	132	114	56.2	61.6	67.6	62.8	58.8	110	133
	PP-58-3f	129	108	51.9	53.0	56.1	53.7	45.8	106	118
	PP-58-8f	125	110	47.7	56.5	65.4	61.6	53.6	110	128
	PP-59-5f	135	104	57.9	62.7	69.3	68.1	62.3	122	137
	PP-58-5f*	134	110	58.4	61.7	64.2	63.3	62.4	110	123
	Average	131	109	54.4	59.1	64.5	61.9	56.5	112	128
150 days	PP-59-3f	104	110	62.0	60.4	60.4	62.8	61.4	118	106
	PP-59-4f	112	112	72.6	75.0	65.1	72.0	76.9	124	111
	PP-59-7f	124	104	53.5	60.3	57.1	63.4	58.1	88.8	107
	Average	113	109	61.7	65.2	60.9	66.1	65.5	110	108

*122 days

Table Seven: Skeletal Parts: Molar Ratio of Calcium to Phosphorus

TABLE SEVEN

Conceptional Age	Fetus No.	Tibial Section								
		1A	1B	2	3	4	5	6	7A	7B
75 days	PP-59-8f	—	—	1.56	1.50	1.57	1.48	1.43	—	—
	PP-59-9f	—	—	1.27	1.33	1.47	1.10	1.23	—	—
	Average	—	—	1.42	1.42	1.52	1.29	1.33	—	—
90 days	PP-58-1f	—	—	1.46	1.60	1.47	1.50	1.51	—	—
	PP-58-4f	—	—	1.52	1.65	1.56	2.26	1.68	—	—
	PP-59-6f	—	—	1.56	1.57	1.44	1.57	1.56	—	—
	PP-59-11f	—	—	1.56	1.51	1.59	1.61	1.40	—	—
	Average	—	—	1.53	1.58	1.52	1.76	1.54	—	—
105 days	CP-58-6f	—	—	1.76	1.58	1.53	1.49	1.51	—	—
	PP-58-7f	—	—	1.63	1.62	1.65	1.61	1.70	—	—
	PP-59-1f	—	—	1.75	1.87	1.79	1.77	1.67	—	—
	PP-59-2f	—	—	1.59	1.45	1.44	1.36	1.39	—	—
	PP-59-10f	—	—	1.52	1.52	1.47	1.50	1.53	—	—
	Average	—	—	1.65	1.61	1.58	1.55	1.56	—	—
120 days	PP-58-2f	—	—	1.58	1.58	1.60	1.62	1.74	—	—
	PP-58-3f	—	—	1.55	1.55	1.55	1.62	1.64	—	—
	PP-58-8f	—	—	1.65	1.65	1.71	1.61	1.63	—	—
	PP-59-5f	—	—	1.82	1.76	1.69	1.67	1.77	—	—
	PP-58-5f*	—	—	1.56	1.54	1.66	1.58	1.49	—	—
	Average	—	—	1.63	1.62	1.64	1.62	1.65	—	—
150 days	PP-59-3f**	1.47	1.54	1.62	1.60	1.62	1.56	1.59	0.81	1.57
	PP-59-4f	1.63	1.04	1.66	1.57	1.90	1.58	1.62	0.71	1.66
	PP-59-7f	1.74	1.01	1.79	1.77	1.76	1.71	1.78	1.22	1.70
	Average	1.58	1.20	1.69	1.65	1.76	1.62	1.66	0.99	1.64

*122 days

**Secondary ossification present

Table Eight: Skeletal Parts: Molar Ratio of Calcium to Nitrogen

TABLE EIGHT

Conceptional Age	Fetus No.	Tibial Section								
		1A	1B	2	3	4	5	6	7A	7B
75 days	PP-59-8f	—	—	1.10	1.06	1.07	0.91	0.92	—	—
	PP-59-9f	—	—	0.91	0.91	1.05	0.70	0.77	—	—
	Average	—	—	1.01	0.99	1.06	0.83	0.85	—	—
90 days	PP-58-1f	—	—	1.03	0.92	0.90	0.83	0.89	—	—
	PP-58-4f	—	—	1.18	1.32	1.36	1.96	1.22	—	—
	PP-59-6f	—	—	1.25	1.33	1.03	1.08	1.13	—	—
	PP-59-11f	—	—	0.91	0.86	0.94	0.97	0.91	—	—
	Average	—	—	1.09	1.11	1.06	1.17	1.04	—	—
105 days	CP-58-6f	—	—	1.28	1.07	1.10	1.23	1.28	—	—
	PP-58-7f	—	—	1.35	0.99	1.02	1.09	1.26	—	—
	PP-59-1f	—	—	1.44	1.42	1.45	1.54	1.61	—	—
	PP-59-2f	—	—	1.16	1.02	0.92	0.93	0.92	—	—
	PP-59-10f	—	—	1.23	1.12	0.98	1.02	0.96	—	—
	Average	—	—	1.29	1.92	1.09	1.16	1.21	—	—
120 days	PP-58-2f	—	—	1.35	1.18	1.05	1.18	1.26	—	—
	PP-58-3f	—	—	1.39	1.38	1.34	1.44	1.79	—	—
	PP-58-8f	—	—	1.69	1.33	1.08	1.15	1.37	—	—
	PP-59-5f	—	—	1.27	1.17	1.02	1.00	1.16	—	—
	PP-58-5f*	—	—	1.19	1.11	1.15	1.09	1.06	—	—
	Average	—	—	1.38	1.23	1.13	1.17	1.33	—	—
150 days	PP-59-3f	0.20	0.06	1.09	1.17	1.20	1.02	1.12	0.04	0.18
	PP-59-4f	0.16	0.04	0.87	0.82	1.10	0.91	0.83	0.03	0.19
	PP-59-7f	0.09	0.06	1.54	1.27	1.43	1.16	1.31	0.13	0.20
	Average	0.15	0.05	1.17	1.09	1.24	1.03	1.06	0.06	0.19

*122 days

Table Nine: Comparison of the mean of section 2 at 75 days to means at succeeding ages. Chemical constituents are expressed in mg./fat-free dry weight (gm.)

TABLE NINE

	P Values: 75 day vs.			
	90 day	105 day	120 day	150 day
Calcium	$> .3$	$> .02$, $< .05$	$> .02$, $< .05$	$> .3$
Phosphorus	$> .7$	$< .01$	$> .2$	$> .9$
Nitrogen	$> .9$	$> .3$	$> .2$	$> .7$
Molar ratio, Calcium/Phosphorus	$> .7$	$> .4$	$> .4$	$> .05$, $< .1$
Molar ratio, Calcium/Nitrogen	$> .5$	$> .02$, $< .05$	$> .05$, $< .1$	$> .5$

Table Ten: Comparison of the mean of section 4 at 75 days to means at succeeding ages. Chemical constituents are expressed in mg./fat-free dry weight (gm.)

TABLE TEN

	P Values: 75 day vs.			
	90 day	105 day	120 day	150 day
Calcium	> .6	> .5	> .02, < .05	> .05, < .1
Phosphorus	> .5	> .3	> .3	> .6
Nitrogen	> .4	> .2	> .2	> .6
Molar ratio, Calcium/Phosphorus	> .9	> .6	> .1	> .1
Molar ratio, Calcium/Nitrogen	> .9	> .8	> .5	> .2

Table Eleven: Comparison of the means of sections 2 and 4 at 75, 90, 105, 120 and 150 days conceptional age. Chemical constituents are expressed in mg./fat-free dry weight (gm.)

TABLE ELEVEN

	75	90	105	120	150
Calcium	> .6	> .9	> .3	> .4	> .6
Phosphorus	> .8	> .8	> .05, < .1	> .4	> .7
Nitrogen	> .9	> .5	> .1	> .05, < .02	> .9
Molar ratio, Calcium/Phosphorus	> .8	> .8	> .4	> .8	> .5
Molar ratio, Calcium/Nitrogen	> .6	> .8	> .1	> .05, < .1	> .7

IV. DISCUSSION

The chemical data presented in the previous section, and certain radiographic and histological findings, will provide the basis for a discussion of several features of fetal skeletal growth and development. Points for consideration will include the maternal dietary, the growth of the total skeleton, the relation of various growth parameters to total skeletal chemical growth and the growth dynamics of the tibia and its regions, including radiographic configuration and morphologic changes. The complexity of the structure, formation and reactivity of skeletal tissue presented in the INTRODUCTION is compounded in the developing monkey fetus by the dynamics of skeletal growth. Interpretations will be made with these concepts in mind.

Any application of these data to other studies must be considered within the total framework of all experimental conditions. These include the environmental factors of diet, caging, temperature, as well as the age, genetic pattern and state of the maternal skeleton.

A. The Maternal Diet

Of obvious, crucial importance is the dietary of the maternal organism. This diet, as listed in the appendix, was felt to be optimal in terms of essential nutrients, minerals and vitamins, including vitamin D. Studies in the

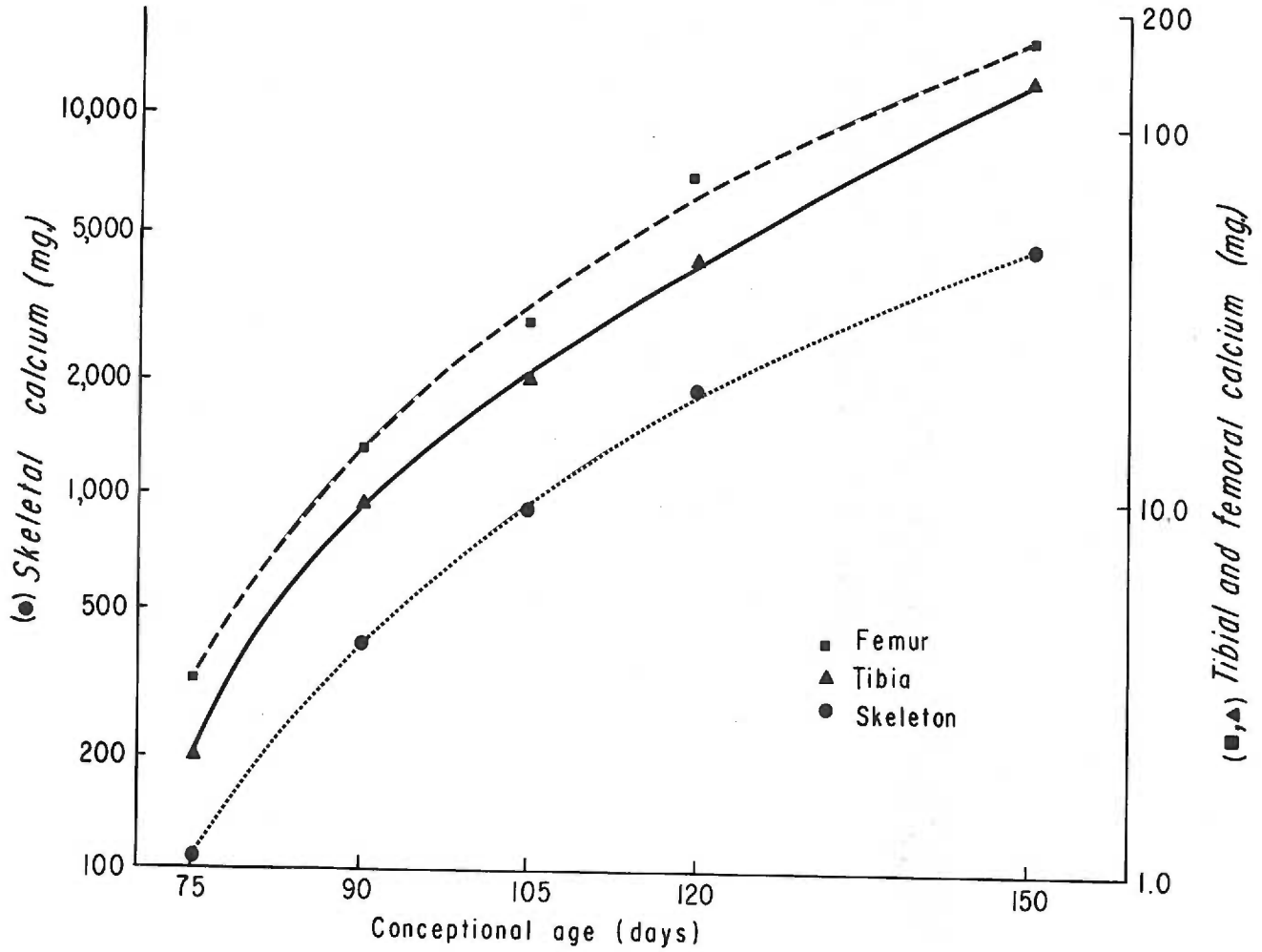
literature regarding the growth, differentiation and composition of the human fetal skeleton as related to the maternal diet are sparse, incomplete and conflicting. That rickets can be observed in newborn infants is a well-known, frequent observation in the Orient, thus demonstrating the occurrence of a dietary deficiency in the fetus.(52) However, other studies relating the composition of a selected fetal skeletal part to a clinical evaluation of maternal diet failed to detect significant changes in mineral composition when correlated with the adequacy of the diet.(74,10) The relationship of well-controlled variations in maternal diet to the growth and maturation of the fetal skeleton, as well as its composition, remains essentially undefined. The Rhesus monkey (Macaca mulatta) would seem a logical experimental animal for use in this regard.

B. Total Skeletal, Tibial and Femoral Calcium Content and Uptake

Figure three relates calcium contained in the total skeleton, tibia and femur to conceptional age. A log scale was used to facilitate the expression of the changes with age. Between ages 75 and 150 days, the incorporation of calcium into the growing skeleton was extremely rapid. During these 75 days of fetal life, skeletal calcium increased from 105 milligrams to over 4700 milligrams. A

Figure Three: Total skeletal, tibial, and femoral calcium (mg.) as a function of conceptional age

SKELETAL, TIBIAL AND FEMORAL CALCIUM
AS A FUNCTION OF CONCEPTIONAL AGE



roughly proportional increase occurred in the tibias and femurs.

The daily rate of calcium uptake by the tibia, femur and total skeleton at the various ages studied has been derived by interpolating the data from the graphs presented in Figure three. These uptake data, presented in Table twelve, demonstrate that the total fetal skeleton's demand for calcium increased with age. Yet if this uptake is expressed on a relative basis, i.e., per unit of existing skeletal calcium, it decreased with age. For example, the average total skeletal uptake of calcium at 90 days was 28.8 mg. (Table twelve) or 7 mg. per 100 mg. skeletal calcium. At 120 days the total skeletal calcium uptake was 79 mg., but only 4 mg. per 100 mg. skeletal calcium.

These uptake values have been compared with other radiocalcium studies performed on the same fetal preparations.(55) Calculations from the 24-hour distribution of maternal IV-injected calcium^{45} gave calcium incorporation values in the femur and tibia very similar to the above values derived from interpolation of chemical data.(55) This important finding suggests that, at least in 24 hours, the calcium that crosses the placenta and is incorporated into the fetal skeleton remains therein, even though the fetal skeletal calcium turnover rate is high.

Table Twelve: Average daily fetal skeletal incorporation of calcium (milligrams)

TABLE TWELVE

Conceptional Age	Total Skeleton mg./day	Femur mg./day	Tibia mg./day
75	11.0	0.402	0.36
90	28.8	1.02	0.636
105	45.6	1.47	1.02
120	79	2.62	2.18
150	118	5.03	4.75

C. Chemical Analyses of the Tibia

Several observations on the chemical analyses of the tibia and its regional parts are presented. These findings will be enlarged upon in subsequent discussion. All chemical values are expressed as the unit content of the element in question (milligram per gram fat-free dry weight).

(1) The total tibial diaphysis at 120 days had a highly significant increase in unit calcium content compared to what was found at 75 days. At 150 days the average content showed a suggestive increase over the 75 day value, having dropped slightly from the mean at 120 days (see Tables two and three).

(2) There was a significant increase in unit calcium contained in areas 2 and 4 at 120 days over their respective 75-day values. At 150 days this increase was not maintained (see Tables four, nine and ten).

(3) The mean nitrogen content per unit bone in the total diaphyseal tibia and in areas 2, 4, and 6 showed no significant changes when 75-day values were compared with their respective means at subsequent ages (see Tables two and six).

(4) The calcium/phosphorus molar ratio did not reveal a statistically significant pattern of change during the period of fetal life studied. This was true for

the total diaphysis, and areas 2, 4 and 6 when the mean values at 75 days were compared to their respective mean values at subsequent fetal ages (see Tables two, seven, nine and eleven).

(5) Values for the molar ratio of calcium/nitrogen per unit bone at 120 days showed increases of suggestive statistical significance when compared to the 75-day values. Such was true of the total tibial diaphysis, and areas 2 and 6. No significant or suggestive differences were found between mean values at 150 days and those at 75 days (see Tables two, three, eight and nine).

(6) When the mean unit calcium, phosphorus and nitrogen values in area 2 were compared with their respective values in area 4 at each of the fetal ages studied, no statistically significant patterns of change were demonstrated. Identical comparisons of the calcium/phosphorus and calcium/nitrogen molar ratios likewise showed no significant differences (see Table eleven).

(7) The cartilage ends of the tibia contained only very small amounts of calcium. Through the first 120 days, less than 10 mg. calcium per gram fat-free dry weight was found in cartilage sections 1A, 1B, 7A and 7B. The greatly increased amounts of calcium and

phosphorus at 150 days in areas 1A and 7B reflect the then well-established secondary centers of ossification. At this age the unit content of both calcium and phosphorus in the proximal secondary center is comparable to that in the distal secondary center (see Tables four and five).

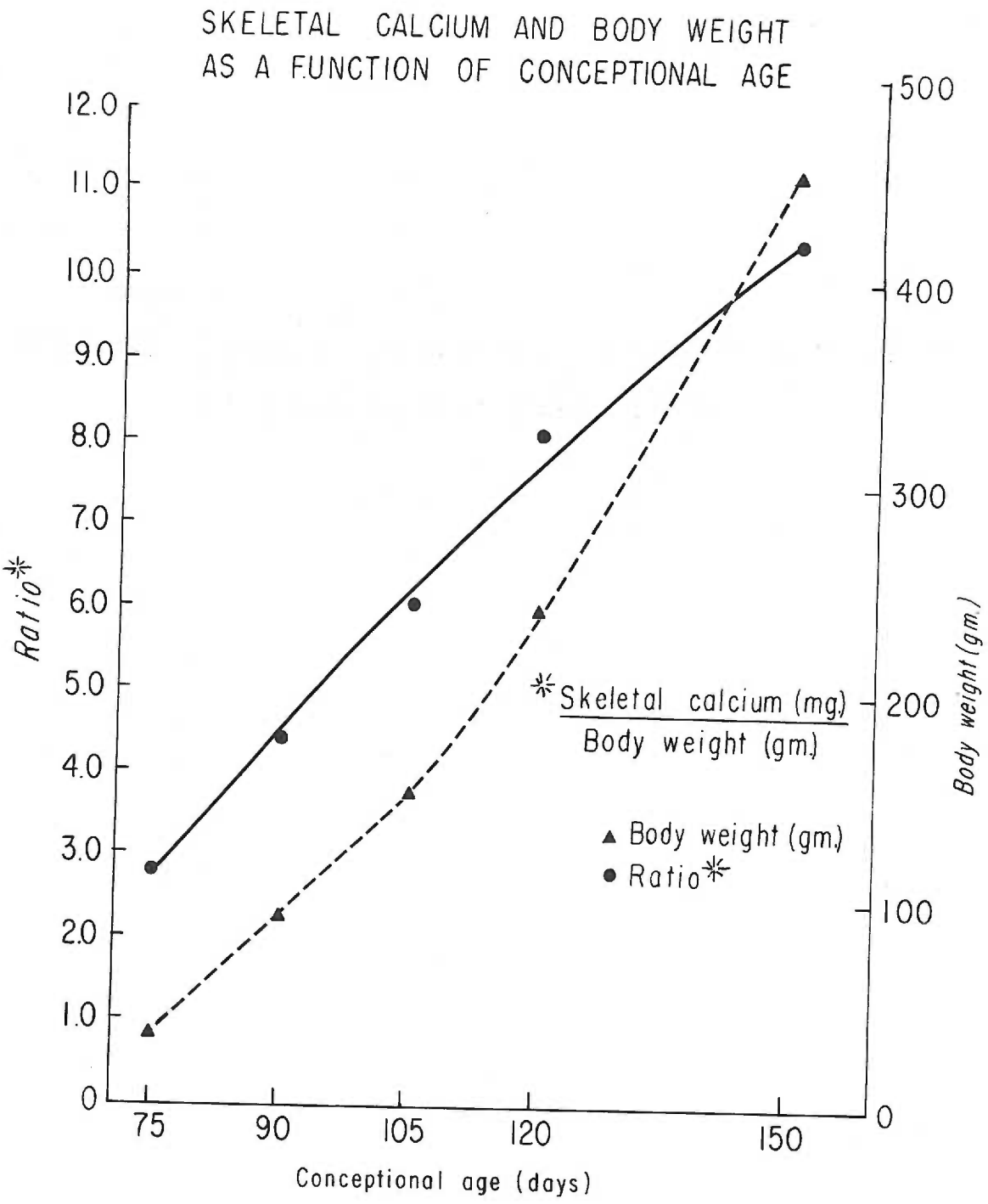
(8) The unit nitrogen content in areas 1A and 7B at 105 and 120 days is greater than the 75-day values in these same areas (see Table six).

D. Estimations of the Growth and Composition of the Total Skeleton from an Analysis of Its Parts

Length measurements and chemical analyses performed on various selected bones and bone samples have been used to interpret over-all skeletal growth.(3,51) Several such comparisons are made in the present study, with the total calcium content at each fetal age being used as a measure of total skeletal growth.

Figure four relates the increasing content of skeletal calcium to increasing body weight over the period studied. The ratio of skeletal chemical growth to somatic growth (body weight) is seen to undergo continuing increases during this period in fetal life. Pickering, et al demonstrated a disproportionate change in skeletal mass with respect to somatic mass in young rats up to 25 days of extra-uterine

Figure Four: Skeletal calcium (mg.) and body weight (gm.) as a function of conceptional age

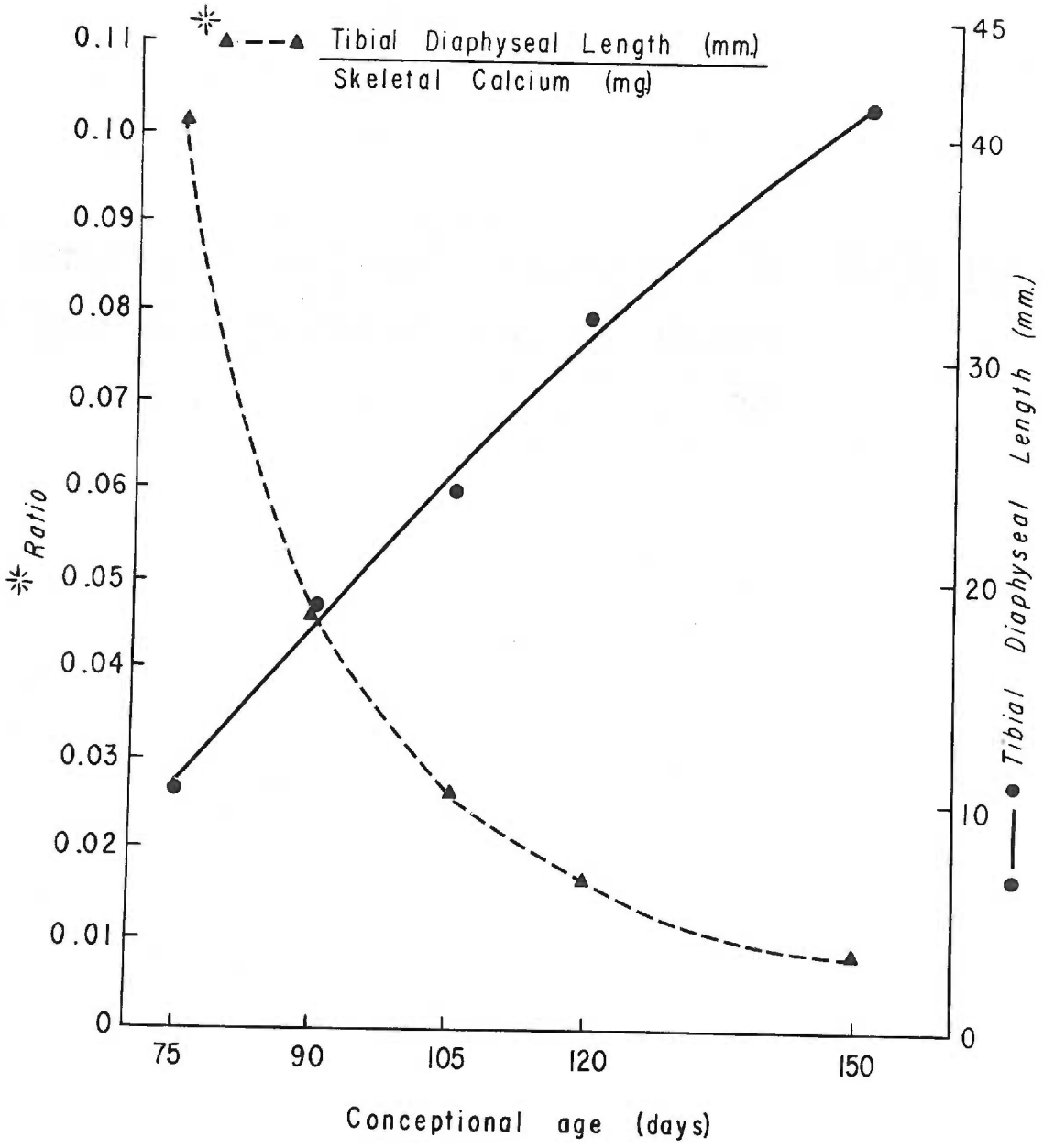

SKELETAL CALCIUM AND BODY WEIGHT
AS A FUNCTION OF CONCEPTIONAL AGE
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
Ratio*
500
400
300
200
100
0
Body weight (gm.)
75
90
105
120
150
Conceptional age (days)
* Skeletal calcium (mg.) / Body weight (gm.)
▲ Body weight (gm.)
● Ratio*

life.[58] After 25 days, the ratio between them tended to be constant. Sherman and MacLeod found that the percentage calcium in the body of the rat underwent a similar increase with age.[62]

An analysis of the relationship between increments of tibial diaphyseal length and calcium content is shown in Figure five. The increase in the length of the shaft of the tibia tended to be linear with age from 75 to 120 days, whereas from 120 to 150 days the rate fell slightly. The average daily growth rate in tibial length is 0.47 millimeters from 75 through 120 days and 0.41 millimeters from 120 to 150 days. The lack of a consistent relationship between growth in length and growth measured by increasing amounts of calcium is also shown in Figure five. This discrepancy is similar to that reported by Pickering, et al for the immature rat tibia.[57] The present study supports their contention that interpretations of skeletal chemical growth may not be satisfactorily implied by an analysis of changes in linear proportions of skeletal parts. The present studies also indicate no consistent relationship when tibial calcium was plotted against the diaphyseal length cubed. Such a correlation was attempted in hopes that it might permit using a key parameter for estimating skeletal mass without chemical analysis. In human prepubertal growth,

Figure Five: Tibial diaphyseal length (mm.) and skeletal calcium (mg.) as a function of conceptional age

TIBIAL DIAPHYSEAL LENGTH AND SKELETAL CALCIUM AS A FUNCTION OF CONCEPTIONAL AGE



the body weight (mass) can be roughly related to the cube of the height (a measure of skeletal growth).(64) The data in this study do not support such a relationship for the monkey fetal tibia, and it is probably true that such a relationship would not hold in equivalent human fetal bone.

An attempt to estimate the total skeletal mass by chemical analysis of a part or sample must also be undertaken. In this study the tibia has been comprehensively analyzed as a representative long bone and will be used for such a comparison.

The total diaphyseal tibia, and areas 2, 4 and 6 were found to significantly increase in calcium content per gram fat-free dry weight from 75 to 120 days. At 150 days the unit calcium content had fallen to values resembling the 75-day fetal tibia. In addition, the total tibial content of calcium, expressed as the per cent of the total skeletal calcium contained in the fetus, varied from 1.9% at 75 days to 2.78% at 150 days. Thus, the chemical growth of the total skeleton does not seem to be a simple multiple of chemical growth in the tibia. Estimations of the total skeleton by chemical analysis of the tibia or its parts thereof without additional data would be inaccurate.

Such a conclusion seems to be the case for the adult skeleton as well as for the monkey fetus. Mitchel, et al

in an extensive chemical analysis of an adult human cadavar found the mineral content in different bones to vary significantly, pointing out the error inherent in predicting total skeletal composition from one bone.(45) Stabino and Farr found that adult ox bones showed marked ash and nitrogen variations in different areas of one bone, as well as in different bones.(70) Recent biophysical techniques have been responsible for demonstrating the complexity of individual Haversian systems in this regard. Newly formed Haversian systems show highest mineral concentrations close to the central canal, with decreases toward the periphery of the system. With maturation, mineralization of the Haversian system becomes evenly distributed.(21) Thus, the complexity of the growth mechanisms and structure of the skeleton preclude any reliable interpretations about total fetal skeletal chemical growth from somatic data, linear measurements of long bones, or selected chemical analyses on single bones. This is not to detract from the value of such measurements as important parameters in estimating effects of experimental variables; however, such a limitation must be kept in mind when interpretations are made.

E. Regional Chemical Analyses and Interpretation

The total diaphyseal tibia, as well as tibial areas 2, 4 and 6, showed a distinct increase in calcium per gram

fat-free dry weight from 75 to 120 days. This pattern was then reversed at 150 days in areas 2 and 6, where new endochondral bone is formed and rapidly remodeled. The calcium/nitrogen molar ratio changed in a parallel fashion over the same time interval. Such a change is paradoxical in the sense that existing bone usually increases in mineral content with age and maturation, as individual Haversian systems incorporate new crystals and increase the size of existing crystals. Some of the many variables involved in producing such a paradox include the placental transfer of calcium, the rate of bone turnover, the rate of long bone growth, and the appearance of secondary centers and small bones. It would appear that up through 120 days, the calcium made available by virtue of turnover of existing skeletal calcium and calcium being added by placental transfer is adequate to permit increasing mineralization of newly formed bone with increasing age. Yet from 120 to 150 days, while the linear growth rate has not accelerated and the total fetal skeletal calcium uptake has continued to increase (Table two), the unit mineral content in the tibial shaft has decreased. During this time a rapid succession of small bones and secondary centers appear in the hand and wrist (see Figures eleven and twelve). In addition, both tibial secondary centers are forming. Therefore,

from 120 to 150 days, the demand for calcium needed to maintain increasing mineralization as growth proceeds and new bones form exceeds the supply from turnover and placental transfer. As a result, the unit mineral content of the growing tibia decreases. Such a finding is illustrative of the fact that the mineral content of one growing bone at any time is a reflection of many rate processes occurring in the total organism.(50)

Although the tibial calcium content per gram fat-free dry weight is seen to be heterogenous with age, no significant changes in unit calcium content occurred among areas 2, 4 and 6 at any <u>one</u> age (Table eleven). The calcium/nitrogen ratio likewise underwent no significant changes among these areas at any one age. Radioisotope studies with calcium^{45} on these same fetuses have shown that bone in areas 2 and 6 is rapidly remodeled, whereas in area 4 it is reformed at a much slower rate.(55) This suggests that new bone matrix, when formed, is quickly mineralized to a degree not strikingly different from "older" fetal bone. Such a finding is in agreement with current ideas on the rate of the mineralization process, which shall now be briefly reviewed.

Crystals of bone have been shown to replace water on a volume basis with intercrystal fluid spaces calculated to

approach atomic dimensions in fully mineralized tissue.[16, 50] Diffusion of ions is therefore restricted as mineralization becomes maximal. Since the driving force for mineralization of osteoid most likely comes from the supersaturated serum,[46] formed matrix rapidly mineralizes until the process is slowed by restricted diffusion of ions. From then on the rate of the mineralization process is very slow until complete. This concept has received support from biophysical studies. Wallgren, analyzing human fetal femurs by microradiography and x-ray diffraction techniques, concluded that following its initial appearance, the mineral content in bone rapidly increases over a period of three weeks to values which are 70-80% of adult bone.[79] Increasing crystal size is associated with this rapid increase in mineral content. Therefore, if new matrix is formed, only a short time is required for incorporation of the bulk of the mineral content.[50] The crucial problem of the <u>initiation</u> of the mineralization process has been discussed in the INTRODUCTION.

Although variable, the calcium/phosphorus molar ratio was found not to change significantly with age. Neither did the ratio show a significant pattern of change when the newly formed bone in regions 2 and 6 was compared with area 4 or with the total diaphyseal tibia at any one age

(Table eleven). Thus, bone mineral formed during fetal life does not appear to manifest statistically significant variation in the relative content of its two major inorganic elements. Formula weights for the apatite crystal have, in the past, been calculated from the calcium/phosphorus ratio,[65,18] but this ratio may vary widely and still represent an hydroxyapatite mineral.[49] The variation is attributed to the extremely small size of the crystals and the correspondingly large influence of the contributions of their surface ions to an analysis of the total mineral.[44]

Surveys of the literature on bone composition pose formidable problems in interpretation and comparison because of the diverse means by which bone tissue is processed and the values expressed.[24] Some data are given in terms of fresh bone tissue,[13,38,78] others as dry weight,[61] or fat-free dry weight,[1,4] and still others in terms of bone ash.[20] Likewise, the results of the analyses of human fetuses are somewhat at variance among different investigators. No study relating an analysis of one bone to total skeletal composition could be found for the fetus. Swanson and Iob analyzed the mineral composition of various long bones of 21 human fetuses of varying weights.[71] They concluded that the composition of the main inorganic elements in these bones remain constant

during intrauterine life. MacDonald, however, analyzed fetal skull bones and found a small but significant increase in per cent calcium between early fetuses and full term infants.(39) Follis, in reviewing the literature, found no appreciable difference between the human fetal rib and adult rib in terms of per cent calcium and phosphorus on a fat-free dry weight basis.(22)

Such findings are of chief value in pointing to the need for a systematic, integrated, standardized approach in analyzing bone and expressing the result obtained.

F. Radiographic Studies of Skeletal Growth

X-rays provide a graphic demonstration of changing length and configuration of bones during growth. Figures six and seven provide a comparative sketch of the tibia and femur at each age studied. These drawings have been taken from representative radiographs of the animals and accurately enlarged. Their length represents the average for each age group (see Table one).

As well as increasing in length, the tibia and femur are seen to undergo changes in configuration with age. They become wider along the shaft with the metaphyseal regions flaring to assume the definitive shape of mature, long bones. Yet, throughout the entire period, both the femur and tibia are recognizable as distinct bones. Such

Figure Six: Right tibial diaphyseal length and configuration, demonstrating secondary centers (magnified 3-1/2 times)

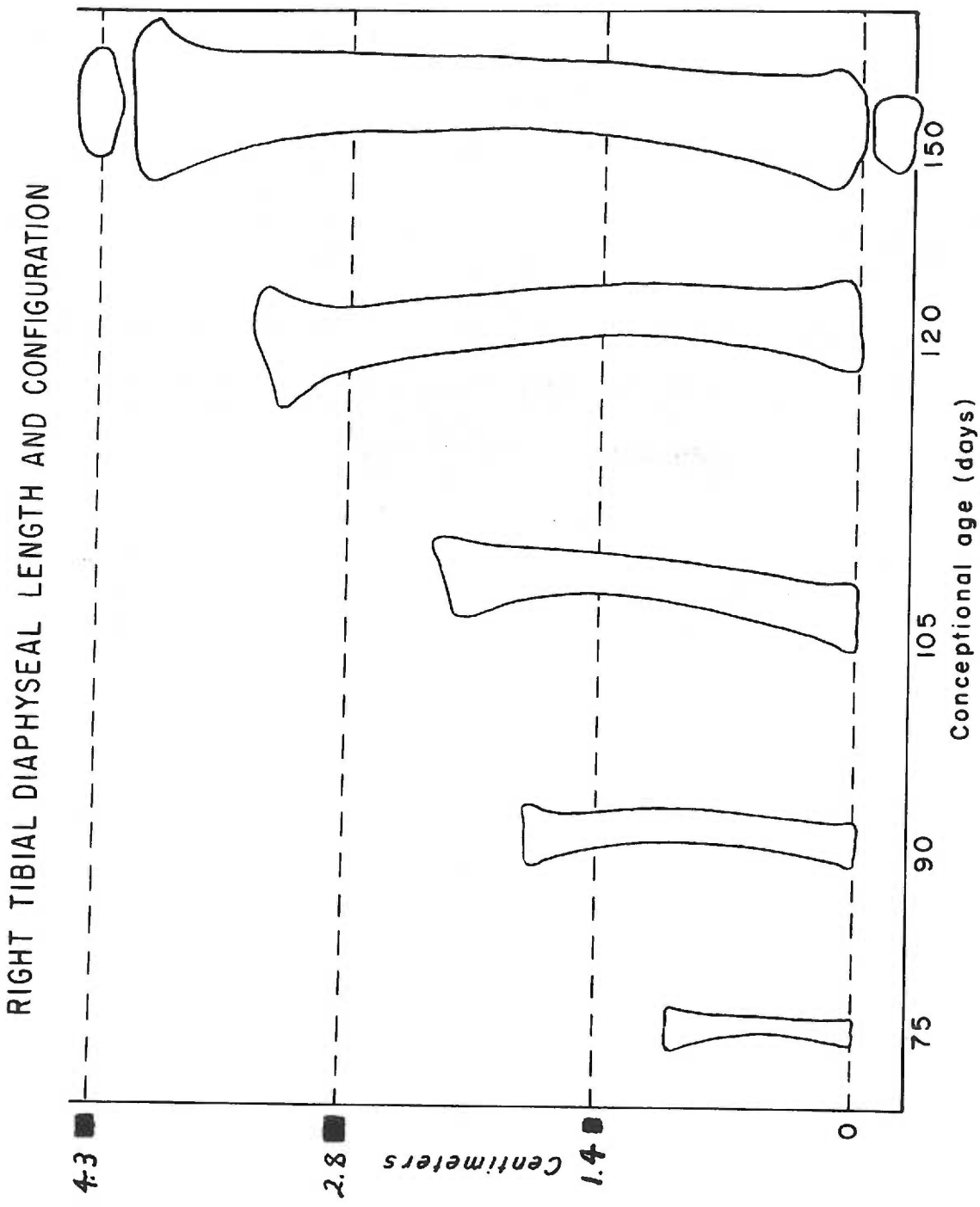
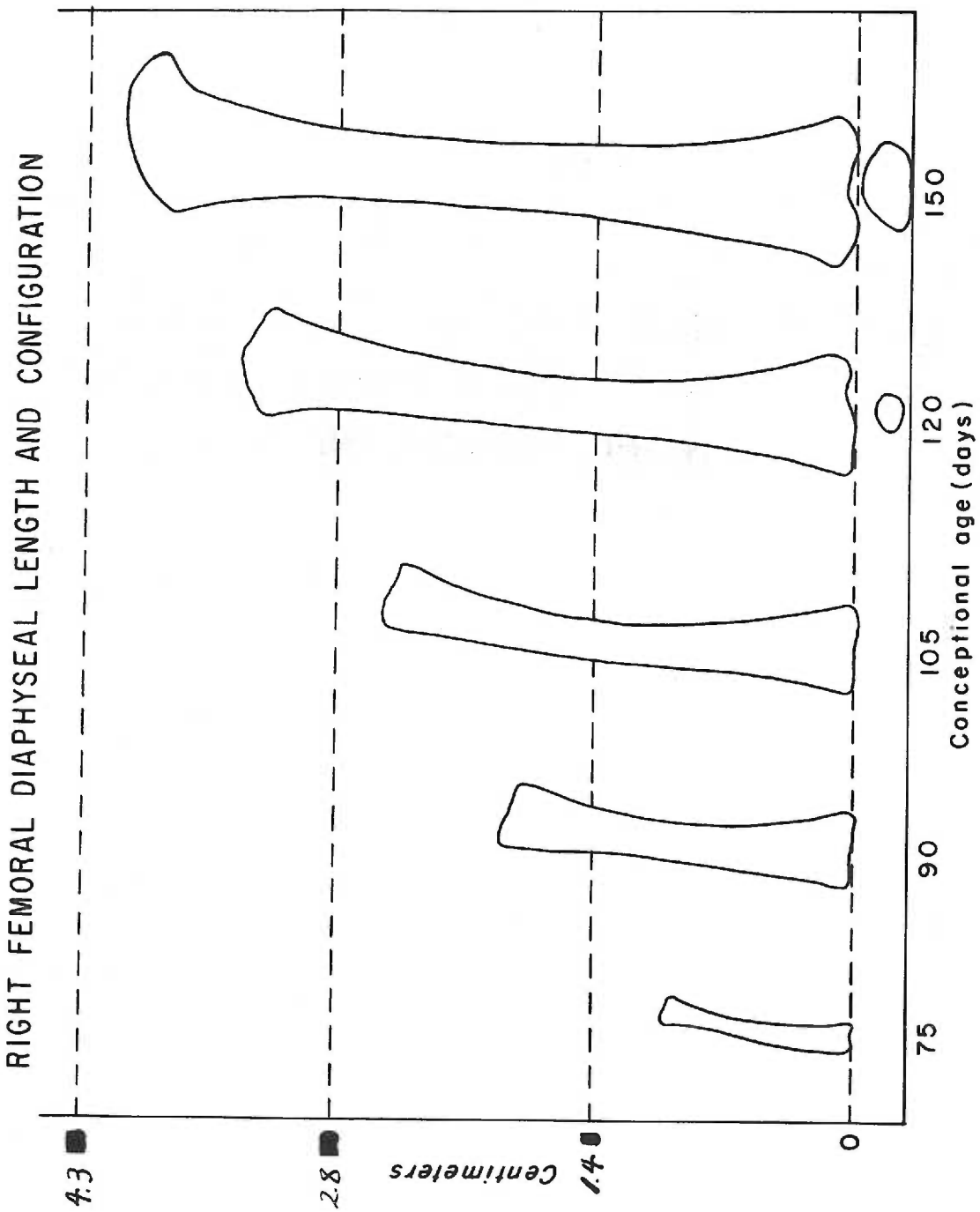

RIGHT TIBIAL DIAPHYSEAL LENGTH AND CONFIGURATION
4.3
2.8
1.4
0
Centimeters
75
90
105
120
150
Conceptional age (days)

Figure Seven: Right femoral diaphyseal length and configuration, demonstrating secondary centers (magnified 3-1/2 times)


RIGHT FEMORAL DIAPHYSEAL LENGTH AND CONFIGURATION
4.3
2.8
1.4
0
Centimeters
75
90
105
120
150
Conceptional age (days)

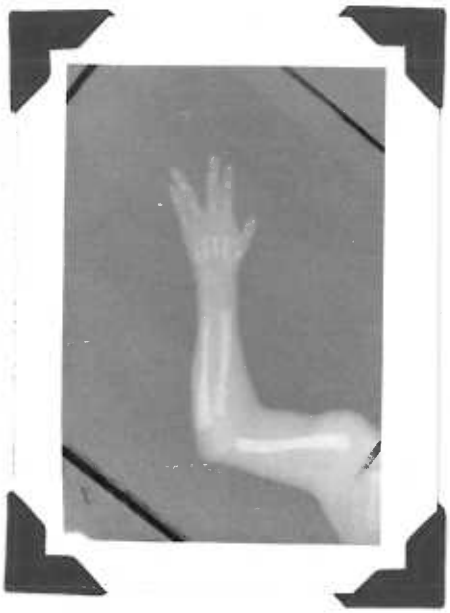
is true for long bones in general in most all vertebrates.(9) This feat is all the more remarkable when one considers that bone tissue with its unyielding mineralized intercellular substance is incapable of expansive or interstitial growth. Growth occurs only at the surface by the apposition of new bone on old.(80) Extensive remodeling must take place whereby long bones increase in length, yet maintain their distinctive configuration. The complexity of this remodeling process has been graphically demonstrated by using radioautographs to trace the deposition and turnover of radioactive materials in long bones at various time intervals after injection.(35)

Radiographs not only record changes in the linear growth of long bones, but also demonstrate skeletal maturation. Figures eight through twelve are radiographs of the hand and wrist at each fetal age studied. Along with a contact photograph of the x-ray, a magnified sketch of the same area is presented to facilitate the visualization of detail in these small fetal bones.

Figure eight shows that at 75 days of age the metacarpals and phalanges have formed but are very small. No secondary centers have appeared.

At 90 days noticeable growth occurred in width and length of these bones in the hand. Again at 105 days

Figure Eight: Left hand and wrist at 75 days conceptional age. (Contact photo and enlarged sketch of same.)



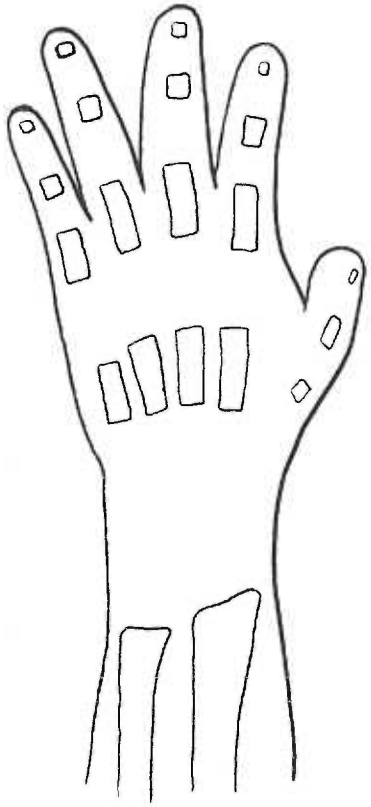
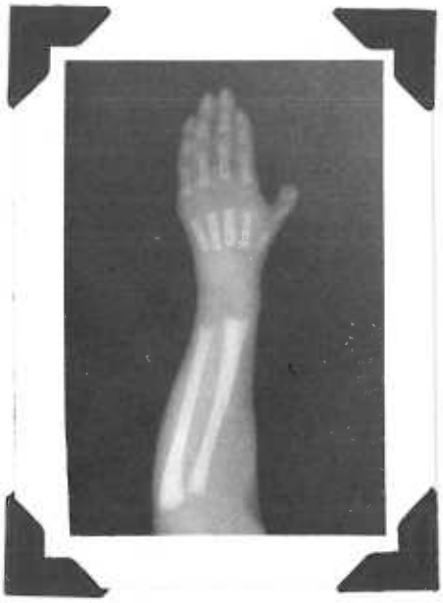


Figure Nine: Left hand and wrist at 90 days conceptional age. (Contact photo and enlarged sketch of same.)



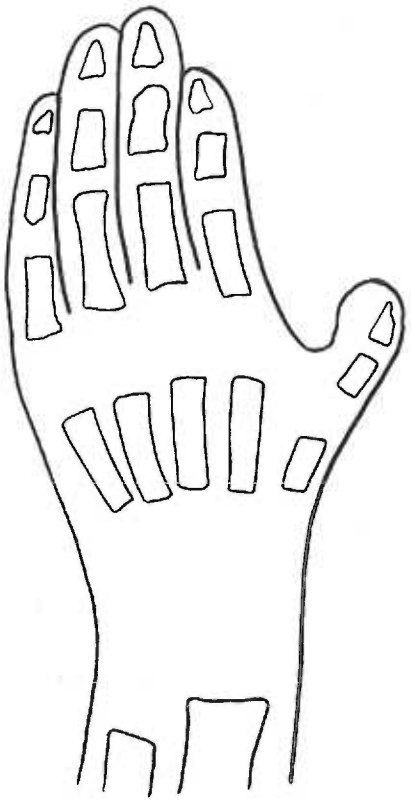
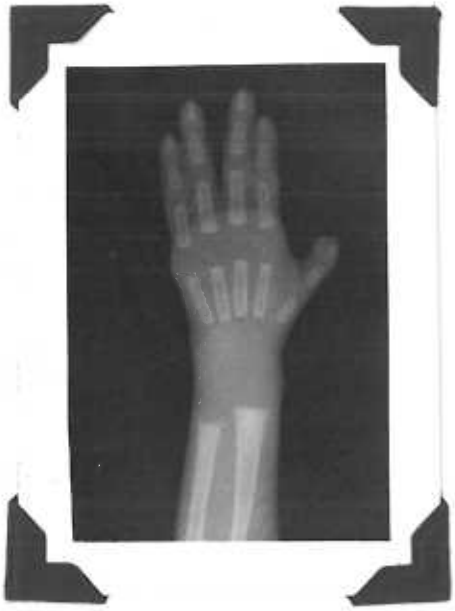


Figure Ten: Left hand and wrist at 105 days conceptional age. (Contact photo and enlarged sketch of same.)



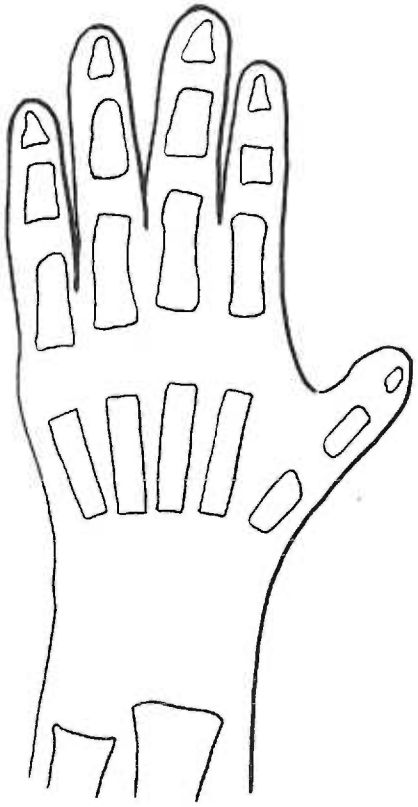
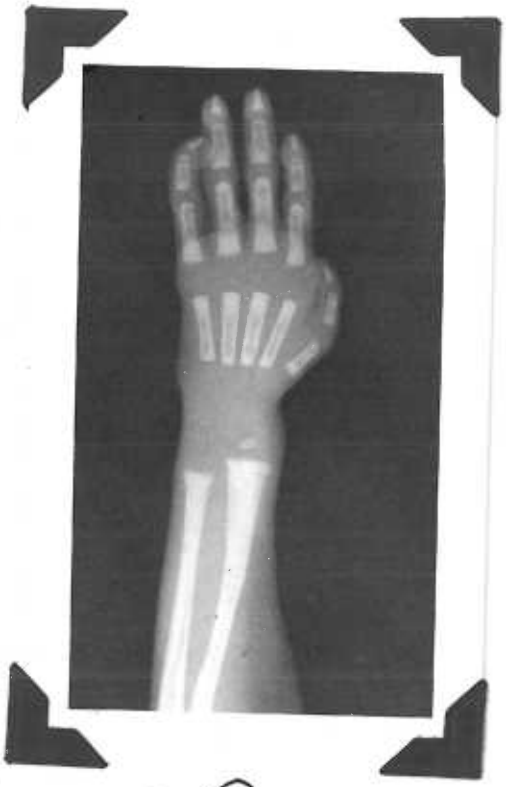


Figure Eleven: Left hand and wrist at 120 days conceptional age. (Contact photo and enlarged sketch of same.)



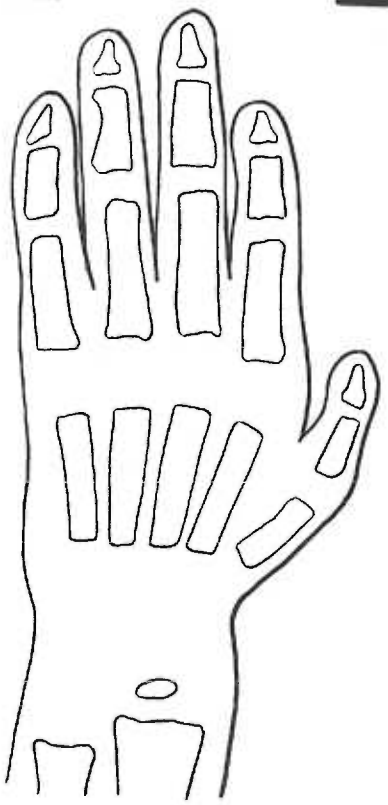
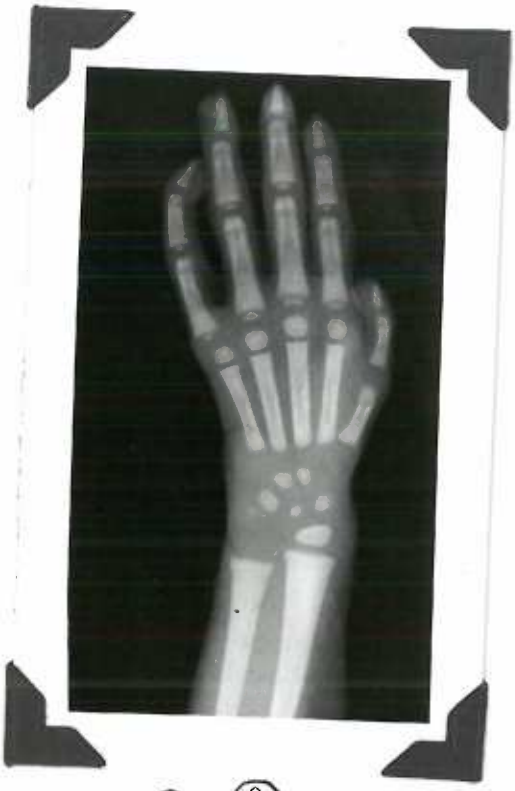
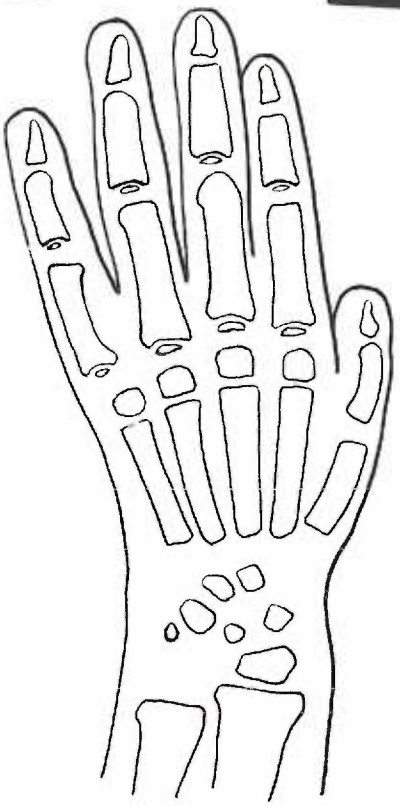


Figure Twelve: Left hand and wrist at 150 days conceptional age. (Contact photo and enlarged sketch of same.)





growth continued to be marked; yet the carpal bones and secondary centers are absent (see Figures nine and ten). The human newborn hand and wrist approximate in general appearance this 105 day monkey fetal hand and wrist.(29)

The radiograph of the hand and wrist at 120 days of age demonstrates a well-formed secondary center in the epiphysis of the distal radius (see Figure eleven). This is the first epiphyseal center to appear in the hand and wrist of the monkey and precedes any of the carpal bones. In the human, this epiphyseal center appears between 12 and 15 months, after the capitate and hamate are well formed.(29) A remarkable maturation of the monkey fetal hand and wrist occurs between 120 and 150 days. The 150 day radiograph (Figure twelve) shows the appearance of many secondary centers and six of the eight carpal bones. The second to fifth middle and proximal phalanges, and the second to fifth metacarpals all demonstrate secondary centers at this age. The distal row of four carpal bones visible at 150 days are, from left to right, the pisiform, the triquestral, hamate, and capitate. The two bones proximal to the three mentioned above, but distal to the radial, epiphyseal center are, from left to right, the lunate and scaphoid.(77) It is now impossible to compare accurately the hand and wrist of the 150 day monkey fetus to the human

hand and wrist because of the different sequence of skeletal maturation processes; however, the general appearance of the human hand and wrist between three and four years roughly resembles this 150 day fetus.(29)

No difference in skeletal maturation was apparent between male and female through 120 days. All the 150 day specimens were males; hence, no definite statement regarding sex differences in rate of skeletal maturation can be made. An early radiographic work done on human fetuses showed definite skeletal differences in males and females at seven months gestation.(32)

The extensive work of Greulich and Pyle(29) in studying skeletal growth and maturation of humans from birth to maturity has made it possible to use skeletal features in clinically assessing the normal growth and development of children. Such an assessment would seem to be desirable in studying the development of the monkey, as an additional parameter in measuring the effects of experimental variables. No comprehensive radiographic study of the monkey fetus and newborn is as yet available, though such a study is now in progress.(55)

G. Histological and Histochemical Observations

Major histological and histochemical features of the right tibia of the 19 monkey fetuses are presented in this

section as selected microphotographs from each age group in Figures thirteen to twenty-one. (Histological details of the specimens in each age group studied were remarkably uniform.) A detailed description of the histology can be found in the appendix.

The microphotographs of the proximal tibia of these fetuses demonstrate classical histological features of endochondral bone formation (Figures thirteen through sixteen.)[8] A distinct increase in nitrogen content was found in areas 1A and 7B at 105 and 120 days when compared to the 75 day value. This chemical finding can be correlated with histological appearances. Unfortunately, no 75 day histological specimen was available. At 105 days one observes a very active zone of proliferating cartilage cells with a large formation of intercellular matrix. Such cellular activity is undoubtedly responsible for the unit nitrogen differences.

A major event in the proximal epiphysis from 120 to 150 days is the appearance and rapid growth of the secondary ossification center. The histological features of this center at 150 days, when combined with its "chemical anatomy" and radiographic appearance should prove to be a valuable and sensitive indicator for alterations in skeletal growth and differentiation induced by experimental

variables. Moreover, intensive studies of the biochemistry of bone formation could logically focus here. The same is true of the distal tibial secondary center which contains comparable amounts of mineral constituents at 150 days.

A distinct, histological transition occurs in the mid-shaft of the tibia from 90 to 150 days (Figures seventeen through twenty). At 90 days a uniformly cancellous pattern of rather delicate bony spicules forming large tunnels is observed. By 150 days the mid-shaft appearance is that of cortical bone with well-developed Haversian systems. The microphotographs at 105 and 120 days demonstrate the intermediate states of this transition.

Sections stained for alkaline phosphatase show the appearance of the enzyme in the zone of cartilage hypertrophy, in vascular endothelial cells, osteoblasts, and possibly newly formed osteoid at all fetal ages studied. Such findings agree with the results of alkaline phosphatase studies in other specimens.(43) Opinion is still divided as to whether the enzyme is actually present in osteoid tissue.(31) The possible role of this enzyme in skeletal processes has been presented in the INTRODUCTION.

Figure Thirteen: Microphotograph of a sagittal section of the proximal tibia at 90 days. (115 x)

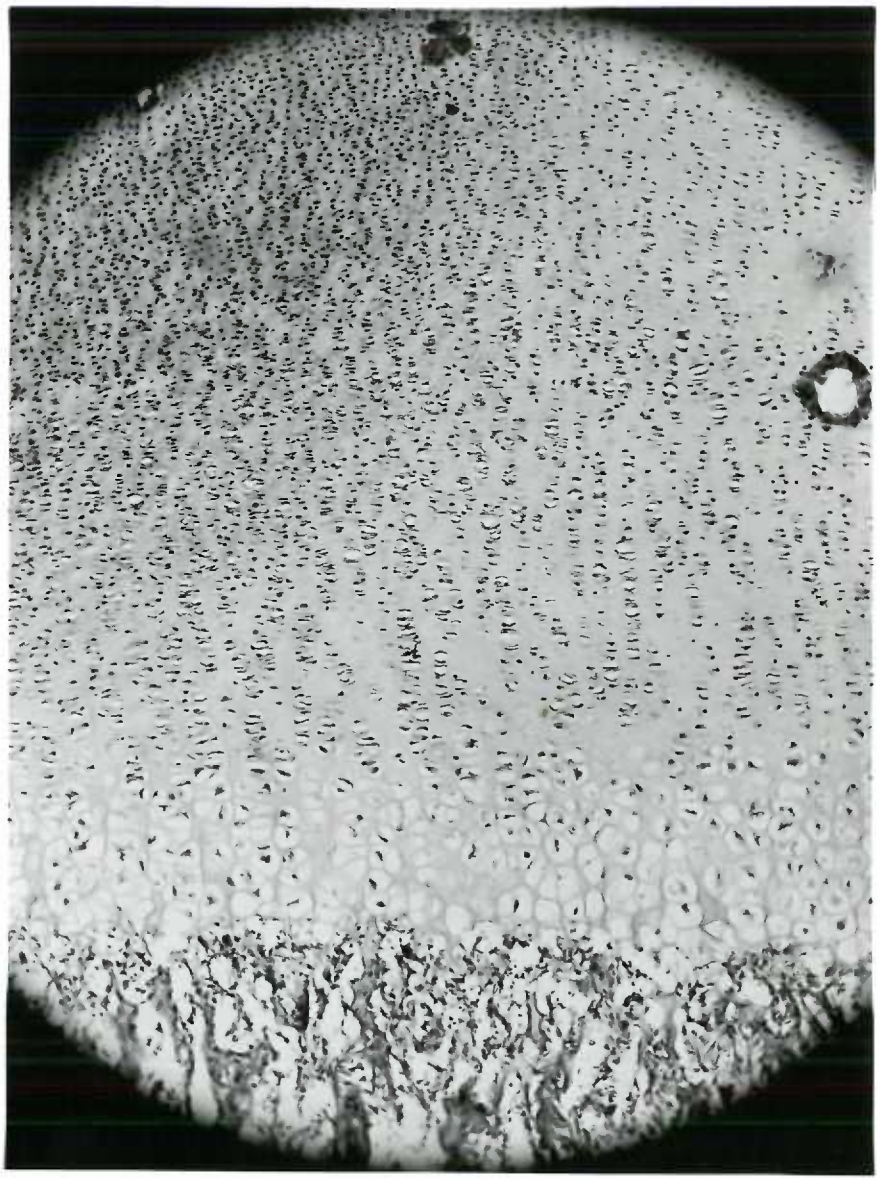


Figure Fourteen: Microphotograph of a sagittal section of the proximal tibia at 105 days (115 x)

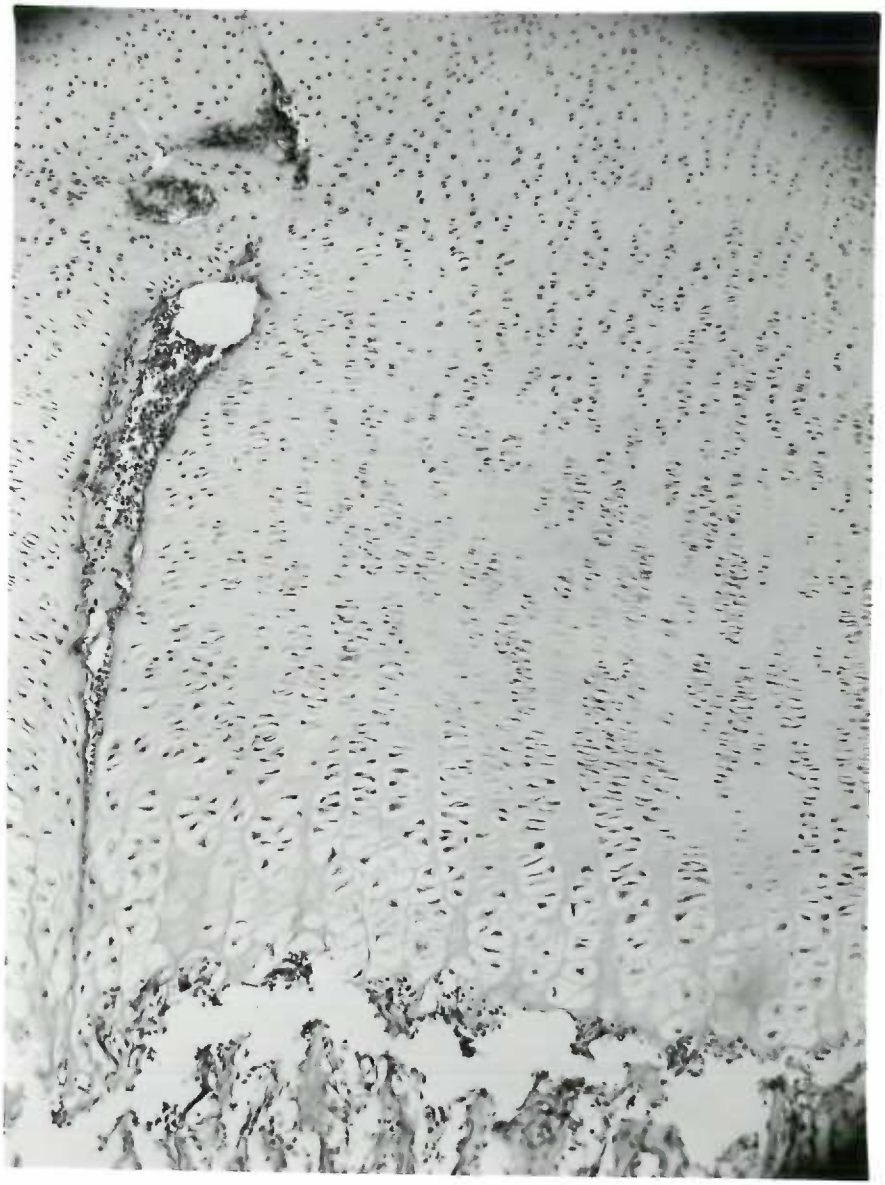


Figure Fifteen: Microphotograph of a sagittal section of the proximal tibia at 120 days (115 x)

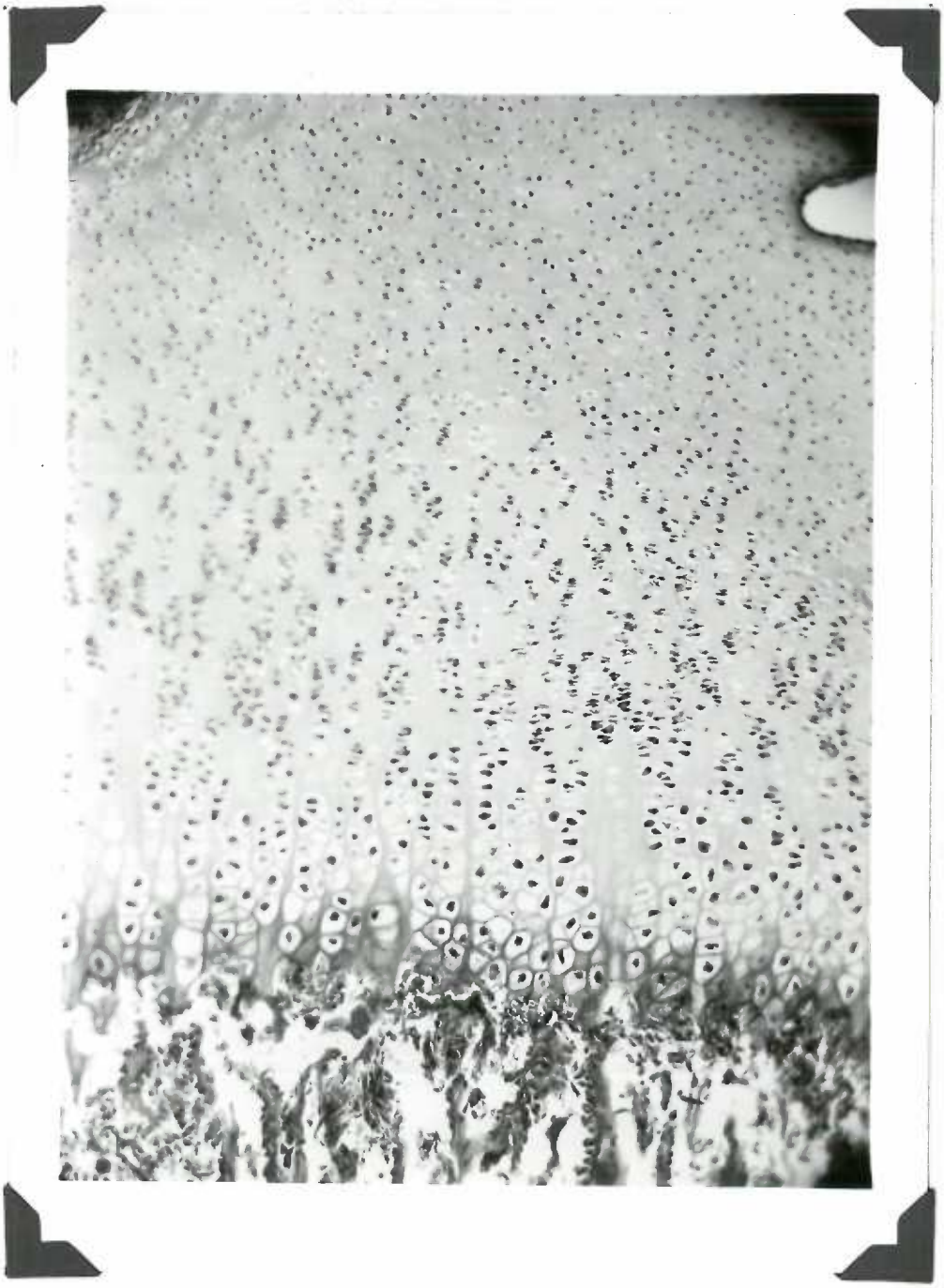


Figure Sixteen: Microphotograph of a sagittal section of the proximal tibia at 150 days (19 x)

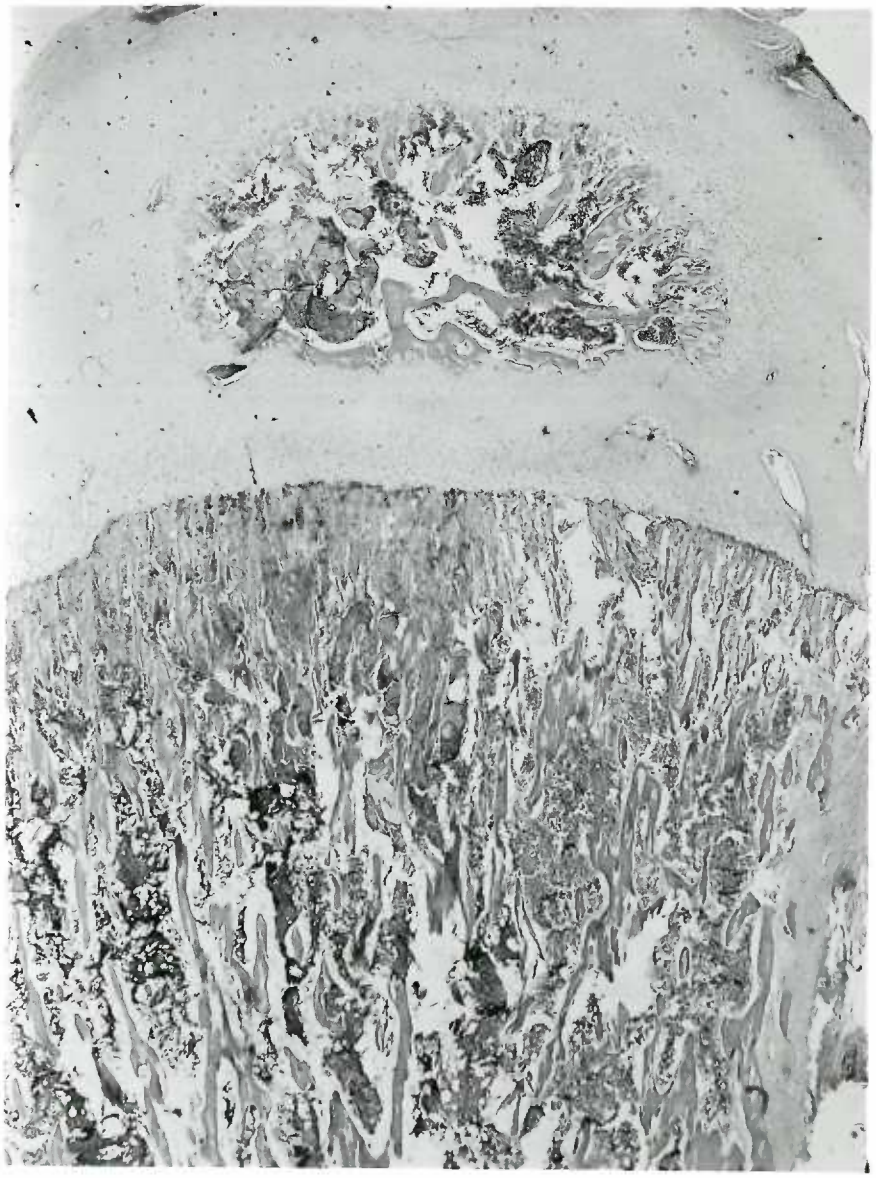


Figure Seventeen: Microphotograph of a cross-section of the mid-shaft tibia at 90 days (95 x)

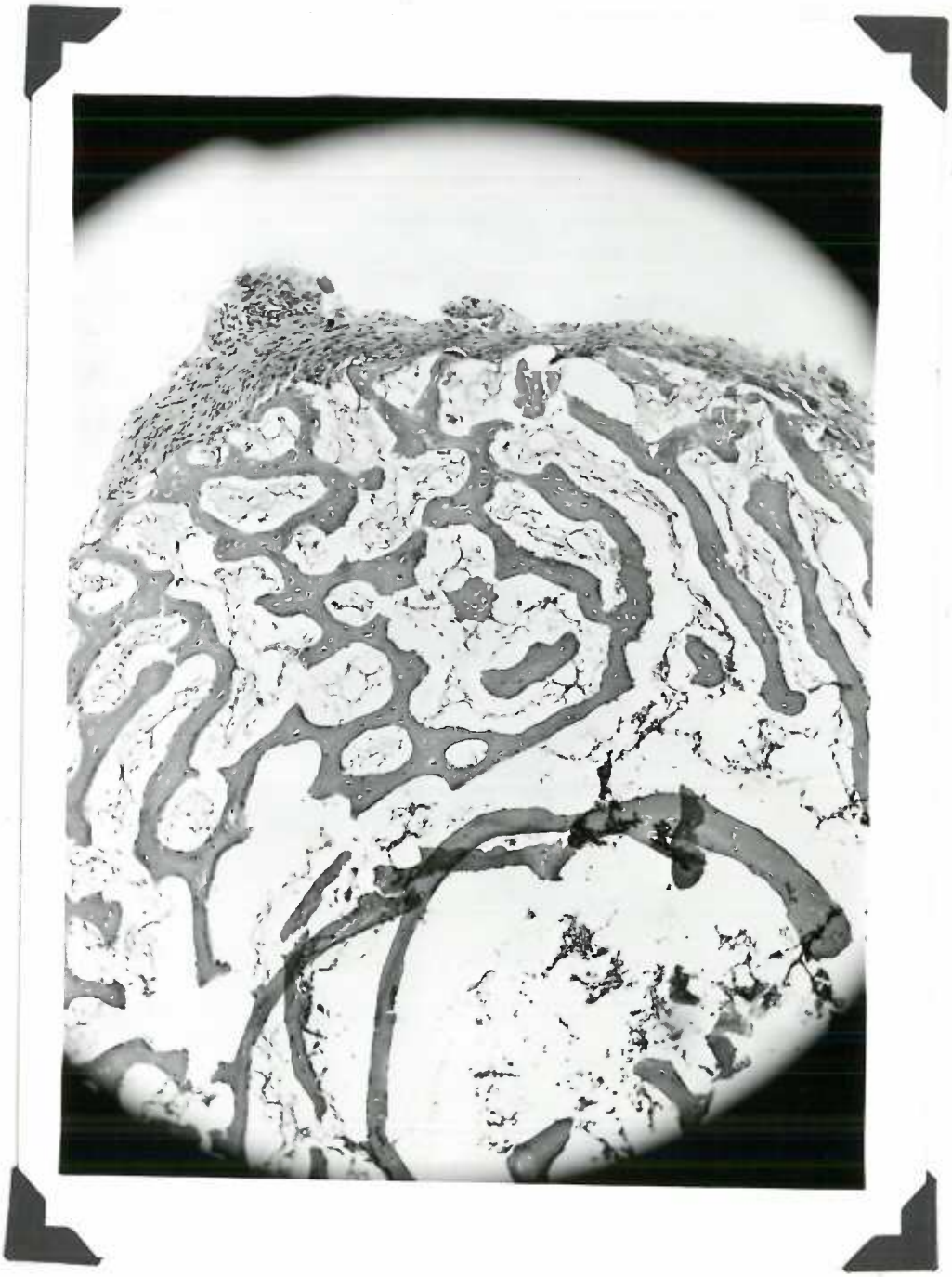


Figure Eighteen: Microphotograph of a cross-section of the mid-shaft tibia at 105 days (27 x)

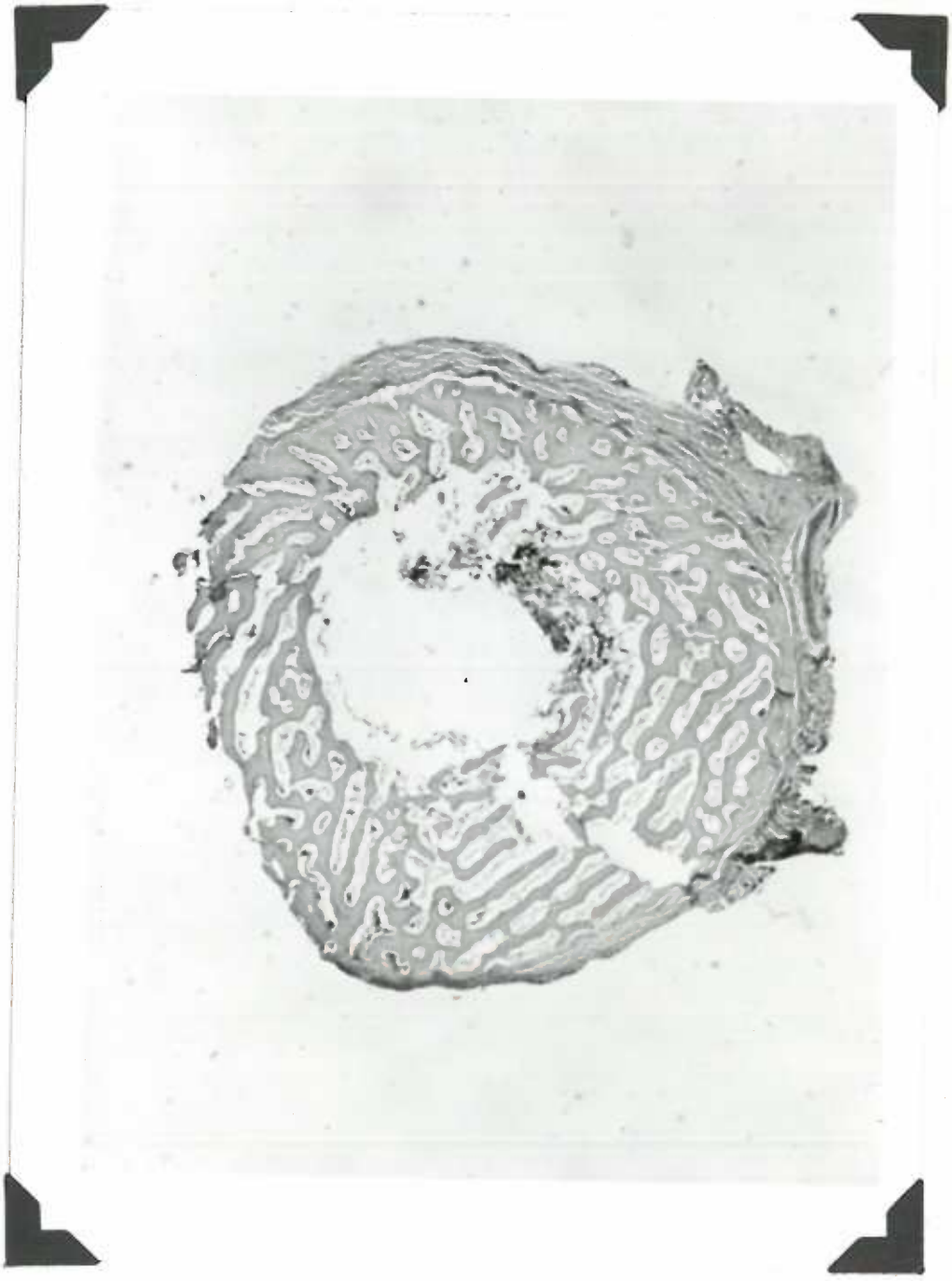


Figure Nineteen: Microphotograph of a cross-section of the mid-shaft tibia at 120 days (22 x)

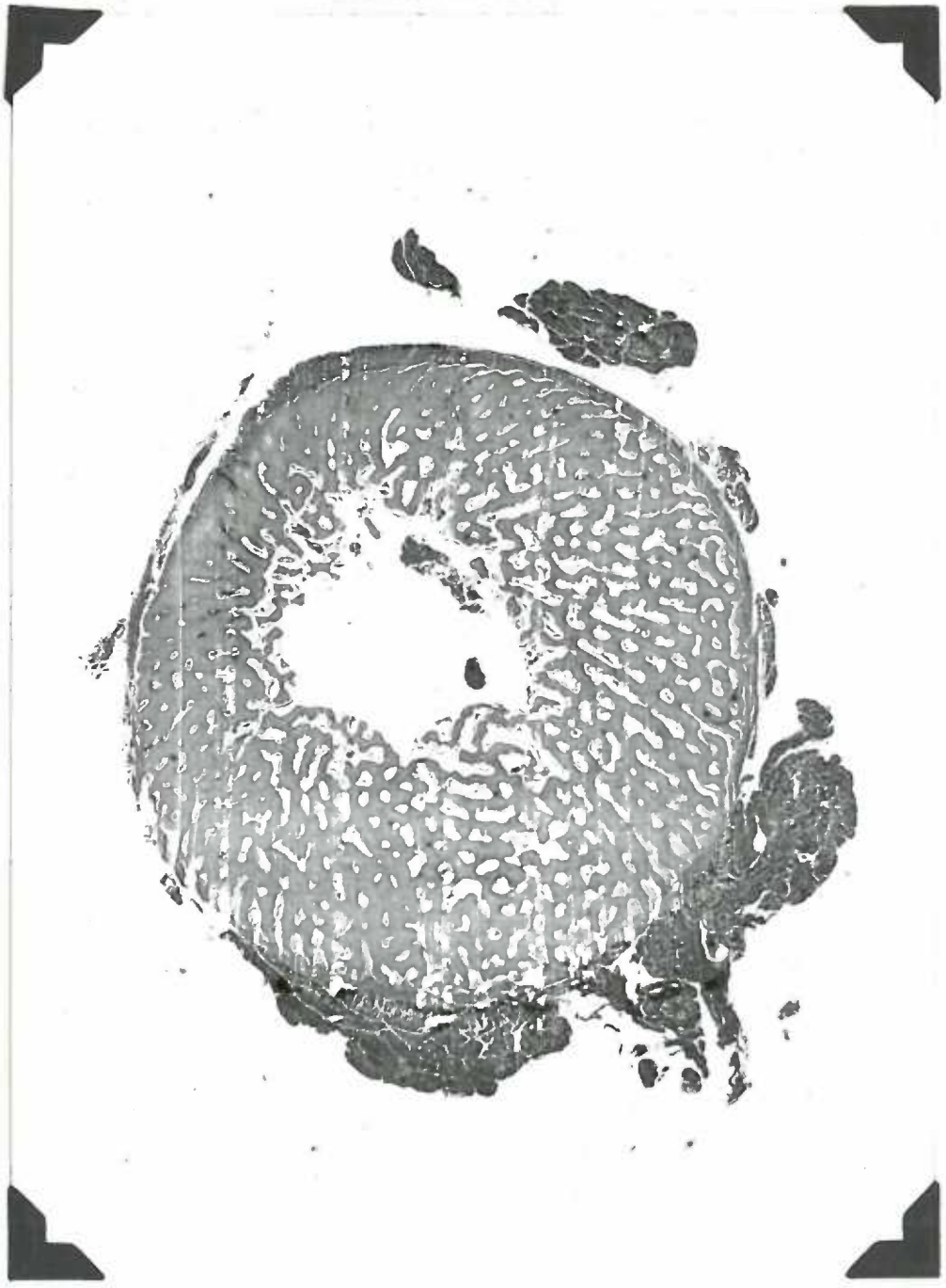
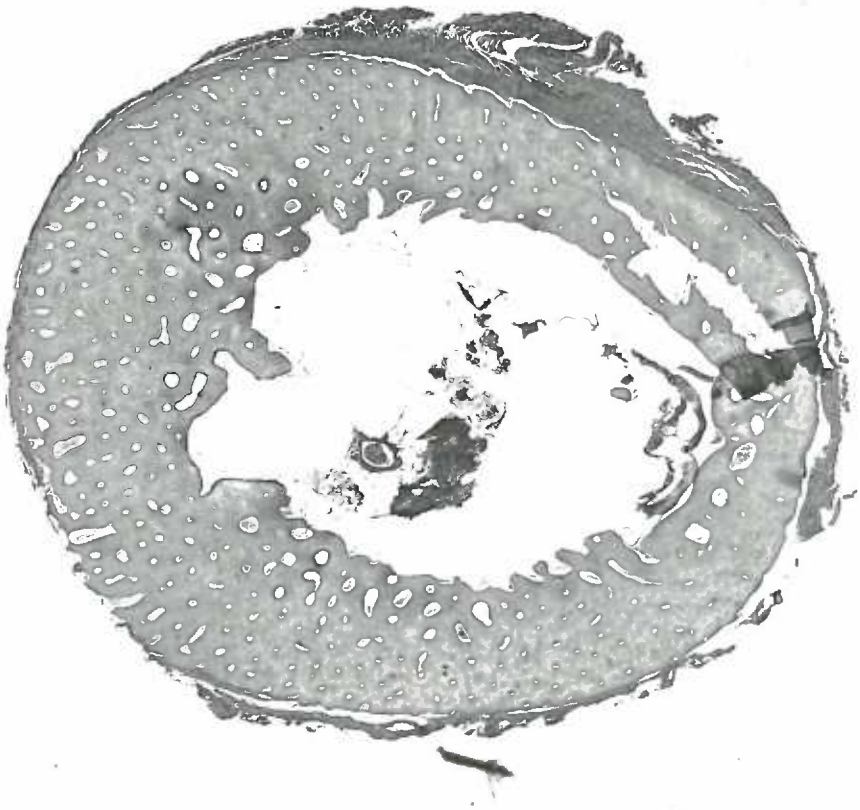


Figure Twenty: Microphotograph of a cross-section of the mid-shaft tibia at 150 days (22 x)



V. SUMMARY AND CONCLUSIONS

A combined study of skeletal growth in the Rhesus monkey fetus (Macaca mulatta) using chemical, radiographic and histological parameters is described in the light of current concepts of skeletal metabolism. The following conclusions are made.

1. Total skeletal, tibial, femoral calcium, and the daily uptake of calcium in the fetal skeleton were found to increase markedly with age.

2. Comparisons of total skeletal calcium to body weight and tibial diaphyseal length revealed a disproportionate relationship during this period of fetal life.

3. The mineral composition of the tibia did not accurately reflect total skeletal chemical growth. The inaccuracy of predicting total skeletal growth from an analysis of one bone was discussed.

4. The unit mineral content of the total diaphyseal tibia and of various regions within this bone were not uniform as growth and development proceeded. The unit calcium content increased significantly from 75 to 120 days of age and subsequently fell at 150 days of age. Such a paradoxical drop of unit calcium content at 150 days is presumably due to an insufficient supply of mineral constituents to meet the demands of continued long bone growth,

and the rapid development of new bones and secondary ossification centers.

5. The unit calcium content and the calcium/nitrogen molar ratio did not show significant patterns of change in different areas of the tibial diaphysis at any one age. Such a finding suggests a rapid rate of mineralization in areas of new bone formation and is compatible with other studies in this regard.

6. The molar ratio of calcium/phosphorus showed no significant changes with age or with a comparison of selected areas along the tibial diaphysis.

7. The radiographic appearance of the femur and tibia demonstrated the preservation of distinct configuration despite rapid changes in length and width. The radiographic appearance of the hand and wrist revealed a rapid appearance of carpal bones and secondary ossification centers from 120 to 150 days conceptional age.

8. Major histological features of the proximal and mid-shaft tibia are presented along with a histochemical study of alkaline phosphatase in these same areas. Alkaline phosphatase was found in the cartilage cells of the hypertrophic zone, in osteoblasts, vascular endothelial cells and possibly osteoid tissue.

9. The proximal and distal tibial secondary ossification centers contain comparable amounts of mineral constituents at 150 days. Their chemical anatomy, the radiographic configuration, and histological features are defined in this study and are suggested as useful growth parameters for future studies.

10. The unit nitrogen content of the proximal and distal tibial epiphyseal areas increased markedly from 75 to 105 days, a chemical feature reflected histologically in the intense proliferation of cartilage cells with increasing amounts of matrix laid down during this period of time.

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APPENDIX A

I. Purina Monkey Chow

a. Guaranteed Analysis

Crude Protein not less than	15.0%
Crude Fat not less than	5.0
Crude Fiber not more than	3.0
N.F.E. not less than	57.0

b. Ingredients

Ground wheat, ground oat groats, ground yellow corn, dried skimmed milk, soybean oil meal, corn sugar, animal fat (preserved with buylated hydroxyanisole), vitamin B_{12}, riboflavin supplement, calcium pantothenate, niacin, folic acid, pyridoxine hydrochloride, thiamin, ascorbic acid, vitamin A feeding oil, D activated plant sterol, vitamin E supplement, 1.5% calcium carbonate, 1% defluorinated phosphate, .5% iodized salt, and traces of iron oxide, manganese sulphate, copper oxide, cobalt carbonate, zinc oxide.

c. Chemical Composition

Protein, %	15.22
Fat, %	6.08
Fiber, %	2.60
N.F.E., %	57.97
Ash, %	5.20

Calcium, %	.97
Phosphorus, %	.54
Potassium, %	.54
Magnesium, %	.15
Sodium, %	.24
Chlorine, %	.28
Iron, p.p.m.	175.89
Zinc, p.p.m.	16.19
Manganese, p.p.m.	44.92
Copper, p.p.m.	13.57
Cobalt, p.p.m.	.26
Iodine, p.p.m.	.80
Carotene, p.p.m.	1.36
Vitamin A, I.U./gm.	13.80
Vitamin D, U.S.P. units/gram	2.20
Vitamin E, p.p.m.	22.95
Riboflavin, p.p.m.	9.00
Thiamin, p.p.m.	7.76
Niacin, p.p.m.	91.41
Pantothenic Acid, p.p.m.	66.02
Choline, p.p.m.	906.29
Folic Acid, p.p.m.	2.92
Pyridoxine, p.p.m.	4.14

Vitamin C, mg./lb. (added)	150.00
Vitamin B_{12} supplement, mcg./lb.	5.13

II. Similac®

Approximate analysis of powder:

Fat	26.85%
Carbohydrate	53.40%
Protein	13.75%
Minerals	4.00%
Calcium	.60%
Phosphorus	.40%
Iron	trace
Moisture	2.00%
Calories per oz. avoir.	145
	per liquid quart
Vitamin A	2500 USP units
Vitamin D	400 USP units
Vitamin B_1 (thiamine)	.65 mg.
Vitamin B_2 (riboflavin)	1.00 mg.
Vitamin C	50.00 mg.

APPENDIX B

I. Microchemical Analysis for Calcium in Bone

A. Principle:

Calcium in bone was determined from a modification of the method for determining serum calcium as described in the Beckman application data sheet DU-9-B.[7] This method employed the Beckman Model DU spectrophotometer with flame and photomultiplier attachments. Each sample was precipitated as calcium oxalate to eliminate the interfering substances.

B. Reagents:

1. Buffered oxalate solution, pH 5. One volume of saturated ammonium oxalate, one volume of molar sodium acetate buffer (pH 5) and three volumes of demineralized water.

2. 2% ammonium hydroxide saturated with calcium oxalate and filtered.

3. Working standard calcium solution: 40 micrograms (µg.) calcium per ml. is prepared using calcium carbonate of primary standard grade.

4. 6N hydrochloric acid.

C. Preparation of the sample:

To an aliquot of ashed, neutralized bone solution containing 20-100 µg. calcium, 4 ml. buffered oxalate

solution was added and allowed to stand at least 4 hours or overnight for complete precipitation of calcium oxalate.

The precipitate was centrifuged at 2200 rpm for 10 minutes. The supernatant was removed, leaving approximately 75 microliters (µl.) solution in the tube over the precipitate.

The precipitate was washed with 1 ml. of 2% ammonium hydroxide solution and centrifuged again at 2200 rpm for 10 minutes. The supernatant was removed and the tube dried in a sand bath.

The calcium oxalate precipitate was then completely dissolved in 0.1 ml. of 6N HCl and finally diluted with 2.0 ml. of demineralized water, care being taken to mix completely.

D. Flame Spectrophotometric Procedure:

An oxygen-acetylene flame was used with the Beckman Model DU spectrophotometer and the emission read on the per cent transmission scale. The emission of a sample was compared to a working standard and the sample concentration was read directly from a prepared standard curve. The response of the calcium emission at 554 mµ was found to be linear over the concentration range used.

Instrument settings were selected as follows:

a. wavelength 554 mμ

b. blue sensitive phototube with a 10,000 megaohm load resistor

c. range 0.1

d. slit width 0.055 mm.

e. photomultiplier sensitivity 3

f. a final oxygen pressure of 10 lbs. per square inch and acetylene pressure of 2 lbs. per square inch.

E. Range and accuracy:

In an analysis of a replicate series of 10 bone samples, a mean variation of 0.73 μg. calcium per ml., and a standard deviation of 0.86 μg. Ca/ml. was found. This represents a maximum error of ± 2.2% in 95% confidence range. In addition, the recovery of standard calcium in 6 bone solutions that had been ashed and analyzed ranged from 99 to 103%.

F. Comments:

Both cations and anions exert significant alterations on the flame emission of calcium.[4,47,73] Hence it was felt desirable in our microanalysis to remove interfering substances by precipitation of calcium as its oxalate, monohydrate salt. Calcium phosphate and calcium

carbonate precipitates were found not to be as desirable as the calcium oxalate. The phosphate anion exerts a powerfully depressant action on the flame emission of calcium.(4) Both calcium phosphate and calcium carbonate are precipitated in alkaline solution. At this pH, magnesium phosphate is also precipitated. Magnesium likewise exerts a depressant effect on the spectral emission of calcium.(73)

It is essential that the pH of the oxalate solution be kept at 5 in precipitating calcium as its oxalate salt. At a pH below 4.5 calcium oxalate is incompletely precipitated, since the salt is acid soluble. At a pH above 5.5, the possibility of bringing down calcium phosphate and magnesium oxalate is greatly enhanced. Hence, the pH of the bone sample in its oxalate precipitating solution was checked.

To remove any possible magnesium oxalate precipitated with the calcium oxalate, the precipitate was washed with two per cent ammonium hydroxide. This removes the alkaline soluble magnesium oxalate.

II. Micromethod for the Determination of Phosphorus in Bone.(72)

A. Principle:

Phosphate reacts with molybdic acid to form phosphomolybdic acid. Reduction of phosphomolybdic acid by

ferrous sulfate produces a deep blue color. The optical density of the color was read in a Beckman DU spectrophotometer. With ferrous sulfate as the reducing agent it is necessary to add sulfuric acid in order to prevent color production through reduction of molybdic acid. The quantity of sulfuric acid used is not enough to cause hydrolysis of labile phosphate esters.

B. Reagents:

1. 10N sulfuric acid

2. Ammonium molybdate, stock solution: 10% in 10N sulfuric acid

3. Ferrous sulfate-ammonium molybdate reagent made fresh before use. 4 ml. of the stock molybdate were delivered into a 50 ml. amber, graduated cylinder and demineralized water was added to the 35 ml. mark. 2.0 gm. of ferrous sulfate was then added. The salt was dissolved by shaking, and the solution made to 50 ml.

4. Potassium acid phosphate, stock solution: 10 mg. phosphorus per 100 ml.

C. Procedure:

Into a suitable size test tube one volume of bone solution was added to one volume of fresh color reagent. As a reference blank, one volume of 0.7 N sulfuric acid was added to one volume of the color reagent. The

color developed within 5 minutes and was stable for at least one hour. The optical density of the solution was read at 720 mμ with a standard and with a reference blank.

D. Range and accuracy:

Construction of a standard curve demonstrated that color development followed Beer's law from one to twelve μg. phosphorus per tube. All determinations were made in this range.

Reproducibility of the method was determined by performing 10 analyses at different days on a phosphorus standard solution. A maximum error of ± 4% in the 95% confidence range was determined for these 10 samples.

Recovery of a standard phosphorus solution from 6 samples of ashed bone ranged from 100.2% to 100.9%.

III. Micro-Kjeldahl Method for the Determination of Nitrogen

A. Principle:

The sample was decomposed by boiling with a digestion mixture consisting of sulfuric acid, potassium sulfate and copper selenite. This digestion converted the nitrogen to ammonium sulfate. With excess alkali, the liberated ammonia is steam distilled into boric acid, then titrated with standard hydrochloric acid.

B. Reagents:

1. Digestion mixture: Sulfuric acid is diluted with an equal volume of water saturated with potassium sulfate and containing 0.1% copper selenite.

2. Indicator mixture:

a. 5 parts 0.1% brom cresol green

b. 1 part 0.1% methyl red

in 95% ethyl alcohol

3. One-half saturated solution of boric acid.

4 ml. of the indicator mixture is added to one liter of half-saturated boric acid solution.

4. Saturated sodium hydroxide solution

5. Standard 0.1N hydrochloric acid.

C. Procedure:

To a 30 ml. digestion flask add 1/2 to 5 ml. of bone sample (50-500 µg. nitrogen), 2 ml. of digestion mixture, a boiling stone, and place on the digestion rack. Digest until 15 minutes after the disappearance of fumes from the neck of the flask. Cool.

Transfer digested solution into the distilling apparatus by washing the flask two or three times with distilled water. Add 3 ml. of saturated sodium hydroxide solution. Distill the ammonia into an Erlenmeyer flask containing 10 ml. of boric acid-indicator

mixture. Distillation takes from 5 - 10 minutes.

Titrate distillate with 0.1 Normal standard hydrochloric acid.

D. Calculations:

ml. HCl x normality HCl x 14 = mgm. nitrogen in sample

E. Range and accuracy:

As little as 50 µg. of nitrogen can be quantitatively analyzed with this method. A series of 5 nitrogen standards containing approximately 200 µg. of nitrogen gave a maximum error of 1% in the 95% confidence range. The digestion step was evaluated by adding nitrogen to 6 bone samples which were then digested and distilled. Recoveries of these nitrogen samples ranged from 98.7% to 101%.

APPENDIX C

This appendix is concerned with histological and histochemical observations on the tibiae of selected monkey fetuses from 90 through 150 days. (Adequate histological specimens were not obtained at 75 days.) The preparation and staining of bone specimens has been previously described in METHODS AND MATERIALS.

90 days

The head of the proximal tibia is entirely cartilage, most of which is embryonic in type. Several vascular channels are seen coursing through this cartilage. The transition to the zone of proliferating cartilage cells is rather irregular and gradual. Proliferating cells are seen to be markedly basophilic, truncated, and surrounded by increasing amounts of intercellular matrix. Below this area, one sees hypertrophied cartilage cells which appear as a distinct zone. However, a gradual enlargement of the lacunae of the proliferating cells is noted as they approach the zone of cartilage hypertrophy. The cells in this area take a light stain and undergo a progressive sequence of cytoplasmic, degenerative changes with accompanying nuclear pyknosis and disintegration. Diaphyseal capillary erosion of the last cell layer is orderly and appears vigorous. The thin matrix between the

hypertrophied cartilage cell columns forms the scaffolding for delicate, regularly spaced primary bone trabeculae. Osteoblastic activity is vigorous, as is true in all the sections studied at these fetal ages. The zone of secondary spongiosa is characterized by shorter, coarser spicules of bone which are found immediately below the more delicate primary trabeculae.

A cross-section through the mid-diaphysis at this age shows the two layers of periosteum surrounding a uniformly cancellous bony pattern. Thin, interconnecting spicules form bony tunnels, which, by appositional growth of new bone, will form the definitive Haversian systems of cortical bone.(29) From the inner cambium layer of the periosteum, capillary tufts and osteoblasts can be seen to extend into uneven grooves of adjacent bone. By further appositional growth, these grooves will enclose periosteal buds to form new bony tunnels, thus increasing the width of the shaft.(29)

105 days

The proximal cartilage head has now become smaller with a relative decrease in the zone of embryonal cell cartilage. Vascular channels are again prominent. The zone of proliferating cartilage now occupies a somewhat larger area of the cartilage head. The area of cartilage hypertrophy is essentially unchanged in general appearance.

The mid-shaft cross-section of the tibia at this age has the same general features described in the 90 day fetus. However, most bony tunnels are formed by shorter, thicker, less delicate trabeculae at this age.

120 days

The cartilage head is now broader and relatively flattened. No secondary center in the epiphyseal area is, as yet, seen, but cartilage cells in the center of the embryonal zone are undergoing vesicular changes that will shortly be followed by a definitive osseous epiphysis. The width and character of the zones of proliferating and hypertrophied cartilage cells remains in general appearance as described previously. Likewise, capillaries tend to be directed against each cell column, and the cartilage-bone margin retains its regularity.

At the mid-shaft, the peripheral surface of bone adjacent to the periosteum is considerably less trabeculated than at earlier ages. Many periosteal buds still extend into shallow bony grooves. The general osseous appearance is still that of a lattice work of cancellous bone, though the diameter of the bony tunnels is still now much smaller in relation to the surrounding lamellae. This appearance foreshadows the subsequent compact Haversian systems of cortical bone.

150 days

The epiphyseal center of ossification is now well established in the proximal tibia and thereby largely replaces the area of embryonal cartilage. The secondary center is surrounded circumferentially by vesiculated cartilage cells which undergo the same maturational and degenerative sequence as described for cartilage replaced by metaphyseal bone. The formed, epiphyseal plate is now mainly composed of proliferating and hypertrophied cartilage cells. The amount of matrix observed between columns of hypertrophied cells remains a thin core. Capillaries are not attacking each individual cell column with as great a regularity as before. The primary trabeculae are now somewhat coarsened, and are not regularly formed around a single cartilage matrix core, but usually occupy the area of two or three cartilage columns. The secondary spongiosa is likewise coarser than at earlier fetal ages.

The appearance of the tibial midshaft at this age approximates typical compact, cortical bone. The exterior of the bony shaft is now relatively smooth, but periosteal vessels with accompanying osteoblasts continue to be seen in their task of forming new periosteal bone. In addition, occasional osteoclasts are seen, apparently eroding the adjacent exterior bone surface. The bony tunnels of

earlier ages now appear as typical Haversian canals of cortical bone.

Histochemical Studies

Sections obtained for alkaline phosphatase showed the appearance of this enzyme in the zone of cartilage hypertrophy, in vascular endothelial cells, osteoblasts, and possibly newly formed osteoid at all fetal ages studied.

In the zone of cartilage hypertrophy, alkaline phosphatase is detected first in the nucleus, then quickly appears in the cytoplasm and the intercellular matrix. Heavy staining in the area of invading capillaries and osteoblasts with the formation of the primary spongiosa is due to the presence of the enzyme in the vascular elements as well as in active osteoblasts. Endothelial cells in the vascular channels of the cartilage head also show the presence of alkaline phosphatase. A positive stain appears as a fine rim along some sections of the primary trabeculae. Whether this is enzyme in the newly forming osteoid or is an artefactual diffusion of stain from closely adjacent osteoblasts is impossible to state categorically. Opinions are divided in the literature as to whether alkaline phosphatase is actually present in osteoid tissue.(31)

In cross-sections of the tibial mid-shaft one sees a heavy positive stain in the vascular and osteogenic cells

of the inner, cambium periosteal layer. Again, the marrow elements in the bony tunnels and the osteoblasts adjacent to bony spicules are seen to stain positively.

No microphotographs of the alkaline phosphatase stain in the tibia are available.