An Experimental Inquiry Into the Possible Role of Neurotrophic Mechanisms in Skeletal Development Following Removal of Neural Crest Precursor Cells of Dorsal Root Ganglia and Sympathetic Ganglia in the Chick Embryo.

Randolph Bernard Malloy
Louisiana State University and Agricultural & Mechanical College

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ABSTRACT

This study was carried out in order to elucidate the influence of neurotrophic (GSA, GVA, or GVE) mechanisms on the developing tibia-fibula in the chick embryo.

In chick embryos at stage 17 (Hamburger and Hamilton, 1951), the neural crest and portions of the alar plate were removed at the leg level. Behavioral parameters, along with gross, ionic, histochemical, and neuro-skeletal parameters were studied in control and experimental embryos from 6 through 12 days of incubation. Also, an assessment of vascular flow parameters and vascular innervation was accomplished over the same embryonic period.

Following incubation, control and experimental embryos were sacrificed for gross and histological studies or utilized for in vivo vascular flow studies.

Analysis of tibial length, width, wet weight, and dry weight showed no statistical differences (p < 0.05) between experimental and control embryos. Assessment of ionic concentrations (Ca++, Mg++, iPO4) showed absence of any significant differences (p < 0.05) between control and experimental embryos. Histochemical analysis showed no observable differences between calcification and collagen parameters in the experimental and control groups, however, marked changes in acid mucopolysaccharides were noted at every age. Vessel reactivity to exogenous catecholamines and a-blockers commenced at 7 days of incubation, and increased to maximum values at 9 days. Neurotransmitter was not found in the nerves of the vascular system until 1 day post-hatch. No synaptic
relationships were found between neural and skeletal elements, although nerve fibers came into close association with periosteal cells.

These findings indicate that the removal of afferent and visceral efferent innervation does not influence the gross development, calcification, or collagenous deposition but does effect acid mucopolysaccharide distribution in skeletal elements of long bones. The results also seem to show that vascular parameters are not influenced by neural crest removal, however, differences in this area of the experiment may have been masked due to the use of homeostatic controls. Since Dawes (1974) and McCuskey (1969) have shown that stressful situations can cause drastic changes in the blood levels of metabolites and in the vascular reactivity of fetal vessels, further studies are indicated to delineate the possible relationship of neural crest removal to the alterations in skeletal constituents (acid mucopolysaccharides) that have been noted in this experiment.
INTRODUCTION

The bony skeleton, which comprises the main supporting structure of vertebrates is composed of three tissues: connective tissue, muscle, and nerve. These tissues are arranged in a unique relationship which enables the bony organ to serve its primary function. Although this organ presents itself as a hard, morphologically constant, and relatively inert substance in post mortem specimens, in vivo it is a highly metabolically active organ and responds dramatically to various environmental influences. Environmental factors (physical and chemical agents) together with genetic factors, control the initiation, differentiation, and maintenance of the skeletal elements.

I. GENETIC FACTORS

Discrepancies in genetic factors produce the most striking abnormalities in skeletal growth and development. For instance, Brachydactyly, in which fingers and/or toes may be small, deformed, or absent can be related to an autosomal dominant genetic defect (McKusick, 1969). The etiology of Parrot's Disease is also expressed via autosomal dominance, and results in impaired development and premature fusion of the epiphyseal plates (Langer, 1973). Autosomal recessive defects can also produce disorders in bone metabolism. Such diseases as Conradi-Hunermann Syndrome and Diastrophic Dwarfism fall into the latter category (Silverman, 1973). An X-linked recessive disease, Hunter's Syndrome, results in kyphosis and numerous skeletal abnormalities (Stevenson, 1973). Although the aforementioned
pathological entities occur in humans, genetic disturbances also occur in lower vertebrates. In the fowl, for instance, an autosomal recessive defect is responsible for the production of a polydactylyous talpid mutant. This mutant has shortening of the vertebral column, polydactyly with shortened limbs, and visceral ectopia (Hinchliffe and Ede, 1967). In the mouse, a short-ear(se) recessive mutant gene produces an animal that has a much smaller skeleton, and a reduced rate of callus formation in healing bone fractures, than an unaffected mouse (Green, 1968). Besides abnormal development, genetic factors can also be manifest via experimental procedures in normal embryos. Several investigators have grafted limb buds to the chorion of chick embryos, or allowed limb buds to develop in tissue culture. These procedures result not only in the development of long bones, i.e. femur and tibia, but also in the development of joints between bones (Murray and Huxley, 1925; Willis, 1936; Fell and Conti, 1954). It should be noted, however, that the grafted limb will not attain the same degree of maturation in several gross parameters as the in situ limb (Hamburger, 1940). The evidence cited above confirms the fact that bone development is controlled to a great extent via genetic factors, and that slight modifications of these factors produce profound alterations in its normal development.

II. ENVIRONMENTAL FACTORS

Although genetic factors are of utmost importance in bone development, environmental factors play a very important role in the ontogeny and maintenance of osseous tissues. Chemical agents are one group of environmental factors that assert a marked influence on bone growth and maintenance. Hormones, vitamins, ions, and fatty acidderivatives
exemplify the wide range of chemical agents that affect osseous metabolism.

A. Chemical Agents

1. Hormones

In general terms, a growth hormone, thyroxine, cortisol, and insulin are essential requisites for normal bone development, while sex hormones (testosterone and estrogen) are important in regulating bone turnover rates (Raisz and Bingham, 1972). Specifically, growth hormone acts indirectly on bone cell metabolism via initiating the production of sulfation factor in the serum. The sulfation factor is a low molecular weight protein produced in the liver and bound to a larger protein in the serum (Salmon and DuVall, 1970); it is produced shortly after growth hormone administration in vivo (Daughaday and Kipnis, 1966). Whereas, growth hormone can stimulate calcification of developing bone in vivo, the effect of sulfation factor on osteoid production and mineralization has not been elucidated (Stem, 1971). Growth hormone also affects phosphate and potassium ion interactions and increases collagen turnover in developing bone. Therefore, the concentration of ions and amino acids is a central consideration when assessing the effects of growth hormone on bone production; and this fact demonstrates that growth hormone's primary action is the result of its indirect effects on cell metabolism (Raisz and Bingham, 1972). Such action by growth hormone results in activating cells of the epiphyseal plate of long bones to increase production of proteins, nucleic acids, and mucopolysaccharides (Daughaday and Kipnis, 1966), also (Salmon and DuVall, 1970). Growth hormone also stimulates cell division in cartilage cells of the epiphyseal plate (Rigal, 1964).
Thyroxine (T₄) and Triiodothyronine (T₃) are essential to normal embryogenesis of the skeleton. Specifically, it is believed that T₃ and T₄ act in vivo via affecting growth hormone release and or parathyroid hormone efficacy (Catt, 1970; Jowsey and Detenbeck, 1969). In vitro, thyroxine stimulates chondrogenesis, but neither T₃ or T₄ have an effect on bone resorption (Pawelek, 1969; Feinblatt and Raisz, 1971). The addition or elimination of thyroxine from the environment of developing chick embryos, produces marked decreases in tibial gross dimensions (weight and length) and results in decreased chondrogenic activity along with defective acid mucopolysaccharide deposition (Hall, 1973).

Generalized growth retardation and osteoporosis are the phenomena ascribed to glucocorticoid action on osseous tissue. However, it has been shown that glucocorticoids inhibit release of growth hormone, which would account for the observed paucity of bone production (Thorn, 1970). This group of hormones also affect calcium metabolism via blocking calcium's absorption in the small intestine. The blockade phenomena can lead to secondary hyperparathyroidism along with defects in bone cell differentiation and maintenance (Kimberg, 1969; Jee et al., 1970). The net result of glucocorticoid action, therefore, is a stasis of osteogenic activity.

Insulin has also been implicated in bone ontogeny. This hormone increases protein polysaccharide and nucleic acid synthesis in cartilage, and also increases collagen and mucopolysaccharide synthesis in bone (Salmon et al., 1968; Wettenhall et al., 1969). However, the dose ranges of insulin, producing increased metabolic effects in vivo, are very high, and for all practical purposes are not comparable to
insulin's in vivo concentrations (Yalow and Berson, 1960). Marked developmental abnormalities of the skeletal system have been noted in insulin treated chick embryos. Growth retardation and abnormal angulation of the tibiotarsus were noted along with degenerative necrosis of the epiphyseal cartilage. The abnormalities were not as pronounced distally as they were proximally, nor were the effects as readily observable in older stages of embryonic development (Zwilling, 1948; Rabinovitch and Gibson, 1972). Clinically, one does not see great differences in bone metabolism between diabetics and non-diabetics, although diabetics with inflammatory diseases, such as periodontitis (Glickman, 1972) sometimes present quite extensive bone loss. Whether this last observation is due to effects of diabetes on the vascular system or is due in part to direct influences of insulin, has not been established.

With the onset of puberty, a temporary cessation of longitudinal growth occurs in the long bones of the body. This occurrence is ascribed to the antagonistic action of estrogen and testosterone upon growth hormone, as well as their effects of increasing bone maturation and decreasing its resorption (Katz and Kappas, 1968; Riggs et al., 1969). It should be noted, however, that estrogens in various species have diverse effects. In birds, for instance, an increase in medullary bone resorption occurs with estrogen application, while in rats a decrease in collagen synthesis is noted in skin and bone (Simmons, 1966; Henneman, 1970). Testosterone deficiency produces a decrease in skeletal mass that is in proportion to body weight; this may represent androgen effects on muscle which in turn alter osseous metabolism via changes in physical stress (Raisz and Bingham, 1972). Estrogen causes
a decrease in mucopolysaccharides but an increase in calcium content of cartilage (Berntsen, 1968). In rats, testosterone administration accelerates the maturation of cartilage and lengthens the calcifying zone of the epiphyseal plate (Fahmy et al., 1971). In summary then, the effects of androgens and estrogens on bone metabolism present many paradoxes when an integration of in vivo and in vitro studies is attempted in various animals.

Parathyroid hormone (PTH) has a very profound effect on calcium metabolism; it stimulates bone resorption (Raisz, 1970). Bone resorption mediated via PTH can be initiated either by osteoclastic resorption or by osteocytic osteolysis (Rowlend et al., 1971). Among experimental animals, the efficacy of PTH varies a great deal. For instance, in cows, PTH administration may have little effect, if any, on calcium mobilization (Mayer et al., 1966), while in the rat, vast quantities of calcium are mobilized in response to PTH administration (Orimo et al., 1972). PTH administration has also been shown to produce a short lived positive calcium balance in the Golden Hamster (Biddulph, 1972). The actual cellular mechanism of PTH is said to be the activation of adenyl cyclase thus producing an increase in 3',5'-adenylic acid in bone and kidney tissue. The increase of cyclic AMP is said to account for the diverse physiological effects of PTH (Aurbach and Chase, 1970). Not only does PTH stimulate calcium mobilization, but it also has inhibitory as well as stimulatory effects on matrix production. The effects vary depending on whether the experiment was done in vivo or in vitro, and whether amorphous or formed components are being analyzed (Rasiz and Bengham, 1972).
The physiological antagonist to PTH is thyrocalcitonin, which stimulates bone formation or inhibits bone resorption. Although calcitonin can stimulate osteoblastic proliferation as well as increased bone production (Matrajt et al., 1968; Wase et al., 1967); it does not increase collagen production (Flanagan and Nichols, 1969). In human subjects with histories of thyroid tumors, where alterations in growth of bones should be affected, high calcitonin levels do not seem to produce any marked abnormalities in bone morphology or mineral metabolism (Melvin and Taskjian, 1968). In pathological processes such as Paget's disease, the response to calcitonin is quite marked. For instance, upon intramuscular injection of porcine thyrocalcitonin there is a prompt fall in blood calcium levels as well as a reduction in alkaline phosphatase and urinary hydroxyproline values (Potts, 1970). Not only is the response to calcitonin variegated in degree among different disease states, but also the structure and efficacy of calcitonin varies among the phylogenetic spectrum of normal animals (Hoyt et al., 1972). Salmon ultimobrachial calcitonin, for example, is approximately thirty times more biologically active than bovine, ovine, porcine or human calcitonin (Potts, 1970). As with many of the other hormones, the \textit{in vivo} and \textit{in vitro} actions of calcitonin have not been completely elucidated and further study is needed to correlate clinical and experimental findings.

2. Vitamins

A second group of chemical agents which have profound effects on bone metabolism is the vitamins. Vitamin D and its various derivatives $D_2$ (Ergocalciferol), $D_3$ (Cholecalciferol), $D_4$ (22, 23 Dihydroergocalciferol), 25-Hydroxycholecalciferol (HCC), and 1,25 Dihydroxycholecalciferol (DHCC) have all been implicated in bone metabolism to some
From the time McCollum et al., (1922) named Vitamin D in 1922, the antirachitic properties of this vitamin have been studied exhaustively. The primary function of Vitamin D was thought to be the intensification of calcium absorption with a secondary effect of increasing phosphate absorption in the small intestine (Nicolaysen and Eeg-Larsen, 1953). However, recent evidence has shown that metabolites of Vitamin D₃ (1 HCC and 1,25 DHCC) may be the most important factors governing calcium ion absorption in the small intestine and resorption of osseous tissue (DeLuca, 1969). Vitamin D deficiency produces a poorly mineralized osseous matrix, widened epiphyseal plates and a decrease in linear growth of long bones (Kember and Walker, 1971). Primarily then, the effects of Vitamin D are related to mineralization and resorption phenomena, although some investigators indicate that Vitamin D may increase collagen production and affect an overall decrease in matrix production (Paterson and Fourman, 1968). Skeletal manifestations, as just described for rickets, will remit if Vitamin D, or one of its analogs, are added to the diet. However, these symptoms may persist after Vitamin D therapy, in a disease termed Vitamin D resistant rickets (VDRR). The etiology of VDRR is a defect of renal tubular cells which leads to hypophosphatemia and ultimately to pathology linked with secondary hyperparathyroidism (DeLuca, 1970). Although the absence of Vitamin D and its derivatives produce numerous effects on osseous tissues, Vitamin D's direct influence on bone metabolism remains a debatable point (Raisz and Bingham, 1972).

Vitamin A has a profound effect on bone metabolism. In excess, Vitamin A results in extensive bone resorption in various laboratory animals. In the rat, hypervitaminosis A produces a marked decrease
in growth at the distal ends of long bones, along with reductions in diaphyseal width. Long bones, however, are not the only osseous structures affected, perforation in the supraspinous and infrapinous fossae occurs, along with extensive perforations of the mandible (Barnicot and Datta, 1971). In animals given an excess of Vitamin A, abnormalities not only occur in the skeletal system, but can also be observed in the parathyroids (Wolbach and Maddock, 1952; Barnicot and Datta, 1971). The mechanism of hypervitaminosis A on osseous tissue is brought about via increased production of lysosomal enzymes by osteoclasts. It should be noted, however, that high doses of Vitamin A do not destabilize lysosomal membranes (Vaes, 1969). Hypovitaminosis A produces quite the opposite effects on osseous tissue from Vitamin A excess. Vitamin A deficiency results in a thickening or overgrowth of osseous tissue in many bones of the body. For instance, in the rat, a lack of bone resorption and thickening of bone causes dislocation of cerebral and brain stem structures along with herniation of nerve roots as they leave the vertebral foramina (Wolbach and Bessey, 1941). In man, dogs, and rabbits low levels of Vitamin A produce hyperostosis of the temporal bone and narrowing of the internal auditory meatus (Gerlings, 1947). In chicks and ducks on low Vitamin A diets, the growth of long bones is decreased due to the fact that epiphyseal cartilage and endochondral bone formation are retarded and not as regular as in control animals (Wolbach and Hegsted, 1952). The process by which low Vitamin A levels produces the previously mentioned defects is related to the spacial organization of osteoclasts and osteoblasts. Through some mechanism, lack of Vitamin A results in increased osteoclastic activity in places where increased osteoblastic activity
occurs in the normal state, and vice versa. Reversal in the location of cells can be demonstrated very consistently in Vitamin A deficient animals, although the exact regulatory mechanism remains obscure (Mellanby, 1950; Barnicot and Data, 1971).

A third vitamin which affects bone metabolism is Vitamin C. The importance of this vitamin in relation to osseous tissue relates primarily to its effect on the formation of collagen. In scurvy, bone formation is markedly decreased although normal resorption phenomena may take place (Rasiz and Bingham, 1972; Bourne, 1971). Many authors have shown in vivo and in vitro evidence which indicates that a decrease in collagen fiber formation, alkaline phosphatase, and calcification occurs in the scorbutic state (Bourne, 1971; Ross, 1968). Concentrations of chondroitin or mucoidin sulfate decrease in scurvy, while glycogen and non-sulfated mucopolysaccharides increase (Van Wersch, 1954). If Vitamin C is added to the diets of normal animals, an increase in calcium and phosphate retention can be demonstrated (Lucke and Wolf, 1938; Lanfod, 1939). However, it has been shown subsequently that only the calcium ascorbate derivative of Vitamin C increases calcium retention and bone calcification (Bourne, 1971). Even the latter investigator indicates that increased calcification may be the result of Vitamin C's effect on matrix formation, rather than being directly attributable to Vitamin C. Although the primary action of Vitamin C has been ascribed to the enzymatic hydroxylation of proline and subsequent production of 'normal' collagen (Ross and Benditt, 1964), it has been shown that the purified enzyme can catalyze the hydroxylation reaction without Vitamin C as a co-factor (Buhler and Mason, 1961; Cardinale et al., 1971). Vitamin C,
therefore, is very closely related to the formed and amorphous components of the ground substance, and variation in the concentration of this vitamin causes marked changes in those elements.

3. Ions

Although hormones and vitamins are essential for normal bone metabolism, their action is mediated through ions. There are several ions which are related to the extracellular and intracellular environment of bone cells. Calcium, phosphate, pyrophosphate, potassium, strontium, magnesium and fluoride ions are just a few of the species which have been implicated in osseous metabolism. Calcium and phosphate ions are the most significant members of this group when reference is made to the 'normal' physiological activity of osseous tissue. The metabolism of calcium, as it relates to bone, has been amply covered in the previous pages of this review, but the direct effect of calcium ions on osseous activity needs further amplification. *In vitro*, addition of calcium, after PTH induced resorption has begun, produces no change in the metabolic activity of bone (Raisz and Niemann, 1969). However, cell division in rat thymocytes, initiation of PTH resorption phenomena, and stimulation of ribonucleic acid synthesis in osseous tissue have been shown to be dependent on calcium ion concentration (Raisz et al., 1972; Raisz and Bingham, 1971; Whitfield et al., 1969). It has also been shown that simultaneous administration of calcium and PTH produces an increase of collagen formation in parathyroidectomized rats (Park and Talmage, 1968). Calcium administration, *in vitro*, can produce an increase in gluconeogenesis by increasing intracellular calcium ion concentration, thus activating PEP carboxykinase and inhibiting pyruvate kinase (Rasmussen et al., 1970). The same authors
have also shown that PTH increases 3',5'-AMP with or without the presence of calcium ions; however, if calcium is not present, gluconeogenesis is not intensified.

Phosphate ion concentration has also been linked to several metabolic events in osseous tissue. When phosphate ions and PTH are administered to thyroparathyroidectomized rats, plasma calcium ion concentration is not significantly affected (Rasmussen et al., 1970). These authors also reported that thyrocalcitonin blocks PTH induced osteocytic osteolysis while phosphate does not. The efficacy of phosphate ions is related to increases in collagen formation and bone mineralization which occur subsequent to its administration (Nichols and Flanagan, 1969). In human subjects with Paget's disease, phosphate therapy results in decreased urinary hydroxyproline levels. This same phenomena has also been reported in patients with malignancies, Cushing's Syndrome, Paraplegia, and Hyperparathyroidism (Goldsmith, 1970). In vitro, it has been demonstrated that phosphate ion administration to chick limb buds increases collagen formation in bone to a greater extent than it does in cartilage (Rasiz, 1970). It has also been reported that growth hormone increases serum phosphate concentration while PTH increases urinary excretion of phosphate ions (Raisz and Bingham, 1972). Therefore, it is very probable that phosphate ions play as central a role in the development and maintenance of osseous tissue as do calcium ions.

Other ionic moieties, such as pyrophosphate (PPI) and Diphosphonate, in low concentrations have very profound effects on bone metabolism. PPI inhibits hydroxyapatite crystal formation while allowing amorphous calcium phosphate to form (Fleisch and Russell,
It is hypothesized that PPi's effects are due to inhibition of adenyl cyclase, resulting in increased intracellular calcium ion concentration (Rasmussen et al., 1970). PPi is deactivated by neutral pyrophosphatase and alkaline phosphatase; therefore, it is possible that hydroxyapatite formation is tied to PPi concentrations (Fleisch and Russell, 1972; Robison, 1932). Diphosphonates differ slightly in chemical structure and are much more resistant to enzymatic breakdown than PPi (LaNauze et al., 1970); in high concentrations, they can produce an increase in bone matrix along with a decrease in mineralization (Fleisch and Russell, 1972). The influence of magnesium ions on bone metabolism relates to PPi action through magnesium's function as a co-factor in phosphatase enzymes (Rasmussen and Tenenhouse, 1967). Magnesium deficiency results in a lag in osteocyte maturation along with a decrease in osteocytic resorption (Bélanger, 1970). The actions of PPi and diphosphonates, which have been reported to date, tend to clarify classical concepts of mineralization; however, further experimentation is needed to elucidate the exact cellular mechanisms and effective dose ranges of these substances.

One of the most controversial aspects of hard tissue metabolism is related to the action of fluoride ions. Fluoride therapy is often used to treat patients with osteoporosis, multiple myeloma and Paget's disease (Shambaugh, 1966). Although the rational behind fluoride therapy has been linked to an increase of bone mass and strength (Hodge and Smith, 1968), some investigators have shown that bone formed under such conditions is poorly mineralized and poorly organized (Baylink and Bernstein, 1967). Administration of fluoride preparations IV, increases serum fluoride concentration 2 to 3 fold up to one hour.
after injection; but subsequently a plateau is reached, and no further increase can be noted (Taves, 1970). The last author has also reported that in rats the serum fluoride concentrations should be kept below 15μM or serum protein concentration, amino acid uptake, and weight gain are significantly decreased. Fluoride treatment results in production of a mineralized matrix which is more resistant to osteoclastic resorption (Rich, 1970); and lack of resorption would ultimately result in more bone mass. However, the primary consideration when attempting fluoride therapy is to maintain fluoride concentrations below the toxic level but yet high enough to yield beneficial osseous effects (Rich, 1970; Taves, 1970). Therefore, it is probable that fluoride ions could have a positive effect on bone maintenance, if safe but effective dose ranges could be determined.

Potassium and strontium ions are present in osseous tissue although their functional significance is poorly understood. Potassium ion concentration is highest intracellularly, but low concentrations have been noted in the extracellular fluid in juxtaposition to the osteocyte plasma membrane (Canas et al., 1969; Geisler and Neumann, 1969). Potassium ion concentrations in bone have also been reported to decrease with age and to fluctuate upon hormonal application (Canas et al., 1969; Rasiz and Bingham, 1972). Although potassium ions make up the majority of intracellular monovalent cations, it is evident their functional role in osseous metabolism needs further elucidation. Strontium, on the other hand, has been studied to some degree with reference to Vitamin D levels and strontium loading. It has been reported that as long as Vitamin D₃ levels are maintained from 500–1000 ICU/kg, Sr can replace up to one-half of the calcium
requirement during the early growth period of young chicks (Creger and Colvin, 1971). Also, investigators have indicated that Sr competes with magnesium for ionic sites in the hydroxyapatite crystal; and in animals where excess Sr has been administered the formation and composition of the inorganic phase of the matrix is abnormal (Johnson, 1972). Even though the latter two ions are minor contributors to the overall ionic environment in osseous tissue, it is important to note the dynamic relationship they have to the cellular and extracellular environment of bone cells.

4. Fatty Acid Derivatives

Another class of compounds which influence osseous maintenance are the fatty acid derivatives termed prostaglandins. In tissue culture, prostaglandins have been shown to stimulate bone resorption (Klein and Raisz, 1970). It has also been reported that HSDM (fibrosarcoma) tumor cells produce a specific prostaglandin (PGE₂) which stimulates bone resorption (Tashjian et al., 1972). The resorptive action of prostaglandins produces a possible explanation why certain malignant tumors in man produce hypercalcemias without bony metastases or increases of PTH, although no in vivo studies have demonstrated this hypothesis.

B. Physical (Biophysical) Factors

Although various chemical factors influence bone metabolism markedly, many physical parameters also produce alterations in osseous development. Muscular activity, vascular flow, piezoelectricity and nerves are just some of the physical entities which comprise this group of physical factors. The four previously mentioned physical factors are functionally almost inseparable; however, for the purposes
of this discussion they will be treated individually, as much as possible.

1. Muscular Activity

The impact of muscular activity on skeletal growth and development has been studied extensively. In 1869, Wolff postulated that bone morphology was directly related to the direction of mechanical stresses imposed on osseous tissue (Wolff, 1869). From that time, up until the present, many studies have been accomplished in embryonic systems (chick, rabbit, rat, frog, etc.), and have attempted to correlate muscular activity with skeletal development. In chorio-allantoic grafts of chick limb buds, it has been shown that quite normal femoral shafts develop (Murray and Selby, 1930). This study indicates that lack of muscular activity and innervation seems to have little effect on the gross morphology of developing long bones. In the toad, grafting experiments have shown that not only do long bone elements develop to normal gross dimensions, but that also joint formation proceeds in an anatomically correct fashion, although joint dimensions may be slightly decreased (Braus, 1910). Complete denervation of embryonic frog legs has been shown to produce skeletal elements which have normal morphology except for a 12% decrease in proximo-distal length (Hamburger, 1928). While embryonic long bone elements seem to develop almost normally in the absence of muscular activity, workers subsequent to Braus have shown that development of joint cavities are impaired, as well as joint cavity occlusion via fibrous connective tissue (Fell and Canti, 1934). Summarizing the literature in this area up to 1936, Murray states that, "the gross form of limb skeletal elements are developed by self-differentiation, that is, under the
direction of factors intrinsic in each developmental element" (Murray, 1936). The latter author goes on to say that intrinsic factors are not sufficient for production of a normal skeleton, since growth pressures of certain elements are necessary for completely normal skeletal relationships, i.e. joint formation. In the late 1930's a series of coelomic grafts in chick embryos were carried out by Hamburger and associates which indicated that distal structures of transplanted limbs may have slight abnormalities, i.e. hypodactyly and hypophalangy and that, overall, skeletal growth is reduced approximately 20%. It should be noted that the limbs studied by Hamburger were poorly innervated and had very low, if any, motility. Hamburger alludes to the fact that decreases in limb dimensions may be due to decreased muscular activity, altered blood supply, or lack of innervation (Hamburger, 1939; Hamburger and Waugh, 1940). Hamburger also noted that chondrification and the first phases of ossification proceed normally in the absence of innervation.

Other investigations have indicated that even drastic changes of muscle action in young animals produce no changes in long bone dimensions. In these studies, rats and dogs were subjected to forelimb amputation shortly after birth. This procedure caused the animals to assume a bipedal stance to locomote, and did not produce differences in length of hind limbs between operated and normal animals (Fuld, 1901; Colton, 1929). The potent intrinsic capacity for skeletal elements to develop autonomously, is demonstrated by studies which show that forces of 500-560 lbs. are required to slightly retard longitudinal growth of the epiphyseal plate (Strobino et al., 1952). Other investigators have reported unsuccessful attempts to retard
longitudinal growth by pinning or stapling the epiphysis to the metaphysis (Blount and Zeier, 1952).

Although the aforementioned studies seem to indicate that muscular activity has no effect on skeletal development, it should be pointed out that no weight measurements (wet or dry) or histochemical analyses were attempted by the authors. Also, the previous studies dealt only with embryonic or young post-natal animals; and it is possible that muscular effects during the initiation and early differentiation stages of skeletal elements may be much different from muscular effects related to maintenance of adolescent or adult skeletal tissue.

More recent investigations as to the muscular systems effect on skeletal development have shown that changes in various skeletal parameters do take place in paralyzed limbs. In rabbits with motor denervation of the lower limb, it was found that tibial dry weights were significantly lower, and tibial bone lengths were significantly longer in denervated specimens (Ring, 1961). In other experiments the same author removed all tendons which act upon the foot of the rabbit. He found, after tendotomy, that experimental tibial bone weights were significantly lower than the control bones. Ring also produced anterior root lesions in kittens and puppies, and found that tibial bone lengths increased in the experimental animals but not significantly. However, in the same experiment, dry bone weight was found to be significantly lower in the experimental tibias of the kitten. Studies on denervated limbs of young rats have shown only slight (non-significant) shortening of the humerus and radius in the experimental animals (Armstrong, 1946). Gillespie in his studies on denervated (efferent) kitten limbs reported considerable decreases in bone weight and density, but no length changes (Gillespie, 1954).
Muscular effects on bone growth and development have also been noted in humans. In children with paralytic poliomyelitis, clinicians have reported an initial lengthening of the affected limb followed by a reduction of limb growth. It has been reported that the change from lengthening to shortening of the limbs takes place during the first two years of the disease (Gullickson et al., 1950). In children, however, the extent of muscle loss cannot be used to predict the degree of limb shortening with any reliability (Ring, 1961). Therefore, muscle action is not the only factor which determines change in limb dimensions (Gullickson et al., 1950; Ring, 1961).

The results of limb immobilization in adult animals produced by application of plaster casts, calcaneal tendotomy, ventral root section or peripheral denervation have been studied by increasingly sophisticated methods in recent years. Experimental procedures carried out on rabbit tibias using immobilization methods have indicated that growth does increase in the affected limbs. The increase in growth of the affected limbs, however, was related to increased blood flow (Sundén, 1967). Other workers have reported initial increases in long bone growth, after immobilization of limbs, followed by a period of decreased bone production and osteoporosis. The reason for this bimodal response was reported to be changes in vascular flow patterns brought about by limb paralysis. Initially, it was determined that an increased blood flow occurs upon immobilization; and the increase in blood flow produces increased bone growth. As immobilization continues, vascular flow decreases and thus bone resorption ensues (Geiser and Trueta, 1958). In a very complete study on metabolic responses of adult bone to limb immobilization it has been reported that bone
calcium and phosphorous levels are increased along with blood flow shortly after immobilization. Also, in the initial period of immobilization pCO₂ levels decrease while pO₂ and venous pH levels increase. Within a week after the initial phase, vascular flow begins to decrease along with calcium, phosphorous, pO₂ and venous pH levels. pCO₂ is the only parameter which increases in this latter phase (Hardt, 1972). It should be noted that the flow rates and metabolite concentrations reported by Hardt are indicative of increased bone production or resorption, respectively. Recently, Swedish workers have shown that bone mineral content of adult human male long distance runners is significantly higher (20% higher in appendicular bone, 10% higher in axial bone) than that of office employees in the same age and physical groups. Also, it was reported that office employees put on a rigid exercise schedule for several months showed no significant differences in their bone mineral content from unexercising office workers (Dalén and Olsson, 1974).

Although the previous text has dealt with muscle influence on long bone development, experiments on embryonic membranous bones of the chick mandible, have shown that secondary cartilage development is very dependent upon muscular activity (Murray and Drachman, 1969; Murray, 1963; Hall, 1972). When paralyzing drugs, chorio-allantoic grafts or organ culture were used as experimental procedures to affect development of the mandible's articular surface, the secondary cartilage of this surface was transformed into bone like tissue. Hall indicated that in normal articular surfaces (where no immobilization has taken place) the cartilage is maintained, and is not converted into bone. The first histochemical change that Hall observed in immobilized
mandibles was a decrease in the acid mucopolysaccharide content of the secondary cartilage matrix. Following a decrease in acid mucopoly-
saccharides the secondary cartilage cells seem to undergo transformation
or dedifferentiation and redifferentiation into bone producing cells. Hall
indicates that immobilization is the stimulus which produces the change
in cell type (cartilage to bone).

2. Vascular System

Although the effects of the vascular system on bone development
are closely tied to muscular and neural activity, many investigations
have attempted to associate vascular phenomena directly with hard
tissue metabolism. The influence of the vascular system on osseous
development can be related to two main areas: 1) metabolite transport
and 2) physical properties of vascular flow.

In relation to the first area (metabolite transport), many
investigations have been carried out. Most of these studies have
dealt with the removal of sympathetic control of the vascular system
and its effect on the development of gross bone dimensions. One case
report in a human with anterior poliomyelitis showed that removal of
the lumbar sympathetic chain produced lengthening in the paralyzed limb;
and at two years post-operation there was only three-fourths of an inch
discrepancy between the normal and affected limb (Harris, 1930).
Subsequently, many lumbar sympathectomy investigations have been
accomplished but none have demonstrated, unequivocally, the effect of
sympathectomy on limb growth. In studies on young goats, sympathectomy
produced no changes in growth between operated and control limbs
(Bisgard, 1933). Similar studies performed on kittens (Cannon et al.,
1929) and rats (Bacq, 1930) reported no differences in limb growth
between sympathectomized and control animals. Bisgard also noted the effect of lumbar sympathectomy on a monkey with poliomyelitis; no dimensional changes were noted between the control and operated limb after three months of observation. Sympathectomies have also been accomplished in adult cats, and it was reported that differences in limb length remained unchanged between control and experimental animals up to three years after the operation (Corbin and Hinsey, 1939). More recently, in 1955, it was reported that lumbar sympathectomy in young rabbits caused a significant increase (>4%) in paw growth (Goetz et al., 1955). In other studies of lumbar sympathectomy in kittens (Gillespie, 1954), it was shown that bone weight and cortical thickness were not affected by sympathectomy, but breaking stress was significantly lowered in experimental groups. Gillespie concluded that the altered properties of bone in a denervated limb are due almost entirely to a loss of muscular activity. However, human studies on sympathectomies in patients with anterior poliomyelitis showed that limb length increased markedly on the operated side (Barr et al., 1950). Subsequent studies on gross changes in limb length following sympathectomy have added to the confusion already present in this area of research. In puppies with a unilateral lumbar sympathectomy, growth of the limb on the operated side was greater than the control limb in three out of eight cases (Ring, 1961). The author notes, however, that no tests of sympathetic function were performed after the operation, and the extent of the sympathectomy was not determined at necropsy.

Histologically and physiologically the effect of the sympathetic nervous system on vascular flow within long bones has been documented with much less controversy than the gross dimensional studies.
previously enumerated. It has been shown in animal studies that sympathectomy leads to interruption of blood flow and decalcification of bone (Zinn and Griffith, 1941). Also, stimulation of the sympathetic chain has been shown to reduce the total volume of bone marrow constituents and increase leukocytes, red blood cells, and reticulocytes in the nutrient vein of the dog tibia (Foa, 1943). Foa also found that stimulation and subsequent volume decrease in the bone marrow was followed by a return to normal values within 20 seconds. Sympathectomy has been shown to produce increased diameters of Haversian systems; this result, according to the author, was attributed to increased blood flow, not to increased osteoclastic activity (Fell, 1949).

The vasoactivity of bone and bone marrow vessels has been amply documented by several other studies (Lowenstein, 1958; Amado, 1947). It has been postulated by some authors that blood flow within the bone marrow itself is not subject to vast changes in pressure or rate, due to the distribution of endarterial sphincters and anterio-venous shunts (Serratrice and Eisinger, 1967). The last two authors believe, therefore, that blood flow within the bone itself can change markedly in rate and pressure, but blood flow within the marrow is kept relatively constant. It has also been postulated that sensory nerves from bone and bone marrow elements take part in the afferent limb of a reflex arc which ultimately affects the metabolism of bony elements by controlling blood flow (Solovoev, 1958). Although much conflict (previously mentioned) is present in the literature as to the effect of the visceral efferent system on gross bone development, information regarding metabolite concentrations and flow rates during bone formation and resorption has more unanimity of opinion. For instance, tissue culture studies in the late 1950's showed that reduction in O₂ levels,
below one atmosphere of pressure, induced bone formation; higher pO₂ levels promote bone removal (Goldhaber, 1958). It has been reported that osteogenesis is not only supported by raising pCO₂ levels (Willmer, 1965), but that increased pCO₂ levels are necessary for osteoblastic differentiation (Richards and Brookes, 1969). In general, it can be stated that bone formation (osteogenesis) takes place in a mildly alkaline environment; the more acidic the pH drift in this alkaline environment the greater the tendency for compact bone formation (Brookes, 1971). On the other hand, the more basic the pH drift in the alkaline environment, the greater the tendency for spongy bone to be formed. The acidic drift in pH can be coupled physiologically with increased pCO₂ levels and a relative decrease in venous return, while basic drift in pH can be coupled with decreased pCO₂ levels and a relative increase in venous return (Brookes, 1971). Other investigators have found in immobilization studies on rabbit hindlimbs, that bone formation occurs simultaneously with an increase in venous pH and pO₂ levels, along with a pCO₂ decrease (Hardt, 1972). The same author found that bone resorption occurs when venous pH and pO₂ markedly decrease, while pCO₂ greatly increases.

In addition to the reports of metabolite concentrations in adult animals (reported previously), studies have also been accomplished relating pCO₂, pO₂ and nicotinamide concentrations to the initiation and differentiation of various cell types during development. It has been demonstrated in the developing chick limb (Caplan and Koutroupas, 1973; Ede, 1971; Pawelek, 1969) that the differentiation of limb tissues is dependent on, or controlled by, the differential vascularization of the limb. Caplan reported that chondrogenesis occurs in
areas of the limb bud with the lowest pO₂, nutrient supply, and
nicotinamide content, while myogenesis occurs in areas of relative
high pO₂, nutrient supply, and nicotanamide concentration. It is very
evident from Caplan's paper that the anatomical position of vascular
elements is the factor controlling the distribution of the various
metabolites mentioned above. Further studies in this crucial area are
needed before interrelationships between the embryonic and adult state
can be elucidated.

Biophysically, vascular elements can also have an affect on bone
metabolism. It has been reported that vascular flow produces what is
known as streaming potentials in osseous tissue. Streaming potentials
arise in tissues due to various charged bodies (ions, dipoles, etc.)
flowing past sites of fixed charge (Bassett, 1971). Several authors
have noted that increases in blood flow lead to a relative electron
negative environment which favors osteogenesis, while decreases in
blood flow lead to a relative electron positive environment favoring
osteoclasis (Gorham and West, 1964; Digby, 1966; Bassett, 1971).
Although metabolite distribution is probably the most important
function of the vascular system, biophysical principles, such as
streaming potentials, cannot be ignored when assessing the total
involvement of the vascular system on bone metabolism.

3. Piezoelectricity

Although muscular and vascular effects on bone development have
been studied quite extensively, other physical parameters which lead
to effects on bone metabolism have also been studied. Piezoelectricity
is one such parameter. Piezoelectricity can be defined as electricity
resulting from stress on crystals (Bassett, 1971). Piezoelectric
potentials have been elicited from a number of biological materials among which are the following: polypeptides, wood, bone, amino acid crystals, collagen, keratin, elastin and dentin (Bassett, 1971). Some investigators have attempted to apply the principle of piezoelectric potentials in reverse; in other words, instead of deforming bone to obtain a potential, a potential was applied to bone to determine its effect on osteogenesis. Lavine et al. created a bony defect in a rabbit femur and implanted two electrodes at equal distances from the defect. Over a three week period of time, a two to four microampere direct current was applied to the electrodes. The results of this study showed that healing of the defect in three week post-operative experimental animals was approximately the same as healing in six week post-operative control animals (Lavine et al., 1971). Although the previous experiment demonstrates the possibility of piezoelectric electricity contributing to bone growth and development, it should be pointed out that other attempts to produce bone growth in vivo with electric currents have not been as conclusive as Lavine's study (Bassett et al., 1964; Minkin et al., 1968).

4. Nerves

The remaining biophysical entity which could affect skeletal growth and development is the nervous system. Although the nervous system has previously been implicated indirectly with skeletal ontogeny via its relationships to the muscular and vascular systems, the direct effect of the nervous system on skeletal development must be analyzed.

Neuro-osseous relationships are not generally acknowledged in gross anatomy and histology books, but their presence has been confirmed by many authors over the past three hundred years. M. du Verney was the first investigator to make note of the fact that nerves do enter
long bones accompanied by blood vessels (du Verney, 1700). A subsequent study of nerve relationships to long bones, reported in some detail the neuroanatomy of ox and horse femurs (Gros, 1846). In the latter paper, the author describes nerves entering the nutrient foramen of long bones accompanied by nutrient vessels and lymphatics. It was also reported by Gros that a neural ganglion was situated near the nutrient foramen. The branching patterns of vascular and neural elements within the long bone were also noted by Gros. It was reported that upon entering the nutrient foramen, arterial and venous elements divide into ascending and descending branches which ramify throughout the entire bone marrow; at the same time it was noted that the main nerve trunk branches profusely, some branches following blood vessels and others taking a random course throughout the marrow cavity. Other workers (Variot and Remy, 1880) noted that myelinated nerve fibers are destined for the substance of the marrow cavity, while non-myelinated nerves innervate the blood vessels. These workers also refuted Gros' observation that a ganglion was situated near the nutrient foramen. They found, instead, that Gros' ganglion was actually a mass of fat cells. In the early 1900's Ottolenghi described nerve-long bone relationships in the tibia of birds. In this study it was reported that the greatest abundance of nerves in the long bone are associated with the blood vessels. Relatively few fibers were noted between the vascular elements. Also, it was indicated that myelinated and unmyelinated nerves form a relatively rich and complex innervation of the bigger vessels, but a much smaller number of fibers envelope the smaller vessels. In this work, myelinated fibers were reported to be associated primarily with blood vessels, while the non-myelinated elements were said to be
concerned with pain perception. Finally, no intimate neural relationships to any cellular or extracellular entities of the bone or bone marrow were noted, nor were there any vast differences noted between blood vessel innervation of bone and other organs (Ottolenghi, 1902). Although the previous workers established the fact that nerves (myelinated and unmyelinated) are present in bone and bone marrow, it was not until the mid 1920's that detailed histological studies of neural relationships to osseous structures were accomplished. DeCastro, working in Cajal's laboratory at the University of Madrid, described basic neural relationships to bone tissue and bone marrow elements. It should be noted that many of DeCastro's observations have been reaffirmed in subsequent years, but certain findings he reported have yet to be proven or disproven. DeCastro divided his discussion on the innervation of long bones into two main categories, the innervation of bone and the innervation of bone marrow. Therefore, for the purposes of this discussion, a thorough literature review of the innervation of bone marrow will be accomplished first; this will be followed by a complete review of neural relationships to bone tissue proper.

DeCastro indicated that nerves follow the blood vessels throughout the extent of the bone marrow. He noted that in juxtaposition to areas of bone formation two nerve branches could be delineated; those nerves which remained in association with the blood vessel and those which entered the pulp tissue of the bone marrow (DeCastro, 1925; DeCastro, 1929). It was also reported that the nerves surrounding the vascular elements consisted of myelinated and unmyelinated fibers, the unmyelinated fibers being more numerous. Both the myelinated and unmyelinated nerves were said to give off branches as they traversed
the marrow, but the non-myelinated fibers terminate on muscle cells of the vessels, while the myelinated nerves end as a branching network of centrifugal fibers. Some of the fibers branching within the marrow terminate as delicate fibers surrounding parenchymal cells. In subsequent work it has been reported that the nerves innervating vascular elements of bone marrow proceed in a spiral course around the vessels (Rossi, 1932). The same author described loose nerve fiber networks enveloping venous sinuses and ending in close approximation to their endothelium. It was also noted in the same study that nerve fibers which do not follow the blood vessels terminate in small brushes, buttons, rings or fine filaments within the cellular elements of the marrow. The same type of afferent specializations have been reported in subsequent work, and in addition, intimate relationships have been noted between parenchymal cells and the terminal specializations (Takeyama, 1936). Other workers have reported that afferent nerve fibers of the bone marrow occur in relationships which indicate they are connected with receptors imbedded in the parenchyma (Kuntz and Richins, 1945). The last two authors concluded that the parenchyma of bone marrow is devoid of direct sympathetic efferent innervation and that unmyelinated fibers, which are not associated with vascular elements, represent unmyelinated afferent fibers or the unmyelinated terminal portions of myelinated fibers. One of the most complete and thought provoking study on the innervation of bone marrow was reported by a Russian investigator in 1959. In this study a detailed description was made of the afferent and efferent innervation in the femur of rats. With special reference to the bone marrow, this investigator reported bush-like endings occurring in juxtaposition to
parenchymal elements. He also noted that terminal twigs of myelinated fibers are in close approximation to cartilage cells undergoing degeneration in the area of the epiphyseal plate. Nerve terminals were also reported to be closely associated with forming bony trabeculae of the metaphysis, epiphysis, and endosteum (Solovoev, 1959). The aforementioned findings have been reported by other Russian workers, but in less detail (Ignatov, 1956; Polacek, 1955). Both Solovoev and Polacek hypothesize that the afferent free endings may be functioning as monitors of position sense or may be sensing load pressures and tensions exerted upon those portions of the bone. Solovoev goes on to say that it is entirely possible that the terminal processes of these afferent nerves are chemoreceptor elements which may be operative in hematopoiesis or internal reconstruction of bone.

Studies on the innervation of bone marrow in the dog and goat confirmed the findings previously mentioned, except for the fact that terminal specializations were not observed in association with parenchymal elements (Kawahara and Osada, 1962). Miller and Kasahara (1963) reported on the innervation of human long bones. They noted the presence of small myelinated and amyelinated nerve fibers entering numerous foramina in the epiphyseal and metaphyseal regions. The myelinated fibers traversed the thin cortex of these regions and wound about trabeculae or spread out beneath the surface of the articular cartilage. Small bulbous terminations were also noted in juxtaposition to the endosteum. Their description of neural relationships in the diaphysis was much like the previous reports, however, no functional significance was attributed to any particular type of afferent fiber or nerve ending. In an exhaustive study of monkey,
rabbit, rat and mouse bone marrow innervation, Calvo (1968) described in detail the concepts of bone marrow innervation as we know them today. He divided the nerve supply of bone marrow into three parts: 1) an arterial component, 2) a sinusoidal component and 3) a parenchymal component. In a description of the arterial component, it was reported that every portion of the arterial tree is closely associated with nerve fibers. The main branches of the nutrient artery are surrounded by a number of nerve bundles each having numerous fibers. The arterioles and capillaries are often accompanied by only one naked fiber. Thin non-myelinated fibers leave the nerve bundles and end on smooth muscle fibers in the vessel wall. Non-myelinated fibers were said to often follow one capillary and then jump to another one. Fibers surrounding the capillaries were frequently observed leaving these vessels and terminating among parenchymal elements.

The sinusoidal component of the bone marrow has myelinated fibers associated with some of its divisions. The central sinus, for instance, has myelinated fibers which either terminate on it at some point, or end blindly in the parenchyma. Other fiber bundles run equidistant between the endosteum and the central sinus; these bundles may divide at certain points in their path and envelope a sinusoid. The naked endings of myelinated and non-myelinated fibers have been described lying in contact with endothelium of the sinusoids.

The innervation of bone marrow parenchyma is formed by a few nerve bundles passing in a proximal and distal direction after entering the nutrient foramen. These fibers do not follow vascular channels and are composed of myelinated and non-myelinated elements. Some of the myelinated elements branch from these bundles and end as naked branches
among parenchymal elements. Non-myelinated fibers follow a more tortuous course than myelinated fibers, and they may end in the wall of an arteriole, on a capillary, or in the parenchyma as a naked fiber. A few of the non-myelinated fibers may be found ending among elements of the endosteum or entering Haversian canals of the diaphysis. Calvo notes that the greatest quantity of nerve fibers are found in the area of the nutrient foramen. He states that nerves may have a direct influence on bone marrow physiology, which would compliment other known regulatory mechanisms of bone marrow function.

Calvo and Forteza-Vila (1969) described the development of innervation in the bone marrow of new-born rats. They noted major signs of differentiation (myelination of axons and increases of RER in Schwann cells) at the end of the second week after birth. The nerve fibers in association with muscle elements of the blood vessels were identified at the EM level as sympathetic elements. These authors noted that the maturation of nervous tissue within the bone marrow coincides with the onset of responsiveness of bone marrow elements to stimulatory and inhibitory conditions related to erythropoiesis.

Recent studies on the innervation of bone marrow in the rabbit, have amplified our knowledge of the functional development of nervous elements in the bone marrow (Miller and McCuskey, 1973). These authors reported that myelination begins on the fifth neonatal day simultaneously with vascular maturation (formation of muscular medias, complete internal elastic laminas, and PAS(+) adventitias). They also noted an increase in sulfated acid mucopolysaccharides in the connective tissue of the developing marrow cavity on the fifth post-natal day. At this same time haemopoietic activity was initiated at some points
in the marrow cavity. Since adrenergic innervation was shown to mature at this same post-natal stage, the authors hypothesized that a mature neurovascular complex is necessary for the establishment of a microenvironment for haemopoiesis to occur.

This brings us to the topic of the innervation of bone proper. DeCastro reported that the nerves of the bony diaphysis can be divided into two main categories: 1) those derived from periosteal nerves, and 2) those derived from bone marrow fibers which become incorporated into Haversian systems or become associated with the endosteum. There is no doubt that nerves supply the periosteum, to that statement all the previous authors, from DeCastro on, agree. Various types of afferent terminals have been described in periosteum from free nerve endings to encapsulated elements.

Within bone itself, the Haversian systems contain nerve fibers; this is general knowledge found in any current histology text. The nerve fibers in Haversian systems arise primarily from periosteal nerves, although some fibers do emanate from bone marrow nerves (Miller and Kasahara, 1963; Calvo, 1968). Cooper et al. (1966) and Milgram (1965) have amply documented the existence of non-myelinated nerves in Haversian systems at the electron microscopic level. The presence of nerves closely related to the endosteum is also generally accepted by the aforementioned authors.

Aside from the general comments previously stated on neural-osseous relationships, some authors, notably DeCastro, believe there are special relationships between nerve fibers and osseous tissue. DeCastro (1929) reported that in non-ossified areas (osteoid matrix) one can delineate fine nerve terminals. Also, he reported nerve
processes terminating in varicosities upon osteoblasts. Hurrell (1937) noted that nerve fibers terminate in close relation to bone cells and also end blindly in bone matrix. Takeyama (1936) and Ignatov (1956) have also described nerve fibers extending between bone lamellae. The last four authors are the only workers who have been able to demonstrate direct neuro-osseous relationships; none of the other investigators previously mentioned in this research area have noted actual neuro-osseous connections.

It can be ascertained from the previous discussion that bones, do indeed, have an afferent and efferent nerve supply. However, the direct effect of nerves on the development and maintenance of the 'normal' anatomy, physiology, and biochemistry of skeletal elements is not well understood. It should be recalled from preceding pages of this discussion, that efferent nerves do effect skeletal muscular activity (GSE modality) and blood flow (GVE modality), which in turn may produce effects on bony elements. However, such neural effects are indirect; they are not mediated via direct relationships of the nervous system on osseous tissue. From the anatomical discussion of neuro-osseous relationships, it is evident that neural effects on skeletal metabolism, if present, must be mediated via GSA, GVA, or GVE modalities. Since the indirect effects of the nervous system on skeletal development have already been enumerated concerning the GVE (sympathetic innervation of blood vessels) modality, the only relationship that remains a question mark is the effect of afferent innervation on skeletal metabolism.

In studies done by Corbin and Hinsey (1939), Gillespie (1954), and Ring (1961) it has been demonstrated that removal of sensory (afferent)
innervation to the limbs of young and adult animals does not produce marked post-operative changes in gross dimensions of skeletal elements. Therefore, it may be possible, that the only function of the afferent portion of the nervous system in bone tissue may be in relation to position sense, or stress and strain parameters (Miller and Kasahara, 1963; Polacek, 1955; Solovoev, 1959). However, it should be remembered that in post-natal studies Miller and McCuskey (1973) related a functional neurovascular system (afferent and efferent components) in bone marrow to initiation of haematopoietic events.

There is another possibility, however, whereby afferent nervous elements could influence metabolism of skeletal elements; this being through a neurotrophic mechanism. Neurotrophism can be defined as an "interaction between nerves and other cells which initiate or control molecular modifications in other cells" (Guth, 1969). Nerves do exhibit trophic affects on various tissues. GSE nerves have been shown to control differentiation and maintenance of skeletal muscle in vivo and in vitro (Muchmore, 1968; Studitsky et al., 1962; Gutmann, 1968; Buller et al., 1969). Gustatory nerves (SVA) have been shown to be necessary for the development and maintenance of taste buds (Zalewski, 1969). GSA nerves have been shown to be important in the maintenance of intact oral epithelium and skin (Winkelman, 1960; Frank and Karli, 1962).

Neurotrophic mechanisms have also been shown to influence the growth and development of regenerating amphibian limbs. This topic has been extensively researched and hundreds of reports have been made relating regeneration phenomena directly to neurotrophic mechanisms taking place within the blastema (Singer, 1952). It has
been reported that once contact between neural and epidermal structures occurs in the regenerating blastema; the epithelial covering of the apical ridge thickens (Hay, 1960). After epidermal thickening occurs, differentiation and growth of a new limb takes place (Singer and Craven, 1948). Other studies have shown that nerves are necessary for synthesis of DNA, RNA, and proteins in regenerating limbs (Robbins, 1967; Dresden, 1969).

Although it has been well documented that neurotrophic effects are necessary for the growth and development of many tissues, the exact nature of the trophic mechanism has not been delineated. Two schools of thought are present concerning this phenomenon. First, Singer (1965) has brought forth a trophic substance hypothesis, which maintains that regeneration depends upon the production of some substance necessary for regeneration. He notes that prior to innervation the cells of the limb produce this substance themselves; after nerves enter the limb, the limb cells lose their capacity to produce this substance and from then on the regeneration of the limb is dependent on innervation. It should be pointed out that this theory does not equate the production of an action potential, and subsequent muscle contraction, with a neurotrophic mechanism. The second theory which has been stated by Becker (1972) is that once nerves have entered a developing area they assume control of the bioelectric field. It should also be noted that this theory does not imply that the action potential and subsequent muscle activity produced by somatic efferent nerves comprise a neurotrophic effect.

If one analyzes the known facts, as have been put forth, on direct effects of the nervous system on skeletal growth and development, it is
very evident that little concrete information is available. Many of
the aforementioned studies have attempted to solve this problem;
Corbin and Hinsey (1939), Gillespie (1954), Solovoev (1959), Ring (1961),
Hurrell (1937), Hamburger (1929, 1939, 1940) to name just a few.
However, none of the investigators were able to answer the question as
to whether direct neurotrophic mechanisms are present during bone
development and maintenance.

The reason no one has been able to answer the proposed question
was stated by Ring in 1961, "it seems almost impossible that one would
be able to devise an experiment to delineate a direct neurotrophic
effect on the skeleton, because inevitably the muscular system (limb
movement) is always involved as a variable." Since 1961, however,
experimental studies on embryonic motility have reported information
which now makes the study of direct neurotropic influences on skeletal
development possible. In these experiments it has been demonstrated
that deafferentation or removal of neural crest cells which give rise
to afferent (GSA & GVA) and visceral efferent nerves produce little
effect, if any, on the development and maintenance of periodic motor
activity in chick embryos (Hamburger, Wenger, and Oppenheim, 1966;
Hamburger and Narayanan, 1969; Decker, 1970; Narayanan and Malloy,
1974). Due to the findings in the previous studies, the problem of
compromising limb movement during experimental deafferentation (neural
crest removal) of skeletal elements has been overcome. Therefore, using
the chick embryo as a model system, I propose to study the following
question, "Do the neural crest progenitors of afferent (GSA, GVA) and
visceral efferent nerves exert an influence on skeletal development?"
In this investigation, tibial growth and development will be studied
in normal embryos and in embryos which have had the neural crest
removed at leg levels. After collection of data relating to develop-
mental parameters of control and experimental groups, the information
will be analyzed in an attempt to answer the proposed question.
STATEMENT OF SPECIFIC AIMS

The question to be studied in this investigation is, "do the neural crest progenitors of afferent (GSA, GVA) and visceral efferent nerves exert a direct influence on skeletal development?" The skeletal member which will be investigated is the tibia-fibula of chick embryos. In this study, the development of the tibia-fibula will be analyzed in control embryos and in embryos which have undergone neural crest removal at the leg level (experimental embryos).

A series of experiments have been devised in order to study development in the tibia-fibulas of control and experimental embryos from 6 through 12 days of incubation. The first experiment will be concerned with studying behavior (leg motility) in control and experimental embryos. The second experiment will be concerned with elucidating neuro-skeletal relationships in the developing tibia-fibula at the light and electron microscopic level. The third experiment will be aimed at determining various gross parameters (weight, length, and width), muscle cross-sectional areas, and ionic concentrations (calcium, inorganic phosphate, and magnesium) in the tibia-fibula of control and experimental embryos. In the fourth experiment various histochemical parameters will be analyzed in the developing tibia-fibulas of control and experimental embryos. The following histochemical tests will be accomplished: 1) Von Kossa's technique for demonstration of calcified tissue, 2) polychrome stain for differentiating 'immature' collagen from 'mature' collagen, and 3) alcian blue stain for demonstration of
acid mucopolysaccharides. The fifth experiment will be devoted to recording vascular flow phenomena in the tibia-fibula of control and experimental embryos. The Falck technique for identification of catecholamines will be used to assess the developmental stage when neurotransmitter first appears in the neuro-vascular system of the tibia-fibula. Also, an in vivo assessment of blood flow rates and vessel diameters will be accomplished. Finally, tests of vessel reactivity to exogenous catecholamines and α-blocking agents will be made in vivo.

By studying the proposed question in the manner outlined above, it will be possible to determine whether the nervous system exerts a direct influence on developing skeletal elements.
MATERIALS, METHODS AND RESULTS

Since this investigation includes five separate studies, which utilize the same basic methodology and materials, a generalized description of the subjects, surgical methods utilized and results of the operation on development of spinal cord elements will be discussed first. Subsequently, a separate discussion of every experiment will be accomplished, noting the specific purpose, materials, methods, and results of that study.

I. MATERIALS AND METHODS (General Description)

A. Animals

The embryos used in this study were obtained as fertile chick eggs (DeKalb strain) from Ken-Roy Hatcheries, (Burger, Missouri). The eggs were refrigerated prior to incubation in order to synchronize their development (Gottlieb, 1963). For incubation, the eggs were placed in a commercial forced draft incubator which was maintained at a temperature of 37.5°C with a relative humidity of 65% to 70%. The eggs were incubated obliquely and turned twice every day.

The embryos were prepared for surgery in the manner outlined by Narayanan (1970). After two days (forty-eight hours) of incubation, eggs were taken from the incubator and candled; candling allows for determination of embryo position. Then an elliptical window was made over the embryo's position by sanding off the candling mark with a power-driven rotating sanding unit; the shell membrane remains intact during this procedure. The region of the shell surrounding the window
was then swabbed with alcohol, and the shell membrane was removed. The shell window was covered with a sterilized cover slip and sealed with paraffin. Following this procedure, the eggs were returned to the incubator and placed in a horizontal position with the window facing upward. The embryos were allowed to develop to stages 16–17 (Hamburger and Hamilton, 1951), and then microsurgical procedures were performed.

B. Surgical Procedure

Before the embryos underwent microsurgical preparation, they were allowed to acclimatize for 30 minutes in a plexiglass observation chamber. After acclimatizing, the vitelline membrane was stained lightly with neutral red. Subsequently, the vitelline membrane was reflected away from the operating field with a watchmaker's forceps. Embryos to undergo the operation were then selected at random, and the remaining embryos (sham operated) served as control. Next, cauterization of the neural crest elements at the leg level was accomplished by using a modified Concept 'C' (Model 140) cauterization unit (Fig. 1). This procedure proved to be very effective for the discrete removal of neural crest elements (Figs. 2 & 3).

All operative procedures were carried out under aseptic conditions. All of the instruments utilized in the surgery were housed in a plexiglass operating hood, which was fitted with a germicidal lamp and necessary dust covers (Narayanan, 1970).

After completing the operative procedure, the glass coverslip was remounted over the shell window and the embryos were returned to the plexiglass egg chamber. Twenty-four hours after the operation, the eggs were returned to the regular incubator. The embryos were allowed to develop until they reached representative stages needed for gross,
histological, chemical, behavior, or vascular flow studies.

C. Condition of Embryos used in all Experiments

Before harvesting the embryos, certain criteria were employed to insure that the control embryos and the experimental embryos were representative of their respective groups. First of all, the developmental age of the embryos was determined according to the criteria reported by Hamburger and Hamilton (1951); this procedure assured age uniformity. Next, any embryos with observable developmental defects were discarded; this factor is important, for a small percentage of 'normal' embryos do present developmental abnormalities, i.e. microphthalmia, anophthalmia, cerebral herniation, etc. After Experiment I of this study was completed, two behavioral tests were added to the criteria above to insure that subsequent control and experimental embryos coincided with established behavioral parameters. For the first test, a one minute observation was made of total leg motility; total leg motility had to fall within one standard deviation unit (as reported in Experiment I) of the established leg motility means for each age group before the embryo (control or experimental) could be chosen for the study. Secondly, a stimulation test was performed on control and experimental embryos; for this test the control embryo's reflex arc must demonstrate functional integrity (response to stimulation) while the experimental embryo's reflex arc must prove to be defective (no response to stimulation). Stimulation utilizing an extremely thin and flexible glass needle was performed by gently stroking the lateral cutaneous area at mid-leg levels.

Upon harvesting the embryos for histology, it was observed that no gross abnormalities of the torso or extremities were observable in
the experimental embryos of any age group (Fig. 4). Also, there was no evidence of joint stiffness or fusion in the lower extremity of experimental embryos, although a slight decrease in muscular tissue could be noticed in some legs by day 12 of incubation.

To confirm the behavioral tests, a gross dissection of the spinal cord was carried out at lumbosacral levels in both control and experimental embryos. Since the ischial plexus supplies afferent and efferent innervation to the chick tibia-fibula, special attention was given to spinal cord segments 25 to 30, which give rise to this plexus (Fouvet, 1974). The lumbosacral enlargement and ischial plexus in a 12 day control embryo is shown in Fig. 5. In experimental embryos of the same incubation age (Fig. 6), there is a reduction in the gross size of both the ischial plexus and the lumbosacral enlargement. Upon gross dissection of the leg in experimental embryos, a normal, but decreased quantity of nerves were noted innervating the muscles.

In addition to gross dissections, histological preparations of the lumbosacral spinal cord were accomplished in both control and experimental embryos. The majority of the specimens (control and experimental) were fixed by perfusion with 10% neutral buffered formalin, routinely processed, and serial sectioned in their transverse axis at 12μ, the sections were then stained with 1% thionin. In addition, a modified Cajal-DeCastro silver technique (Levi-Montalcini, 1949) was used in some embryos (control and experimental) for better delineation of fiber tracts. A cross-section through the lumbosacral level of a 12 day control embryo is shown in Fig. 7; note the presence of dorsal root ganglia, sympathetic chain ganglia, dorsal root, ventral roots, and ventral motor horn cells. In experimental embryos, due to
removal of neural crest elements, there is a loss of dorsal root ganglia, sympathetic chain ganglia, and dorsal roots; only the ventral horn with its motor cells remains intact (Figs. 8, 9, 10). The histological preparations made it possible for tracings to be prepared showing the development of the lumbosacral spinal cord, associated ganglia, and nerve rootlets in control and experimental animals.

The tracings were recorded in semi-diagrammatic reconstructions on graph paper, following the method described by Hamburger (1939) and Hamburger and Narayanan (1969). The reconstructions were made from serial sections, and are accurate in their cephalo-caudal dimension, while the distances in the mediolateral direction are arbitrary. Segment boundaries of the spinal cord were determined on the basis of motor rootlets being the midpoint between the last motor rootlet of the following nerve (Hamburger, 1948). The motor rootlets were traced on both sides along with the other structures found in the control embryo (dorsal root ganglia, sympathetic chain ganglia, sympathetic chain, and associated nerve fibers). The results of the reconstructions are shown in Figure 11; the reconstruction of the spinal cord and associated elements of a control embryo demonstrate the presence of the dorsal root ganglia, sympathetic ganglia, and associated fiber elements, from spinal cord segments 23 through 30 (vertebral level L1 + S1). However, an absence or reduction of dorsal root ganglia, sympathetic chain ganglia, and associated nerve fibers occurs in corresponding neural segments of experimental embryos (Fig. 11). No attempt was made to do a cell count of the ventral motor horn cells in the experimental embryos, however, future investigations will be concerned with this problem.
II. SPECIFIC MATERIALS, METHODS AND RESULTS OF INDIVIDUAL EXPERIMENTS
A. Experiment I - Behavioral Testing

Although other investigations have studied the effect of deafferentation and neural crest removal on motility patterns (Hamburger, Wenger, and Oppenheim, 1966; Hamburger and Narayanan, 1969; Decker, 1970; Narayanan and Malloy, 1974), none of these studies have reported motility patterns in control and experimental limbs prior to 9 days of incubation. Since the embryos in this study range from 6 days through 12 days of incubation, Experiment I was designed in order to determine a quantitative and qualitative measurement of total motility in the legs of control and experimental embryos.

Animals:

The type of embryos and procedures associated with their maintenance have been enumerated in the Materials and Methods - General Description portion of this section.

Surgical Procedure:

The methods and materials utilized to perform the surgical removal of neural crest elements have been described in the Materials and Methods - General Description portion of this section.

Experimental Analysis:

The purpose of this experiment was to determine whether neural crest removal at leg levels affected total leg motality in embryos from 6 through 12 days of incubation. To accomplish this goal, observations of total leg motality in control and experimental embryos were made over a fifteen-minute time period. Before the observation period was begun, experimental and control embryos were given a number by an impartial technician. The technician was instructed to cover
any identifying marks on the experimental shells with opaque tape, and then tape a control egg shell in the same manner. Next, numbers were marked on the tape and this was used as the embryo's identification until the embryo was sacrificed. After numbers had been assigned, the embryos were allowed to acclimitize for fifteen minutes in a temperature and humidity controlled observation chamber.

The embryo was then observed using a stereobinocular microscope and all visible motor movements of the leg were recorded over a fifteen minute period. Movements were tabulated by using a digital hand counter. All recordings were made by the same observer (RBM) every day from 6 through 12 days of incubation in control and experimental embryos.

Prior to sacrifice and after leg movements had been recorded for a specific day, the tape was removed from the egg shell. The initial embryo identification number was noted, and gross observations and stimulation procedures were carried out as previously mentioned in the Materials and Methods - General Description portion of this section. Gross dissections of the lumbosacral cord were performed routinely on all embryos, while histological preparations were done at random. These last two procedures confirmed the effectiveness of the deafferentation procedure.

After all the data had been gathered from 6 through 12 days of incubation, the mean number of leg movements per minute was calculated for each embryo. A statistical comparison between control and experimental means was accomplished via analysis of variance. Differences between the means were assumed to be significant at $p \leq 0.05$.

Results:

The quantitative results of Experiment I are graphically depicted.
in Figure 30. An almost linear increase in total leg motality occurred from 6 through 12 days of incubation; during this period, there was a nine fold multiplication of motility. No significant differences were detected between control and experimental embryos at any age group (Table I).

Qualitatively, leg movements during this experimental period evolved from a slight twitch of the limb bud at 6 days to a full range of flexion and extension in the knee and ankle joints at 12 days of incubation. During the evolution of motility in the hind limb, a gradual increase in movement of distal elements was observed from 6 to 12 days. However, no differences in the range or type of movements were detectable between control and experimental limbs from 6 to 12 days of incubation. Since movement evolves from negligible levels to very sophisticated movements over this time period, qualitative differences in limb motility would have been discernible if they had been present.

B. Experiment 2 - Light Microscopy and Electron Microscopy of Neuro-Skeletal Relationships

The main purpose of this experiment was to determine what type of relationships, if any, exist between nerve and skeletal elements during early stages of development in control tibia-fibulas. Although there is some information on the development of innervation in the chick hind limb (Fouvet, 1974), no studies have been reported which are specifically concerned with relationships between neural and skeletal elements in early stages of development. Also, this investigation was undertaken to function as an added criterion to establish the effectiveness of neural crest removal in experimental
TABLE I

MEAN FREQUENCY OF MOVEMENTS PER MINUTE FOR
CONTROL AND EXPERIMENTAL EMBRYOS¹

<table>
<thead>
<tr>
<th></th>
<th>6d</th>
<th>7d</th>
<th>8d</th>
<th>9d</th>
<th>10d</th>
<th>11d</th>
<th>12d</th>
</tr>
</thead>
<tbody>
<tr>
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<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Experimental</td>
<td>9</td>
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<td>10</td>
<td>10</td>
<td>8</td>
<td>8</td>
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Control

<table>
<thead>
<tr>
<th>Days</th>
<th>Frequency ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>6d</td>
<td>4.0±0.8</td>
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<tr>
<td>7d</td>
<td>9.9±1.6</td>
</tr>
<tr>
<td>8d</td>
<td>20.0±6.7</td>
</tr>
<tr>
<td>9d</td>
<td>24.7±5.2</td>
</tr>
<tr>
<td>10d</td>
<td>28.0±5.5</td>
</tr>
<tr>
<td>11d</td>
<td>34.4±11.2</td>
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<td>12d</td>
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</table>

Experimental

<table>
<thead>
<tr>
<th>Days</th>
<th>Frequency ± S.D.</th>
</tr>
</thead>
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<td>8d</td>
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<td>29.7±6.3</td>
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<td>11d</td>
<td>36.5±8.7</td>
</tr>
<tr>
<td>12d</td>
<td>38.4±11.9</td>
</tr>
</tbody>
</table>

*n = Number of specimens observed.

Values are mean frequency of movements per minute ± S.D.

¹ = Nonsignificant differences between control and experimental embryos at p ≤ 0.05 according to Analysis of Variance.

d = Days of incubation.
embryos. Finally, as an adjunct to motility studies, histological material in this experiment was used to study muscle integrity in control and experimental animals.

**Animals:**

The type of embryos and procedures associated with their maintenance have been reported in the Materials and Methods - General Description portion of this section.

**Surgical Procedure:**

The methods and materials utilized to perform the surgical removal of neural crest elements have been described in the Materials and Methods - General Description portion of this section.

**Preparation of Light and Electron Microscopic Tissues:**

Prior to fixation for microscopy the control and experimental embryos from 6 through 12 days of incubation were analyzed using the criteria enumerated in the Materials and Methods - General Description portion of this section.

Fixation was accomplished for light microscopic purposes by perfusion. Perfusion was accomplished by inserting a 27 gauge needle in the left ventricle and injecting a chick Ringer's solution (1-5 ml. depending on embryo's age) followed by injection of 10% neutral buffered formalin (5-15 ml. depending on embryo's age).

The pressure of the perfusing medium was produced and maintained by placing the solutions in bottles 4 feet above the specimen. By the use of polyethylene tubing and two direction stop cocks, it was possible to inject one perfusant directly after the other. During perfusions, the umbilical vessels were clamped in order to prevent perfusant from flowing from the heart through the abdominal aorta and out the umbilical arteries.
After perfusion, the embryos were placed in 10% neutral buffered formalin for at least two days. Following fixation, the tissue was processed routinely and blocked in paraffin. Sections of the lumbo-sacral cord and tibia-fibula were oriented in the transverse plane (cross-section) and serial sectioned at 12 microns; sections of the tibia-fibula were also cut in the longitudinal plane. After sections were mounted on glass slides, they were stained using the Bodian method as modified by Gelderd (1972). No counterstains were used. Once staining was completed, the sections were dehydrated and cover-slipped.

Perfusion for electron microscopy was accomplished in the same manner as described above, except Karnovsky’s fixative (1965) was used in place of the 10% neutral buffered formalin. After perfusion, the leg was cut in small sections (proximal + distal) and placed in Karnovsky’s fixative for 4 hours; then, the tissue was washed in cacodylate buffer overnight at 4°C. The buffer rinse was followed by 4 hours post-fixation with 1% osmium tetroxide. The tissue was then dehydrated and embedded in Epon. Subsequently, the tissue was cut on an ultramicrotome; sections were placed on copper grids and stained with uranyl acetate and lead citrate (Reynolds, 1963). Specimens were observed and photographed using a Philips 300 electron microscope.

Materials and Methods used in Muscle Integrity Analysis:

The material for this study was the same as that prepared and utilized for the light microscopic portion of this experiment. The Bodian cross-section preparations of control and experimental tibia-fibulas were observed under a microscope, and two camera lucida drawings were made of each tibia-fibula. The first drawing was made at the level of the tibia’s primary nutrient foramen (periosteal bud), and the second
drawing was made 10 sections distally from the first. In embryos from 7 and 8 days of incubation, the first drawings were made as close as possible at the junction of the upper and middle third's of the tibia and the second drawing was made 10 sections distally to the first. The 7 and 8 day landmark corresponds to the area where the periosteal bud will be formed at 9 days.

From the two drawings of each leg, planimetric measurements in square centimeters were made of two muscle groups, the flexor digitorum longus m. and tibialis anterior m. These measurements were reported as mean square centimeters of muscle area per leg in each age group. The means of control and experimental embryos were subjected to statistical analysis and any differences at p < 0.05 were considered statistically significant. The statistical test used was the Analysis of Variance. It should be noted that complete reconstructions of entire muscle groups in control and experimental legs will be accomplished in subsequent studies.

Results:

Light microscopic observations on neuro-skeletal relationships showed that the median and lateral tibial nerves innervate the tibia and fibula. The median tibial nerve gives off smaller branches to skeletal elements along its path (Figs. 20, 21). The greatest branching of fibers from the main nerve trunk occurs in the area of the nutrient foramen (Figs. 20, 21, 22, 23), although some fibers can be seen in the periosteum throughout the whole length of the tibia. By day 7 of incubation, the gross pattern for the innervation of the leg is established. It is not until days 11 and 12, however, that nerve fibers can be frequently observed in periosteal areas. At no time were nerve
fibers observed in contact with osteocytes, osteoblasts, or chondrocytes. However, in periosteal areas near the nutrient foramen, nerve fibers were frequently found enveloping or passing in close approximation to cells of the periosteum (Figs. 23, 24, and 25). The nerve processes were observed coming into close approximation to periosteal cells, but no junctional complexes or terminal specializations were observed up to 12 days of incubation (Figs. 27, 28, 29).

Nerve fibers could also be identified in close approximation to the blood vessels of the developing tibia-fibula (Fig. 20). At the nutrient foramen, nerve fibers come into close association with the vascular elements, but no synaptic specializations were observed (Fig. 26).

In experimental embryos, the main nerve trunks of the leg were reduced in their cross-sectional diameters from control levels (Figs. 12 through 19). The size reduction, however, was more apparent in the older embryonic stages, i.e. 11 and 12 day animals. This observation may be related to altered embryogenesis of ventral motor horn cells in experimental embryos. Nerve fibers were not found in association with the periosteum or blood vessels in the limbs of embryos with total neural crest removal; but even in experimental embryos which had remnants of dorsal root ganglia or sympathetic chain ganglia, very few nerve fibers were observed in the vicinity of periosteal or vascular elements.

The results of muscle area measurements are depicted graphically in Figure 39. As can be seen from the graph, differences between the mean cross-sectional areas of the muscle groups from control and experimental embryos were not evident until 10 days of incubation.
After ten days of incubation, however, there was a noticeable decrease of muscle area in the experimental embryos. When the area values were subjected to statistical analysis, significant differences were found between control and experimental values on the 10th, 11th and 12th days of incubation (Table IX). Some of these findings correlate with gross changes that were observable at 12 days of incubation.

The results of this study, are by no means meant to infer any effect of GVA, GSA, or GVE modalities on myogenesis in the embryonic chick leg.

C. Experiment 3 - The Effects of Neural Crest Removal on Gross and Ionic Parameters of the Tibia-Fibula During Development.

Many investigations, which were enumerated in the Introduction, attempt to determine the effect of deafferentation or sympathectomy on long bone growth and development. However, no studies have been accomplished in this area which 1) did not interfere with limb movement, 2) adequately investigated gross and ionic parameters and 3) investigated the effects of afferent and visceral efferent modalities on limb development in situ. Therefore, the following experiment was carried out in order that gross and ionic parameters could be analyzed in the developing tibia-fibula of embryonic limbs which were devoid of afferent and visceral efferent innervation.

Animals:

The type of embryos used in this experiment as well as their preparation and maintenance was covered in the Materials and Methods - General Description portion of this section.

Surgical Procedure:

The materials and methods used to perform the surgical procedure
### TABLE IX

**MUSCLE AREA OF CONTROL AND EXPERIMENTAL EMBRYOS**

<table>
<thead>
<tr>
<th></th>
<th>7d</th>
<th>8d</th>
<th>9d</th>
<th>10d</th>
<th>11d</th>
<th>12d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td>4</td>
<td>6</td>
<td>6</td>
<td>6</td>
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</tr>
<tr>
<td>(n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control</th>
<th>1.89±0.32</th>
<th>2.45±0.37</th>
<th>5.92±3.25</th>
<th>6.75±1.56</th>
<th>15.61±2.02</th>
<th>25.80±2.96</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td>1.99±0.48</td>
<td>2.12±0.50</td>
<td>4.50±0.84</td>
<td>4.21±0.66*</td>
<td>8.77±0.79*</td>
<td>19.72±4.60*</td>
</tr>
</tbody>
</table>

n = Number of tibial specimens observed from different embryos.

Values are mean area in cm.$^2 \pm$ S.D. of two muscle preparations per tibia.

* = Significant difference between control and experimental embryos at $p \leq 0.05$ according to Analysis of Variance.

d = Days of incubation.
are explained in the Material and Methods - General Description portion of this section.

**Experimental Analysis:**

Control and experimental embryos were collected from 7 through 12 days of incubation using the criteria established in the Materials and Methods - General Description portion of this section. Subsequently, each leg was carefully dissected under a stereobinocular microscope; the tibia-fibula was removed and cleared of soft tissue. Then, the tibia-fibulas were immediately weighed on a Mettler balance, and their wet weights recorded in milligrams. After weighing, the length and mid-diaphyseal width of each tibia was measured using a vernier caliper; length and widths were recorded in centimeters and millimeters, respectively. Following gross measurements, each tibia-fibula was placed in a clean glass beaker and dried in a 100°C oven until a constant weight was attained. After a constant weight was reached, the dry weight of each specimen was recorded in milligrams.

Subsequently, the tibia-fibulas were ashed at 600°C for 24 hours. The residue was then weighed and the ash weight was recorded in milligrams. The ash was dissolved in 3M HCl to a final concentration of 1 mg. ash/ml. This concentration was maintained throughout the experiment. The dilutent was then analyzed for calcium, magnesium, and inorganic phosphate using an Ind. Lab. atomic absorption spectrophotometer. The concentration of each element is reported as millimoles per liter of solution.

The lengths, widths, weights (wet and dry), and ionic concentrations were subjected to statistical analysis (Analysis of Variance). Differences between control and experimental means were considered to be significant at $p \leq 0.05$. 
Finally, to demonstrate the gross morphology of the tibia-fibula from 6 through 12 days of incubation, the Lundvall technique (Lundvall, 1905) of cartilage staining in toto was carried out on two experimental and two control animals in each age group. In this technique, formalin fixed tissues were stained in toto with .25% methylene blue, dehydrated, and cleared with a mixture of oil of wintergreen and benzyl benzoate. Cartilage is stained blue, but bone is not stained. It should be noted that 6 day tibia-fibulas were not studied in the analytical portion of this experiment due to a physical inability to acquire uniform specimens by dissection.

**Results:**

Tibial lengths and widths increased markedly from 7 through 12 days of incubation (Figs. 32, 33). The length of the tibia increased approximately four fold, while the width of the tibia increased approximately two fold. The greatest amount of growth in length occurred over the last three days of the study, but growth in width was almost linear throughout the experiment. When tibial lengths and widths of control and experimental groups were subjected to statistical analysis, no significant differences were found in any age group (Tables II, III).

Observations on the gross morphology of control and experimental tibia-fibulas during the experimental period, showed no salient differences (Fig. 31). The morphology of the diaphysis and epiphysis showed no size or shape incongruity over the duration of the study. The knee and ankle joints of control and experimental embryos showed no tendencies towards fibrotic union, and no abnormalities in the growth of distal segments of the lower extremity occurred, i.e. hypodactyly, hypophylangy, accentuated curvature of the metatarsals, and shortening of metatarsals and phalanges.
TABLE II

TIBIAL LENGTH OF CONTROL AND EXPERIMENTAL EMBRYOS

<table>
<thead>
<tr>
<th></th>
<th>7d (n)</th>
<th>8d (n)</th>
<th>9d (n)</th>
<th>10d (n)</th>
<th>11d (n)</th>
<th>12d (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6(12)</td>
<td>8(16)</td>
<td>7(14)</td>
<td>6(12)</td>
<td>7(14)</td>
<td>7(14)</td>
</tr>
<tr>
<td>Experimental</td>
<td>4(8)</td>
<td>5(10)</td>
<td>3(6)</td>
<td>4(8)</td>
<td>4(8)</td>
<td>5(10)</td>
</tr>
<tr>
<td>Control</td>
<td>0.34±0.03</td>
<td>0.47±0.02</td>
<td>0.70±0.04</td>
<td>0.80±0.09</td>
<td>1.07±0.05</td>
<td>1.16±0.06</td>
</tr>
<tr>
<td>Experimental</td>
<td>0.36±0.02</td>
<td>0.48±0.06</td>
<td>0.72±0.04</td>
<td>0.82±0.07</td>
<td>1.04±0.03</td>
<td>1.19±0.06</td>
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</tbody>
</table>

n = Number of embryos in each group (Number of tibia = 2n).

Values are mean tibial lengths in cm. ± S.D.

1 = Nonsignificant differences between control and experimental embryos at p <0.05 according to Analysis of Variance.

d = Days of incubation.
<table>
<thead>
<tr>
<th></th>
<th>7d</th>
<th>8d</th>
<th>9d</th>
<th>10d</th>
<th>11d</th>
<th>12d</th>
</tr>
</thead>
<tbody>
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<td>6(12)</td>
<td>8(16)</td>
<td>7(14)</td>
<td>6(12)</td>
<td>7(14)</td>
<td>7(14)</td>
</tr>
<tr>
<td>Experimental</td>
<td>4(8)</td>
<td>5(10)</td>
<td>3(6)</td>
<td>4(8)</td>
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Control

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<th>12d</th>
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</thead>
<tbody>
<tr>
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<td>0.46±0.03</td>
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<td>0.66±0.05</td>
<td>0.73±0.03</td>
<td>0.81±0.02</td>
<td>0.82±0.07</td>
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</table>

Experimental

<table>
<thead>
<tr>
<th></th>
<th>7d</th>
<th>8d</th>
<th>9d</th>
<th>10d</th>
<th>11d</th>
<th>12d</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0.45±0.04</td>
<td>0.51±0.02</td>
<td>0.62±0.03</td>
<td>0.70±0.02</td>
<td>0.79±0.02</td>
<td>0.85±0.04</td>
</tr>
</tbody>
</table>

n = Number of embryos in each group (Number of tibia = 2n).

Values are mean tibial widths at mid-diaphysis in mm. ± S.D.

\( ^1 \) = Nonsignificant differences between control and experimental embryos at \( p \leq 0.05 \) according to Analysis of Variance.

d = Days of incubation.
A graphic representation of tibia-fibula wet and dry weight in control and experimental animals is presented in Figs. 34 and 35. Both wet and dry weights increased markedly during the last three days of the experimental period; dry weights increased almost three fold during this time period, while wet weights increased only two fold. When control and experimental mean tibia-fibula weights were subjected to statistical analysis, no significant differences in dry or wet weights were found in any age group (Tables IV, V).

Ionic determinations of calcium, inorganic phosphate, and magnesium in both control and experimental groups are depicted graphically in Figs. 36, 37, and 38, respectively. Calcium ion concentration was very low on the seventh day of incubation in both experimental and control groups. On day eight, the concentration of calcium ions in both groups increased markedly. However, after an almost two fold increase on day nine, calcium concentrations in experimental and control groups remained relatively constant for the following three days. The actual millimolar concentration of calcium ions was less than 1mM on day seven, approximately 2 mM on day eight. From the 9th through 12th days of incubation the mM concentration of calcium ranged from 5 to 7 mM, with calcium values showing a slight daily increase over the four day period. Statistical analysis showed no significant differences between control and experimental values from 9 through 12 days of incubation (Table VI). Although statistical procedures were not applied to 7 and 8 day calcium concentrations, it is doubtful that real differences exist between the experimental and control values reported.
### TABLE IV

**TIBIA—FIBULA WET WEIGHTS OF CONTROL AND EXPERIMENTAL EMBRYOS**

<table>
<thead>
<tr>
<th></th>
<th>7d</th>
<th>8d</th>
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<th>10d</th>
<th>11d</th>
<th>12d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7(14)</td>
<td>8(16)</td>
<td>8(16)</td>
<td>6(12)</td>
<td>7(14)</td>
<td>7(14)</td>
</tr>
<tr>
<td>Experimental</td>
<td>4(8)</td>
<td>5(10)</td>
<td>3(6)</td>
<td>4(8)</td>
<td>4(8)</td>
<td>5(10)</td>
</tr>
</tbody>
</table>

- **Control**
  - 7d: 1.0±0.2
  - 8d: 2.1±0.3
  - 9d: 4.5±0.5
  - 10d: 7.4±1.2
  - 11d: 13.1±1.7
  - 12d: 14.5±1.8

- **Experimental**
  - 7d: 1.1±0.3
  - 8d: 2.4±0.5
  - 9d: 4.9±0.8
  - 10d: 7.8±2.6
  - 11d: 12.7±1.3
  - 12d: 15.9±1.1

- **n** = Number of embryos in each group (2n = Number of tibia-fibula).
- Values are mean tibia-fibula weights in mg. ± S.D.

1. Nonsignificant differences between control and experimental embryos at p ≤ 0.05 according to Analysis of Variance.

- **d** = Days of incubation.
TABLE V

TIBIA-FIBULA DRY WEIGHTS OF CONTROL AND EXPERIMENTAL EMBRYOS

<table>
<thead>
<tr>
<th></th>
<th>7d</th>
<th>8d</th>
<th>9d</th>
<th>10d</th>
<th>11d</th>
<th>12d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7(14)</td>
<td>8(16)</td>
<td>8(16)</td>
<td>6(12)</td>
<td>7(14)</td>
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<tr>
<td>Experimental</td>
<td>4(8)</td>
<td>5(10)</td>
<td>3(6)</td>
<td>4(8)</td>
<td>4(8)</td>
<td>5(10)</td>
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Control

<p>| | | | | | | |</p>
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</thead>
<tbody>
<tr>
<td>7d</td>
<td>0.30±0.03</td>
<td>0.38±0.04</td>
<td>0.54±0.03</td>
<td>0.74±0.05</td>
<td>1.56±0.25</td>
<td>2.01±0.26</td>
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<tr>
<td>8d</td>
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<td>9d</td>
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<td>10d</td>
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<td>11d</td>
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Experimental

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<tbody>
<tr>
<td>7d</td>
<td>0.33±0.03</td>
<td>0.42±0.04</td>
<td>0.60±0.05</td>
<td>0.80±0.08</td>
<td>1.58±0.13</td>
<td>2.04±0.10</td>
</tr>
<tr>
<td>8d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9d</td>
<td></td>
<td></td>
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</tr>
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<td>10d</td>
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<td>11d</td>
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</tr>
<tr>
<td>12d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n = Number of embryos in each group (2n = Number of tibia-fibula).

Values are mean tibia-fibula weights in mg. ± S.D.

1 = Nonsignificant differences between control and experimental embryos at p < 0.05 according to Analysis of Variance.

d = Days of incubation.
TABLE VI

mM OF CALCIUM IN CONTROL AND EXPERIMENTAL EMBRYOS

<table>
<thead>
<tr>
<th></th>
<th>7d</th>
<th>8d</th>
<th>9d</th>
<th>10d</th>
<th>11d</th>
<th>12d</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n)</td>
<td>1(16)</td>
<td>1(16)</td>
<td>2(6)</td>
<td>2(8)</td>
<td>3(6)</td>
<td>4(4)</td>
</tr>
<tr>
<td><strong>Experimental</strong></td>
<td>1(8)</td>
<td>1(10)</td>
<td>2(3)</td>
<td>2(4)</td>
<td>2(4)</td>
<td>2(5)</td>
</tr>
<tr>
<td>Control</td>
<td>0.04</td>
<td>1.57</td>
<td>5.09±1.65</td>
<td>5.61±1.50</td>
<td>5.69±0.07</td>
<td>6.54±0.60</td>
</tr>
<tr>
<td>Experimental</td>
<td>0.57</td>
<td>2.42</td>
<td>4.34±0.99</td>
<td>5.76±0.55</td>
<td>6.09±0.35</td>
<td>7.61±0.67</td>
</tr>
</tbody>
</table>

n = Number of groups analyzed.
( ) = Number of tibia-fibulas analyzed in each group.
Values are mM calcium ± S.D.
1 = Nonsignificant differences between control and experimental embryos at p ≤ 0.05 according to Analysis of Variance.
d = Days of incubation.
Inorganic phosphate values were not assessed on the seventh and eighth days, however, nine day control and experimental specimens contained concentrations of approximately 0.65mM. An increase to 1.00mM occurred on day 10, followed by very slight increases up to a 12 day concentration of approximately 1.15mM. No statistically significant differences were found between control and experimental values in any age group (Table VII).

The ionic concentrations of magnesium were determined from 7 through 12 days of incubation in control and experimental tibia-fibulas. It is evident from Figure 38, that magnesium ion concentrations change very little over the time period studied in this experiment. On day seven the magnesium concentration is 0.15mM in control specimens, while it is 0.27mM in experimental specimens. The concentration of control specimens increases on day eight to 0.32mM, while experimental values rose to only 0.28mM. Although there are slight differences in the mM concentrations in both groups on the 7th and 8th days of incubation, it is doubtful that these differences are significant when the average standard deviation of the 9, 10, 11, and 12 day values are considered. In this study, the magnesium ion concentration reached its highest levels (0.35mM) at 9 days of incubation (Fig. 38). Subsequently, a slight decrease in the concentration of magnesium ions occurred at 10 days. From 10 through 12 days of incubation values remained at a relatively constant 0.2mM level.

When values for magnesium ion concentration were subjected to statistical analysis, no significant differences were found between the experimental and control means of any age group (Table VIII).
### TABLE VII

**mM OF INORGANIC PHOSPHATE IN CONTROL AND EXPERIMENTAL EMBRYOS**

<table>
<thead>
<tr>
<th></th>
<th>9d</th>
<th>10d</th>
<th>11d</th>
<th>12d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n)</td>
<td>2(6)</td>
<td>2(8)</td>
<td>3(6)</td>
<td>4(4)</td>
</tr>
<tr>
<td>Experimental</td>
<td>2(3)</td>
<td>2(4)</td>
<td>2(4)</td>
<td>2(5)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.67±0.02</td>
<td>1.01±0.03</td>
<td>1.05±0.19</td>
<td>1.14±0.04</td>
</tr>
<tr>
<td>Experimental</td>
<td>0.63±0.05</td>
<td>1.02±0.03</td>
<td>1.03±0.07</td>
<td>1.19±0.01</td>
</tr>
</tbody>
</table>

n = Number of groups analyzed.
( ) = Number of tibia-fibulas analyzed in each group.
Values are mM Inorganic Phosphate ± S.D.

1 = Nonsignificant differences between control and experimental embryos at p ≤ 0.05 according to Analysis of Variance.

d = Days of incubation.
<table>
<thead>
<tr>
<th></th>
<th>7d (n)</th>
<th>8d (n)</th>
<th>9d (n)</th>
<th>10d (n)</th>
<th>11d (n)</th>
<th>12d (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>1(16)</td>
<td>1(16)</td>
<td>2(6)</td>
<td>2(8)</td>
<td>3(6)</td>
<td>4(4)</td>
</tr>
<tr>
<td><strong>Experimental</strong></td>
<td>1(8)</td>
<td>1(10)</td>
<td>2(3)</td>
<td>2(4)</td>
<td>2(4)</td>
<td>2(5)</td>
</tr>
</tbody>
</table>

**Control**

- 0.15
- 0.32
- 0.4±0.16
- 0.25±0.02
- 0.21±0.08
- 0.21±0.02

**Experimental**

- 0.27
- 0.28
- 0.29±0.16
- 0.25±0.02
- 0.16±0.02
- 0.16±0.03

- Number of groups analyzed.
- ( ) = Number of tibia-fibulas analyzed in each group.
- Values are mM Magnesium ± S.D.
- Nonsignificant differences between control and experimental embryos at p < 0.05 according to Analysis of Variance.
- Days of incubation.
D. Experiment 4 - Histochemical Observations on the Effects of Neural Crest Removal at Leg Levels on Development of the Tibia-Fibula

No previous histochemical studies have reported on embryonic skeletal elements which have developed in the absence of GSA, GVA and GVE innervation. Therefore, the following experiment was designed to study the distribution of calcium phosphate, collagen, and acid mucopolysaccharides in control and experimental tibia-fibulas from 6 through 12 days of incubation. This experiment will aid in determining the direct effect the nervous system may have, if any, on skeletal growth and development.

**Animals:**

The type of embryos used in this experiment as well as their preparation and maintenance was covered in the Materials and Methods - General Description portion of this section.

**Surgical Procedure:**

The materials and methods used to perform the surgical procedure are explained in the Materials and Methods - General Description portion of this section.

**Experimental Analysis:**

Control and experimental embryos were harvested from 6 through 12 days of incubation using the criteria established in the Materials and Methods - General Description portion of this section. Subsequently, three different methods of fixation and staining were used to demonstrate constituents of the inorganic and organic matrix of the tibia-fibula. The inorganic matrix was analyzed by a modification of Von Kossa's stain; this method delineates deposits of calcium phosphate. The organic matrix was studied using a Polychrome stain for collagen
and an Alcian blue stain for demonstration of acid mucopolysaccharides.

Lillie's modification of the Von Kossa method was utilized in this experiment (Lillie, 1965). Fixation of embryos was achieved by perfusion with 10% neutral buffered formalin. The perfusion technique was the same as that described in Experiment 2 for the preparation of light microscopic specimens. However, chick Ringer's (which contains CaCl₂) was not perfused prior to 10% neutral buffered formalin due to the possibility of a false positive Von Kossa reaction.

Following fixation for at least 48 hours, the specimens were washed in distilled water, dehydrated in ascending alcohol solutions, and blocked in paraffin. The tibia-fibulas were then serial-sectioned in their transverse axis (cross-section) at 12μ. Subsequently, the sections were mounted on slides. Since the primary nutrient foramen is a consistent anatomical landmark, slides with sections from this area were chosen for staining. The slides were then grouped in staining dishes so that control and experimental tissues from each age group would be stained together. Tissues were hydrated and then stained for 45 minutes in 5% silver nitrate. Subsequently, they were rinsed in water, treated for 3 minutes with sodium thiosulfate, dehydrated, cleared, and coverslipped using Gurr's Depex mounting medium and No. 1 coverslips.

Collagen was stained by utilizing a Polychrome stain developed by Herovici (1963). Fixation of embryos was achieved by perfusion with Chick Ringer's followed by a solution of formalin-acetic-alcohol (10:5:85). The technique for perfusion was the same as that described in Experiment 2 for the preparation of light microscopic specimens. Following 24 hours of fixation, the tibia-fibulas were routinely...
processed, blocked in paraffin, serial sectioned at 12μ in their transverse axis (cross-section), and mounted on glass slides. The slides were then grouped in staining dishes so that control and experimental tissues from each age group would be stained together. The slide specimens were then hydrated, stained for two minutes with a solution containing .1% methyl blue, .2% acid fuchsin, and 50% picric acid. After staining was accomplished, the sections were rinsed in 1% acetic acid, dehydrated, cleared, and then coverslipped using Gurr's Depex mounting medium and No. 1 coverslips.

An Alcian blue stain was used to analyze acid mucopolysaccharide distribution. Lillie's procedure for Alcian blue staining was utilized (Lillie, 1965). Fixation of embryos was achieved by perfusion with chick Ringer's solution followed by 10% neutral buffered formalin. The perfusion technique was the same as that described in Experiment 2 for the preparation of light microscopic specimens. Following fixation for 48 hours, the specimens were washed in distilled water, and routinely processed and blocked in paraffin. The tibia-fibulas were then serial sectioned in their transverse axis (cross-section) at 12μ. Subsequently, the sections were mounted on glass slides. Since the primary nutrient foramen is a consistent anatomical landmark, slides with sections from this area were chosen for staining. The slides were then grouped in staining dishes so that control and experimental tissues from each age group would be stained together. After sorting, sections were hydrated and then washed for three minutes in 3% acetic acid. Next, the tissue was stained for 2 hours with a solution of 1% alcian blue in 3% acetic acid. After staining, the sections were destained for 5 minutes in 3% acetic acid, washed for 3 minutes in distilled water, dehydrated in
ascending alcohols, cleared in xylene, and coverslipped using Gurr's Depex mounting medium and No. 1 coverslips.

Representative sections from all three histochemical preparations were photographed on High Speed Ektachrome (3200K) color film using a Leitz photomicroscope.

Results:

A summary of the results of all three histochemical tests is reported in Table X. Note the criteria listed at the bottom of Table X; these same parameters will be utilized in a detailed account of the results. The results of each histochemical test will be reported separately in the following order: 1) Von Kossa, 2) Polychrome, and 3) Alcian Blue.

1. Von Kossa Results

The Von Kossa method produces a brown to black-brown color in calcified tissues subsequent to the staining procedure; no color reaction indicates an absence of calcium phosphate from the tissue being analyzed.

Positive staining of calcified elements was not present until eight days of incubation (Figs. 40 through 45). At eight days of incubation there was a small diaphyseal collar of bone surrounding the cartilagenous model of the tibia; the fibula showed only small areas of calcification in the disphyseal collar of the same histological section (Figs. 44, 45). No differences in the quantity or quality of staining between eight day control and experimental tibia-fibulas were observed. In nine day specimens, a double lamella of diaphyseal bone was present in the tibia, while only one lamellae of bone surrounded the cartilagenous model of the fibula. Although the quantity of calcified tissue was constant in control and experimental tibia-fibulas, the
### TABLE X

**QUALITATIVE HISTOCHEMICAL ANALYSIS OF TIBIA-FIBULA DEVELOPMENT**

<table>
<thead>
<tr>
<th>Days of Incubation</th>
<th>Specimen</th>
<th>n</th>
<th>Histochemical Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Von Kossa</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>8</td>
<td>A,2</td>
</tr>
<tr>
<td></td>
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<tr>
<td>7</td>
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<td>6</td>
<td>A,2</td>
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<tr>
<td></td>
<td>Experimental</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Control</td>
<td>8</td>
<td>B,2</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Control</td>
<td>6</td>
<td>B,2</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Control</td>
<td>6</td>
<td>B,2</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Control</td>
<td>8</td>
<td>A,2</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Control</td>
<td>8</td>
<td>A,2</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

n = Number of tibia-fibulas analyzed in each group.

A = Color intensity of substance analyzed was the same in control and experimental groups.

B = Color intensity of substance analyzed was different between control and experimental groups.

1 = Distribution of substance analyzed was greater in controls than experimentals.

2 = Distribution of substance analyzed was the same in controls and experimentals.

3 = Distribution of substance analyzed was less in controls than experimentals.
staining quality was less intense in some experimental specimens (Figs. 46, 47). However, when viewed under a higher power objective, little difference can be observed between the staining qualities of nine day tibia-fibulas (Figs. 48, 49). The findings in the 10 day experimental and control tibia-bibulas are very similar to the 9 day results (Figs. 50, 51). The staining intensity is decreased in some control specimens, while the quantity of stained material was constant in both groups. At 10 days, however, more numerous and thicker trabeculae are found between the diaphyseal lamellae. Near the nutrient foramen, three lamellae of bone are sometimes observed. When viewed under a higher power, the difference in staining intensities between 10 day experimental and control groups is almost negligible. Analysis of 11 and 12 day specimens showed no marked differences in bone quality or quantity between experimental and control tibia-fibulas (Figs. 54 through 61). During the last two days, the number and thickness of trabeculae increased along with the number of diaphyseal lamellae.

In summary then, assessment of the calcium phosphate content in tibia-fibulas by Von Kossa's method, showed no discernable differences in distribution of this substance between control and experimental embryos in any age group (Figs. 40 through 61). However, differences in intensity of the staining reaction could be noticed on the ninth and tenth days of incubation. On these days, the experimental specimens seemed to be stained less intensely than the controls.

2. Polychrome Results

The Polychrome stain for collagen differentiates between 'immature' and 'mature' collagen by staining the former blue and the latter red. Throughout this experiment, no 'mature' (red staining) collagen was
distinguished in bone or cartilage by this staining procedure; only 'immature' (blue staining) collagen was observed. Also, no differences in the quantity or quality of collagen staining were observed between control and experimental specimens in any age group (Figs. 62 through 75).

By seven days of incubation a small dark blue collar of collagen could be seen surrounding the cartilagenous elements of the tibia and fibula (Figs. 64, 65). At eight days, when diaphyseal bone is first formed, the outer aspect (periosteum) of the bony lamella exhibits light blue staining, while the bone matrix itself stains dark blue (Figs. 66, 67). Collagenous elements of the cartilage were never stained by the Polychrome procedure in any of the age groups investigated. From 9 through 12 days of incubation the outer aspect of the diaphysis (periosteum) stained light blue and the bone matrix stained dark blue. Although the number of trabeculae and quantity of bony lamellae increased in the diaphyseal area from 9 through 12 days, no differences in collagen distribution or staining intensity were observed between control and experimental tibia-fibulas in any age group.

3. Alcian Blue Results

The Alcian Blue method stains acid mucopolysaccharide deposits turquoise-blue. The results of this histochemical procedure are represented pictorially in Figures 76 through 89. During the late stages of anlage development in the tibia-fibula (6 days of incubation), very few differences in acid mucopolysaccharide distribution can be observed between control and experimental tibia-fibulas. However, the staining intensity was somewhat decreased in the 6 day control embryos
when compared to 6 day experimental embryos. From 7 through 12 days of incubation there were marked differences in acid mucopolysaccharide distribution and staining intensity between control and experimental specimens.

Seven day control tibia-fibulas had a very uniform distribution of acid mucopolysaccharides throughout their cartilage matrix; no cartilagenous areas were devoid of stain (Fig. 78). However, experimental specimens of this same age group showed cartilagenous areas that were devoid of acid mucopolysaccharides (Fig. 79). Not only was the acid mucopolysaccharide distribution altered in experimental embryos, but also the color intensity of the stain was affected. In experimental tibia-fibulas the cartilage matrix which did stain was a deep blue color while the control matrix was turquoise. Also, cartilage of 7 day experimental specimens was less cellular than the cartilage of the corresponding controls.

Many of the differences noted between control and experimental tibia-fibulas at 7 days, continued to be present at 8, 9, 10, 11, and 12 days of incubation. Control specimens at 8 days of incubation had a uniform distribution of acid mucopolysaccharides in their cartilage, much like the 7 day controls (Fig. 80). Eight day experimental tibia-fibulas had cartilagenous areas which were devoid of stain; in areas which did stain, the color was much darker than that observed in the control cartilage (Fig. 81). Many empty cartilage lacunae can be observed in 8 day experimental tibias, but they are not present in the corresponding control tibias (Figs. 80, 81). The nine day control and experimental specimens showed changes in cartilage which have been noted on the two proceeding days (Figs. 82, 83). However, no
differences were noted relative to the acid mucopolysaccharide
distribution in bone of 9 day control and experimental tibia-fibulas
(Figs. 82, 83).

Although the marrow cavity becomes enlarged and more trabeculae
and lamellae of bone are formed, 10, 11, and 12 day control and experi­
mental specimens exhibit the same cartilage characteristics which were
noted at seven and eight days of incubation (Figs. 84 through 89). For
example, the cartilage of control specimens is stained very uniformly;
no areas devoid of acid mucopolysaccharides were observable. Also, the
color of the cartilage and bone matrix was stained with the same
intensity throughout the three control groups. The highly cellular
nature of the cartilage matrix was maintained in the 10, 11, and 12
day control groups; very little intercellular material was present.
Experimental tibia-fibulas from 10, 11, and 12 day age groups exhibit
cartilagenous areas which are devoid of stain; however, in areas that
do stain, the color is much darker than in the corresponding controls.
Many empty lacunae and relatively high quantities of intercellular matrix
can be observed in the cartilage of the experimental specimens. In the
diaphyseal bone of 10, 11, and 12 day control and experimental specimens
no marked differences were noted in acid mucopolysaccharide distribution
(Figs. 84 through 89). However, in the experimental tibia-fibulas,
a few lamellae and/or trabeculae of bone had small areas which were
devoid of stain; this finding was not observed in the control specimens.
E. Experiment 5 - The Effects of Neural Crest Removal at Leg Levels on
the Developing Vascular System in the Tibia-Fibula of Chick Embryos

Since no studies have been reported on blood flow rates, vessel
reactivity, or vascular innervation in the hindlimb of the chick embryo,
this experiment was designed to determine whether the nervous system might indirectly influence skeletal growth and development through its effects on the developing vascular system. In this study, the development of the vascular system (flow rates, vessel reactivity, and vascular innervation) will be studied in normal tibia-fibulas and in those which have developed without GSA, GVA, and GVE innervation.

**Animals:**

The type of embryos used in this experiment as well as their preparation and maintenance was covered in the Materials and Methods - General Description portion of this section.

**Surgical Procedure:**

The materials and methods used to perform the surgical procedure are explained in the Materials and Methods - General Description portion of this section.

**Experimental Analysis:**

Control and experimental embryos were harvested from 6 through 12 days of incubation using the criteria established in the Materials and Methods - General Description portion of this section.

The harvested embryos were used for histochemical analysis of the neurovascular system or for an *in vivo* analysis of vascular flow parameters.

For histochemical analysis of the neurovascular system, a modification of the Falck technique (Falck and Owman, 1965) was used to demonstrate adrenergic neurons. A modification of Koelle's thiocholine technique (Weitsen, 1970) was used to study acetylcholinesterase activity at the light microscopic level, while the Lewis and Schute technique (Lewis and Schute, 1968) was used for electron microscopic investigation of acetylcholinesterase activity.
The embryos, utilized for the study of adrenergic innervation to the blood vessels of the tibia-fibula, were taken from the eggs and decapitated. Four control and four experimental embryos were studied at 6, 11, and 12 days of incubation. Further studies on normal 16, 19, 20, and 21 day old embryos were carried out, along with studies on normal 1 day post-hatch, and 2 day post-hatch chicks. Three control embryos were used at each time point from 16 days of incubation through 2 days post-hatch. The whole leg was taken from the embryo and dropped immediately into a flask containing isopentane (2-Methylbutane) and dry ice. After freezing for a few minutes at -85°C, the tissue was placed in a Virtis freeze drying apparatus for three days. Then, the tissue was placed in a closed flask containing paraformaldehyde which had been kept for at least one week at 60% relative humidity. The flask containing the tissue and paraformaldehyde was heated for 1 1/2 hours at 80°C. The tissue was placed directly into paraffin, infiltrated until tissues dropped to the bottom of the paraffin container, and then blocked. Specimens which had not been treated with paraformaldehyde, as well as sections of the sympathetic chain ganglia served as controls. Sections were made in the transverse axis (cross-section) of the tibia-fibula, mounted on clean glass slides with xylene, and observed using a Leitz photomicroscope equipped with a mercury lamp and necessary barrier filters. Photographs were taken on Kodak Panatomic-X and Kodak High Speed Ektachrome (3200K) film. Fluorescent nerve fibers appeared yellow-green.

Four control and four experimental tibia-fibulas of 12 day old embryos have been studied using the acetylcholinesterase techniques, therefore this phase of the experiment should be considered a pilot
experiment. Tibia-fibulas to be used for light microscopic processing were taken from decapitated embryos, portions of the tibia-fibulas were then placed on a cryostat chuck and coated liberally with Tissue-Tek O.C.T. embedding compound. The chuck with tissue was plunged immediately into cold isopentane (2-methylbutane in dry ice) at -85°C to insure quick freezing. The chuck was then placed in a Harris International cryostat model CT at -25°C. The tissue was cut at 14μ and mounted on clean glass slides. The sections were fixed in cold (4°C) 4% Neutral buffered formalin for 20 minutes. Next, the sections were washed in distilled water for one minute and placed in an incubation medium containing the following chemicals: 0.05% acetylthiocholine iodide, 0.82% sodium acetate, 0.6% acetic acid, 2.94% sodium citrate, 0.75% cupric sulfate, 0.137% iso-OMPA, 0.165% potassium ferricyanide, and distilled water. The sections remained in the incubation medium for 45 minutes at 37°C. They were then rinsed with distilled water, dehydrated, cleared, and mounted with Permount. The specimens were observed under a Leitz photomicroscope and recorded on Kodak Panatomic-X and High Speed Ektachrome (3200K) film.

Embryos used for the electron microscopic determination of acetylcholinesterase were fixed and processed in the same general manner as described in Experiment 2 of this section. However, following fixation with Karnovsky's fixative, this tissue was rinsed in an isotonic buffer and placed in the following incubation medium for 4 hours at 4°C: 100 mg. acetylthiocholine iodide, 8 ml. distilled water, 7.0 ml. of 0.1M CuSO₄, 50 mg. of glycine, 1 ml. of 1M sodium acetate, and 29 ml. of isotonic Na₂SO₄. The tissues were then rinsed in two changes of a isotonic buffer for a total of one hour. Next, the
Specimens were transferred to a buffered sulfide solution containing 2 grams of sodium sulfide and 0.9 ml. of 0.2M calcium acetate in 90 ml. of .2N acetic acid. Tissues were placed in an isotonic buffer overnight, and post-fixed the next day with Dalton's fixative (4% K₂Cr₂O₇ @ pH = 7.2 adjusted with KOH; 3.4% NaCl; 2% osmium tetroxide) for 1 1/2 to 2 hours. Following post-fixation the tissue was dehydrated, embedded and sectioned as usual. Sections were mounted on copper grids and stained with 3% aqueous uranyl acetate and lead citrate (Reynolds, 1963). Specimens were observed and photographed using a Philips 300 electron microscope.

The gross development of the vascular system of the tibia-fibula was documented by injecting sonicated India ink into the chorio-allantoic veins with a 32 gauge needle. Six day old specimens were then fixed in 70% alcohol for 24 hours, dehydrated, and blocked in paraffin. Sections were cut at 12μ in the longitudinal axis of the limb bud. After mounting, dehydrating, clearing and coverslipping, the six day old specimens were observed and photographed using a Leitz photomicroscope. Other specimens from 7, 8, 10, and 12 days of incubation were injected with India ink (7, 8, and 10 day specimens) or white latex (12 days). They were then dehydrated, and cleared in a solution of oil of wintergreen and benzyl benzoate.

Vascular flow parameters were monitored in vivo on a total of 15 control tibia-fibulas and 14 experimental tibia-fibulas from embryos ranging in age from 6 to 12 days of incubation. The embryos hindlimb was exteriorized while its chorio-allantoic circulation remained intact. Homeostasis was maintained by constant irrigation with Ringer's solution to the leg and to gauze sponges covering the egg (Fig. 108).
The temperature of the Ringer's solution was maintained at 37°C by regulating heaters. Observations of the vascular system in the legs were accomplished by transillumination of the tissue with monochromatic light (580nm) conducted to the limb by a hollow, fused quartz-rod (Knisely, 1954) and examination with a Leitz stereobinocular microscope. Magnification was maintained at 64X. Measurements of the internal diameters of vessels were accomplished by use of an eyepiece micrometer installed in one of the oculars of the stereobinocular microscope. Blood flow rates were recorded using a scale of 0 to 4 with 0 representing no flow and 4 representing very rapid flow, where the individual outlines of cells are not observed. Normal flow rates and vessel diameters were monitored for at least 20 minutes (average) before vasoactivity experiments were carried out. To study vasoactivity in the embryonic vessels 1/1000 epinephrine (50µg) was applied topically to vessels of the tibia-fibula; this procedure was followed 5 minutes later by application of 250µg of phentolamine (α-blocking agent).

Results:

1. Analysis of Neurovascular System

Although nerve fibers have been reported in association with vascular elements of the developing tibia-fibula in Experiment 2, their physiological status and functional capabilities were not delineated at that time. Assessment of the sympathetics vascular innervation of legs up to 12 days of incubation indicated an absence of catecholamines in both control and experimental embryos (Figs. 90, 91, 92, 93, 94, 95). Therefore, according to the Falck technique, sympathetic (GVE) nerve fibers to vessels of the tibia-fibula are not capable of influencing vasoactivity up through 12 days of incubation. Further studies on
control legs at 16, 19, 20, and 21 days of incubation showed a continued absence of catecholamine from the peripheral sympathetic fibers innervating vascular elements. Not until 1 day post-hatch were faint signs of catecholamine fluorescence observed in the sympathetic fibers which innervate leg blood vessels. At 2 days post-hatch, strong catecholamine fluorescence could be identified in nerve fibers of the adventitia and outer media of arteries and veins (Figs. 96, 97).

Analysis of acetylcholinesterase (AChE) preparations at the light microscope level showed no AChE positive nerve fibers in association with vascular or skeletal elements. These findings were the result of studies on two control and two experimental embryos at 12 days of incubation, therefore, further investigations must be accomplished in order to verify or refute the results of the pilot study. Light microscopic preparations did show positive AChE staining in the periosteal cells of the tibia and fibula (Figs. 98, 99).

Acetylcholinesterase preparations at the electron microscopic level, showed AChE localization at the periphery of some nerve fibers and in juxtaposition to the nuclear envelope of periosteal cells (Figs. 100, 101, 102). Specialized, ultrastructural relationships were not observed between nerve fibers and skeletal elements in the AChE preparation. However, more studies must be done in order to verify or refute the findings of this experiment.

2. In Vivo Analysis of Vascular Flow Parameters

Assessment of blood vessel distribution throughout tibia-fibula development indicated that the position and arrangement of major vascular channels in the leg are established at very early stages (Figs. 103, 104, 105, 106). The gross vascular parameter which changes
most remarkably during development is, of course, the total quantity of vessels in the limb.

From 6 through 12 days of incubation, an analysis of flow rates and vessel diameters in the legs of control and experimental embryos showed no observable differences in base line values (Table XI). Flow rates in arteries were generally higher than those in the veins. However, both vessels had relatively high flow velocities, where the blood cells are blurred, at very early stages of development. The diameters of arteries and veins did not change during the observation period when base line data was being gathered (Table XI).

The topical application of epinephrine and phentolamine had no effect on flow rates or vessel diameters in either 6 day control or experimental animals. However, the vasculature of 7 day legs was affected by the application of epinephrine and phentolamine. The arterial system of 7 day animals showed a greater change in flow rates and diameter after drug administration than the venous system. No differences in vascular parameters between control and experimental groups were discernable on day 7. The vessels of 8 day animals were also responsive to drug administration. However, the magnitude of the response to epinephrine and phentolamine was greater on day eight than seven. Arterial flow and vessel diameters were affected more by drug administration than venous parameters on the eighth day of incubation. No differences in vascular physiology or anatomy were noted between control and experimental embryos on day 8. Responsiveness to epinephrine and phentolamine was even more marked at 9 days than it was at 8 days. Flow rate and vessel diameter changes in 9 day old specimens were approaching maximum levels. In other words, after epinephrine
<table>
<thead>
<tr>
<th>Incubation Age in Days</th>
<th>n</th>
<th>Average Observation Time Per Vessel</th>
<th>Average Flow Rate(\text{in Microns/Sec}) Arteries</th>
<th>Average Flow Rate(\text{in Microns/Sec}) Arteries</th>
<th>Average Vessel Diameter in Microns</th>
<th>(\Delta) in Vessel Diameter in Microns</th>
<th>Average Vessel Diameter in Microns</th>
<th>(\Delta) in Vessel Diameter in Microns</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Control (11 vessels)</td>
<td>4</td>
<td>32 minutes</td>
<td>3.00±0.56 No A No A</td>
<td>1.50±0 No A No A</td>
<td>71.13±35.0 No A No A</td>
<td>7.1±23.4 No A No A</td>
<td>93.5±23.4 No A No A</td>
<td>93.5±23.4 No A No A</td>
</tr>
<tr>
<td>6 Experimental (13 vessels)</td>
<td>4</td>
<td>28 minutes</td>
<td>2.73±0.41 No A No A</td>
<td>2.35±1.06 No A No A</td>
<td>65.2±12.5 No A No A</td>
<td>6.5±23.4 No A No A</td>
<td>93.5±23.4 No A No A</td>
<td>93.5±23.4 No A No A</td>
</tr>
<tr>
<td>7 Control (7 vessels)</td>
<td>3</td>
<td>29 minutes</td>
<td>2.70±0.67 1.40±1.14 1.30±1.30</td>
<td>4.00±0 2.50±1.41 2.75±1.06</td>
<td>68.0±14.6 39.6±7 21.8±20.4</td>
<td>93.5±23.4 9.5±14.8 27.4±18.4</td>
<td>93.5±23.4 9.5±14.8 27.4±18.4</td>
<td>93.5±23.4 9.5±14.8 27.4±18.4</td>
</tr>
<tr>
<td>7 Experimental (5 vessels)</td>
<td>2</td>
<td>24 minutes</td>
<td>3.00±0.71 1.50±1.41 2.50</td>
<td>4.00±0 3.00±0 2.75±1.35</td>
<td>49.5±7.8 55.0±7.1 37.1±7.1</td>
<td>77.0±15.6 64.5±13.5 41.5±12.1</td>
<td>77.0±15.6 64.5±13.5 41.5±12.1</td>
<td>77.0±15.6 64.5±13.5 41.5±12.1</td>
</tr>
<tr>
<td>8 Control (7 vessels)</td>
<td>2</td>
<td>27 minutes</td>
<td>3.17±0.29 0.33±0.50 2.00±1.73</td>
<td>3.00±0 0.50±0.71 3.00±1.00</td>
<td>31.3±12.7 66.7±28.9 12.5±17.7</td>
<td>66.0±10 83.5±23.4 16.5±23.4</td>
<td>66.0±10 83.5±23.4 16.5±23.4</td>
<td>66.0±10 83.5±23.4 16.5±23.4</td>
</tr>
<tr>
<td>8 Experimental (6 vessels)</td>
<td>2</td>
<td>23 minutes</td>
<td>2.83±0.76 1.33±1.15 0</td>
<td>3.50±0.50 1.75±1.28 3.00±1.00</td>
<td>44.0±11.0 67.3±27.5 Vein Collapsed</td>
<td>69.7±38.6 87.7±210.8 &lt; 3.2±2.5</td>
<td>69.7±38.6 87.7±210.8 &lt; 3.2±2.5</td>
<td>69.7±38.6 87.7±210.8 &lt; 3.2±2.5</td>
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<tr>
<td>9 Control (1 vessel)</td>
<td>1</td>
<td>27 minutes</td>
<td>2.50 0 0</td>
<td>3.00 0 2.50</td>
<td>44.0 100X Vein Collapsed</td>
<td>55.0 &lt;80X GZ</td>
<td>55.0 &lt;80X GZ</td>
<td>55.0 &lt;80X GZ</td>
</tr>
<tr>
<td>9 Experimental (1 vessel)</td>
<td>1</td>
<td>30 minutes</td>
<td>0 - -</td>
<td>3.00 1.00 2.50</td>
<td>- - -</td>
<td>- - -</td>
<td>- - -</td>
<td>- - -</td>
</tr>
<tr>
<td>10 Control (4 vessels)</td>
<td>2</td>
<td>32 minutes</td>
<td>3.75±0.25 0.75±0.06 2.75±1.06</td>
<td>4.00 0 3.00</td>
<td>116.5±54.4 86.5±18.0 19.0±18.5</td>
<td>110.0 &lt;80X GZ</td>
<td>110.0 &lt;80X GZ</td>
<td>110.0 &lt;80X GZ</td>
</tr>
<tr>
<td>10 Experimental (4 vessels)</td>
<td>2</td>
<td>28 minutes</td>
<td>3.38±0.23 1.88±0.23 3.13±0.25</td>
<td>- - -</td>
<td>66.0±23.4 64.0±16.8 &lt; 1.23±1.5</td>
<td>- - -</td>
<td>- - -</td>
<td>- - -</td>
</tr>
<tr>
<td>12 Control (9 vessels)</td>
<td>3</td>
<td>94 minutes</td>
<td>3.25±0.61 0.50±0.26 2.60±1.14</td>
<td>2.83±0.76 0 2.00±1.40</td>
<td>111.2±45.3 &lt;72.2±25.5 &lt;47.3±14.5</td>
<td>84.3±12.8 &lt;44.3±12.8 &lt;61.3±12.6</td>
<td>84.3±12.8 &lt;44.3±12.8 &lt;61.3±12.6</td>
<td>84.3±12.8 &lt;44.3±12.8 &lt;61.3±12.6</td>
</tr>
<tr>
<td>12 Experimental (5 vessels)</td>
<td>3</td>
<td>30 minutes</td>
<td>3.00±0 0.67±0.76 1.83±0.76</td>
<td>2.75±1.06 0 1.00±0</td>
<td>110.0±0 66.7±15.9 43.3±20.8</td>
<td>60.5±7.8 100X</td>
<td>49.5±14.9</td>
<td>49.5±14.9</td>
</tr>
</tbody>
</table>

\(n\) = Number of embryos observed (number of vessels observed in particular age group).

\(A\) = Change.

Values are means ± S.D. unless otherwise indicated.

E.E. = Before application of 50 \(\mu\)g of epinephrine.

A.E. = After application of 50 \(\mu\)g of epinephrine.

F.R. = Five minutes after epinephrine, 250 \(\mu\)g of phenylephrine was applied.

Flow rate values: 0 = No flow.
1 = Very slow flow, the blood oscillates with individual cells seen.
2 = Slow flow, steady forward progression of blood cells with individual cells seen.
3 = Moderate flow, blood cells are blurred.
4 = Rapid flow, the individual outlines of cells are not observed.
application the flow rates approached zero and the lumens were almost closed. At 10 days of incubation the results were very similar to 9 day observations. Once again, action of the drugs on arteries seemed to produce more marked changes than on veins. No differences between experimental and control vascular parameters were obvious in the leg at 9 or 10 days of incubation. On the last day of the investigation, arteries and veins reacted to topical application of epinephrine and phentolamine, but the responsiveness was no greater than that observed on the 9th and 10th days. Arteries continued to show slightly more vascular reactivity in terms of changes in flow rates and vessel diameters than veins. Analysis of vascular anatomy and physiology in control and experimental legs at 12 days of incubation showed no noteworthy differences.
DISCUSSION

The effects of neural crest removal on the development of the chick tibia-fibula have been analyzed by a series of five experiments. Each experiment has contributed valuable information as to the role of the nervous system in limb ontogeny. Numerous similarities exist between the results of these experiments and the findings of previous investigations, even though experimental parameters were dissimilar. However, certain findings were elucidated which are worthy of further comment.

The Effect of Neural Crest Removal on Behavior

The behavioral data obtained in Experiment 1 adds to previously reported findings on development of limb motility in the absence of afferent innervation. No differences were found in the qualitative or quantitative aspects of total leg motility between control and experimental embryos from 6 through 12 days of incubation (Table I, Fig. 30). These findings indicate that from their onset, motility patterns in the hind limbs are not influenced by afferent input.

Embryos which had undergone neural crest extirpation were analyzed by gross dissection and histological means. It was determined that dorsal root ganglia, sympathetic chain ganglia, and the dorsal half of the spinal cord were absent or severely reduced in all experimental embryos, while the ventral half or one-third of the spinal cord and the ventral roots remained intact (Fig. 11). Through comparisons with control embryos, it is very evident that 1) proper cauterization methods...
are very effective in the elimination of neural crest elements, and 2) the presence of very few ventral horn cells maintains normal motility patterns in the leg from 6 through 12 days of incubation. No cell counts were carried out on the number of ventral horn cells which remained in experimental embryos, but further studies are contemplated which would focus on the probable mechanisms involved in the differentiation and maintenance of the cells in the basal plate. Transneuronal trophic affects may well be playing a role in the development of the basal plate; this possibility has been mentioned by Hamburger et al. (1966). Hamburger and Oppenheim (1967) have classified the predominant motility patterns of chick embryos between 9 and 17 days of incubation as being spontaneous (non-reflexogenic); these movements are jerky, uncontrolled, and intermittent movements of the limbs. This type of motility is said to result from autonomous discharges of cells in the ventral portion of the spinal cord or from discharges in suprasegmental structures that diffuse throughout the cord (Provine, 1971). The role that sensory input plays in the development of motor activity in the chick embryo was studied at lumbosacral levels by Hamburger et al. (1966). Results of this investigation showed that radical extripation of the dorsal half of the spinal cord did not interfere with the normal motility patterns (quantitatively or qualitatively). This study indicated that afferent stimuli does not control the development of motor activity in the chick embryo. A similar study on deafferentation of the V cranial nerve (Hamburger and Narayanan, 1969) showed once again that afferent input is not necessary for development of motor activity in the chick. Oppenheim (1972) performed stimulation experiments on chick embryos from 5 through 16 days of incubation. Results
of this study indicated that proprioceptive or tactile stimuli do not modify the quantitative or qualitative aspects of embryonic motility in the age groups studied.

In other embryonic species, the effects of deafferentation have shown similar results to those mentioned above. In *Ambystoma maculatum* removal of the neural crest bilaterally in neurulae produces no changes in walking or swimming movements (Yntema, 1943; Detwiler, 1947). Other studies in frogs (Taylor, 1944) showed that removal of dorsal root ganglia at early tapole stages (before innervation of limb buds) resulted in no interference with normal leg motility patterns. The last three studies did not, however, monitor behavior at stages when motility is just beginning in the limbs.

Through the contribution of information from Oppenheim's (1972) study and the present experiment, it seems very probable that Hamburger's statements on the lack of influence of afferent stimuli on motility in chick embryos can be expanded to include the initial stages of motor activity in the limbs.

**Neuro-skeletal Relationships in the Tibia-Fibula of Control and Experimental Embryos with Some Comments on Muscle Integrity.**

Numerous nerve fibers were found in close association with periosteal cells of the tibia-fibula from 8 through 12 days of incubation; however, no synaptic relationships were observed. Branching of the main nerve trunk (median tibial n.) occurred throughout the length of the tibia-fibula, but was most evident in the area of the primary periosteal bud (nutrient foramen). Nerve fibers were also in close approximation to the blood vessels of the periosteal bud (nutrient foramen); however, no specialized junctional complexes were noted.
between neural and vascular elements. No nerve fibers were observed to form junctional complexes with cartilage or bone cells; and no terminal processes were observed within the cartilage or bone matrix.

Analysis of nerve fiber quantity in the limbs of experimental embryos (where neural crest had been removed at leg levels) indicated that the cross-sectional diameter of major nerve trunks was much smaller than in control specimens. It was noted in most experimental specimens that very few, if any, nerve fibers were found in the periosteum.

Previous investigations have shown conflicting evidence on the innervation of neo-natal and adult skeletal elements. DeCastro (1929) reported that nerve fibers come into contact with bone cells and also are found entrapped in bone matrix. Hurrell (1937) noted that nerve fibers come into close approximation to bone cells and terminate in bone matrix. However, no other investigators (Rossi, 1932; Kuntz and Richins, 1945; Miller and Kasahara, 1963; Milgram, 1965; Cooper et al., 1966; Calvo, 1968) have noted any specialized neuro-skeletal relationships. The present study, on embryonic tissues, agrees with the latter position.

An attempt was made in the present investigation to determine whether the cross-sectional area of leg muscles was altered in experimental embryos. When the cross-sectional areas of two muscle groups adjacent to the tibia were measured; significant differences between control and experimental values were noted on the 10th, 11th, and 12th days of investigation (Table IX). The decrease of muscle cross-sectional area in experimental embryos does not necessarily imply that afferent or visceral efferent innervation is necessary for normal differentiation of muscle to take place. It might indicate that the number or metabolic activity of ventral motor horn cells may have been affected due to the
operative procedure, the loss of afferent input from spinal ganglia (transneuronal degeneration), or the interruption of normal vascular patterns.

Previous studies have reported that innervation is not necessary for initial stages of myogenesis to occur (Muchmore, 1968; Studitsky, 1962). However, studies in regenerating frog limbs have indicated that the quantity and not quality of nerve fibers is the most important parameter concerned with limb growth and development (Singer and Craven, 1948). Whether any of these principles are applicable to embryonic muscle differentiation in higher forms, such as the avian species, is a question which warrants further investigation.

The Effect of Neural Crest Removal on Gross and Ionic Aspects of Skeletal Development

Findings of the present experiment indicate that length, width, wet weight, and dry weight measurements of the tibia-fibula are not affected by the absence or reduction of afferent and visceral efferent innervation during early stages of development (Tables II, III, IV, V). Also, the gross morphology of the experimental tibia-fibulas showed no discernible differences from the morphology of control specimens (Fig. 31). Likewise, analysis of ionic concentrations (calcium, inorganic phosphate, and magnesium ions) in the skeletal elements from 6 through 12 days of incubation showed no significant differences between control and experimental values (Tables VI, VII, VIII).

The previous results indicate that an absence or a marked decrease in innervation (afferent and visceral efferent) to developing long bone elements does not alter the gross morphology or the primary ionic constituents during the stages preceding and during periosteal bud formation.
It should be remembered that the experimental embryos in this investigation maintained a motility pattern which was quantitatively and qualitatively comparable to the control animals. Since the denervated limbs were not immobilized, joint formation was not impaired. Neither the knee nor the ankle joint had fibrotic adhesions between their articulating members; this fact was evident up through 12 days of incubation. These findings seem to indicate that joint formation can take place normally in the absence of afferent and visceral efferent innervation.

Since we know of no other experiments where embryonic skeletal development has been extensively analyzed after the in situ removal of specific neural elements, comparisons with other studies are not possible. However, certain analogies can be drawn between previous studies and the present investigation. A series of experiments by Hamburger (1929, 1939, 1940) led to our investigation, so it is only proper that some comparisons be made with these studies. In experiments on complete denervation of embryonic frog legs, Hamburger found a 12% reduction in the overall growth of the skeletal elements of the leg. At the same time he noted some abnormalities in joint development; fibrotic unions between articulating members and a decrease in size of proximal articulating structures were reported. As Hamburger alluded to in this work and in other subsequent studies (1939, 1940), the reason for a decrease in size of skeletal elements and abnormal joint development was probably the lack of motor activity. In a series of coelomic grafting experiments (Hamburger, 1939, 1940), chick hind limb buds were transplanted to the flank of host embryos and allowed to develop. It was found that skeletal elements of the grafts were reduced
20% in length and width and that some joint irregularities were present. Also, abnormalities such as hypodactyly and hypophalangy were noted in the experimental limbs. Since grafting was employed by Hamburger, he necessarily disrupted the normal environment of the developing limb bud; blood supply and innervation of the grafted bud were altered from its normal state. Although Hamburger was attempting to discern whether the nervous system had a direct neurotrophic effect on skeletal development, his experimental methods did not allow him to distinguish between neurotrophic, muscular, and vascular effects.

Many deafferentation and sympathectomy studies have been carried out at lumbar levels using neo-natal and adult animals; several of these have been reported in the introduction section of this thesis. However, none of these investigations have been able to assess the direct effects of deafferentation on skeletal development, because limb movements were inevitably impaired. The effects of sympathectomy on skeletal growth have been studied in many animals and humans, but the majority of these studies either did not confirm the absence of sympathetic elements properly, or did not use very sensitive analytical methods for their assessment of skeletal parameters. Even though none of the lumbar deafferentation investigations have offered conclusive results, it is important to note that experimental animals in these studies showed no marked post-operative changes in gross dimensions of skeletal elements (Corbin and Hinsey, 1939; Gillespie, 1954; Ring, 1961). The effects of lumbar sympathectomy on long bone growth range from reports of limb lengthening (Goetz et al., 1955; Barr et al., 1950; Ring, 1961) to no effects at all (Corbin and Hinsey, 1939; Bisgard, 1933; Cannon et al., 1929; Bacq, 1930).
Although deafferentation and removal of the visceral efferent modality did not influence the gross development or ionic concentrations of the tibia-fibula in our investigation, two more experiments were deemed necessary before any concrete statements could be made as to the effect of neural crest removal on early skeletal development. The first experiment is concerned with analysis of matrix elements in cartilage and bone, and the second is concerned with assessment of vascular parameters.

**Effect of Neural Crest Removal on Matrix Components of Cartilage and Bone**

The present study has shown that calcification proceeds at comparable levels in both control and experimental tibia-fibulas. The first observable histochemical signs of calcification are observed on the 8th day of incubation. From the ninth through the twelfth day of incubation the distribution of calcification in control and experimental specimens showed no observable differences (Table X). The differences in color intensity of calcification between experimental and control specimens at 9 and 10 days of incubation are probably non-significant. Such color differences were not observed between all control and experimental tibia-fibulas on those days. Also, the color difference seemed to diminish when viewed with a higher power objective; this seems to indicate that the thickness of the control and experimental sections may have been slightly different, not that real differences in the quantity of calcified tissue were present. When the conclusions of this histochemical experiment are coupled with the ionic determinations of the last experiment, it is evident that removal of afferent and visceral efferent innervation has no effect
on calcification of the tibia-fibula in early developmental stages.

Analysis of collagen development in both control and experimental animals showed no observable differences in the quantity or quality of cartilage in any age group (Table X). Collagen distribution from nine through twelve days of incubation consisted of a dark blue band in calcified bone, while periosteal areas were stained a light blue. No staining of the collagen in the cartilage matrix was noted in any age group. This latter finding seems to indicate the Polychrome method is specific for osteoid collagen and not cartilaginous collagen. Also, no 'mature' collagen was identified by the Polychrome method. Therefore, it is probable that the collagen present up until 12 days of incubation is not highly crosslinked. If it were highly crosslinked, the Polychrome method would have stained those elements red. Since the results of this test were very uniform, it can be concluded that lack of afferent and visceral efferent innervation does not interfere with collagen production in early stages of skeletal development.

Assessment of acid mucopolysaccharide distribution in control and experimental animals showed marked differences from seven through twelve days of incubation (Table X). The total distribution of acid mucopolysaccharides were greatly altered (decreased) in the cartilage and bone of experimental specimens. Also, it was observed that degeneration of cartilage cells was more marked in experimental tibia-fibulas. It is important to note that the change in cartilage matrix occurred early on the seventh day of incubation and remained through the twelfth day. This finding was a consistent finding among all the experimental specimens.
The conclusion one must draw from these findings is that removal of afferent and/or visceral efferent modalities does effect early development of the tibia-fibula. It is possible that an actual neurotrophic mechanism is present which is mediated through the afferent component of the nervous system; this in turn may be involved with the initial stages of skeletal development. Alternatively, it is also probable that the visceral efferent portion of the nervous system is controlling skeletal metabolism indirectly by its effect on the vascular system. By modulating blood flow, it is very probable that changes in nutrient concentrations could be affected; this might in turn lead to metabolic changes in the developing skeletal elements. Of course, it is entirely possible that both the above mechanisms may be operative in early skeletal development.

Although we know of no other studies which we can directly compare our results with, Hall (1972) has indicated that any variable which lowers the level of matrix acid mucopolysaccharide in cartilage cells might well result in loss of hypertrophy and leave the cells susceptible to modulation to other skeletal cell types. The results of his studies over the past years, on membrane bones of the chick embryo, has lead him to the conclusion that in his experimental system, mechanical stress is required to initiate and replace cartilage on joint surfaces of membrane bones (Hall, 1972). It should be noted, however, that Hall observes changes which he terms metaplasia or differentiation and redifferentiation of cartilage tissue, after the immobilization of membranous bones. His results are not derived utilizing the same methods or tissues as our findings, and therefore no comparisons in fact can be drawn between the two studies. However, Hall's general
principle of acid mucopolysaccharide loss may apply to our study. Other studies have also indicated that loss of proteinpolysaccharides are necessary before the general scheme of ossification can proceed (Baylink et al., 1972; Dearden; 1974).

It is very evident that the change in acid mucopolysaccharide distribution is the most important finding in this study, however, it must be reiterated that this study was terminated at 12 days of incubation. No data is available, as of now, on the effects of neural crest removal on older stages, however, such studies are in process.

**Effect of Neural Crest Removal on Vascular Parameters**

The findings of this study indicate that neurotransmitter is not present in the sympathetic nerve fibers innervating blood vessels of the leg until one day post-hatch. This indicates that functional sympathetic innervation to leg vessels doesn't occur during the time period of our present study. This would lead one to believe that the loss of GVE modalities to the leg prior to hatching would not produce deficits in vascular function. However, the Falck technique, which was used to demonstrate catecholamines, may not be sensitive enough to pick up low quantities of vasoconstrictor which are present. Many investigators have shown that catecholamines are present in the chick embryo from two or three days of incubation up through hatching (Lawrence and Rutden, 1973; Girard, 1973; Enemor et al., 1965; Ignarro and Shideman, 1967). Therefore, further studies are warranted to determine the functional status of the neurovascular system at early stages.

Findings from the acetylcholinesterase preparations are too pre-mature to discuss here, but it is interesting that AChE activity can be
demonstrated in periosteal cells at early stages of skeletal development.

The most interesting findings of this study were the in vivo results of vascular recordings of the leg vessels in embryos from six through twelve days of incubation. No differences in blood vessel distribution, flow rates, or diameters were found to exist between experimental and control embryos. However, the embryos were all maintained at normal physiological levels during the in vivo recordings. Therefore, it is possible that differences between experimental and control embryos may have been masked.

Limb veins and arteries began to respond to topical application of epinephrine and phentolamine on the seventh day of incubation. The response to drugs, which consisted primarily of vasoconstriction and vasodilation, increased until 9 days of incubation. After 9 days, little change in the response to epinephrine and phentolamine was noted. (Table XI).

The vasoactivity of vessels in the early developing limb bud has many implications. First, it implies that modulation of nutrients, ions, etc. could occur if the proper vasopressor or vasodilator was present. Since vasopressors are present in the chick embryo during these stages, as was mentioned in the previous paragraph, blood pressure changes should also be present. This last statement has been confirmed in the chorio-allantoic circulation by several authors (Hoffman et al., 1971; Lodewyek et al., 1967).

It has also been established that pO₂, pCO₂, and pH values fluctuate greatly during development of the chick embryo (Dawes, 1974; Dawes and Simkiss, 1969). Fluctuation of pO₂ and pCO₂ values in other embryonic systems has been shown to have a marked effect on
fetal blood flow, which is mediated by an $\alpha$-adrenergic mechanism (McCuskey et al., 1969). Therefore, because the baroreceptor and chemoreceptor elements are the first neural reflexes to form in the chick embryo (Kuntz, 1910; Lodeqyk, 1967) it is very possible that differentiation of skeletal and other elements depends on the functional integrity of this system. Most of our efforts in the future will be aimed at studying this aspect of embryonic development.
PLATE 1

EXPLANATION OF FIGURE

1 A line drawing of surgical procedure used during the investigation.
Procedure for Neural Crest Removal

Embryo (Stage 17)

Concept, Cautery

Somites

Posterior Limb Bud

Neural Tube

Neural Crest
PLATE 2

EXPLANATION OF FIGURES

2 An in vivo photograph of a Stage 17 (Hamburger & Hamilton, 1951) chick embryo before surgical operation. White arrows delineate neural crest elements at the leg level. Stained with Neutral Red. Magnification X10

3 Same embryo as Figure 2 after surgical removal of neural crest at the leg level (white arrows). Magnification X10
EXPLANATION OF FIGURE

4 A photograph representation of gross specimens in control and experimental groups from 6 days to 12 days of incubation.
PLATE 4

EXPLANATION OF FIGURES

5  A ventral view of a dissection showing the ischial plexus in a 12 day control embryo. Cr = Crural plexus; Is = Ischial plexus.
Magnification X28

6  A ventral view of a dissection showing the ischial plexus in a 12 day experimental embryo. Is = Ischial plexus.
Magnification X28
PLATE 5

EXPLANATION OF FIGURES


PLATE 6

EXPLANATION OF FIGURES

9  A photomicrograph of a cross-section through the lumbosacral spinal cord of a 12 day experimental chick embryo. White arrow indicates ventral motor horn cells. Thionin preparation. Magnification X48

10 A photomicrograph of a cross-section through the lumbosacral spinal cord of a 12 day experimental chick embryo. White arrow indicates ventral motor horn cells. Thionin preparation. Magnification X48
PLATE 7
EXPLANATION OF FIGURE

11 A series of schematic reconstructions of the lumbar spinal cord in normal and experimental chick embryos. Note the absence of GSA, GVA, and GVE elements in the experimental embryos.
PLATE 8

EXPLANATION OF FIGURES

12 A photomicrograph of a cross-section through the tibia-fibula of an 8 day control chick embryo. White t = tibia; White f = fibula; Black arrows indicate nerve bundles. Bodian preparation. Magnification X108

13 Same preparation as Figure 12. Magnification X268
PLATE 9

EXPLANATION OF FIGURES

14 A photomicrograph of a cross-section through the tibia-fibula of an 8 day experimental chick embryo. White t = tibia; white f = fibula. White arrows indicate nerve bundles. Bodian preparation. Magnification X108

15 Same preparation as Figure 14. Magnification X268
PLATE 10
EXPLANATION OF FIGURES

16 A photomicrograph of a cross-section through the tibia-fibula of a 12 day control embryo. $t =$ tibia; $f =$ fibula. Black arrows indicate nerve bundles. Bodian preparation. Magnification X108

17 Same preparation as Figure 16. Magnification X268
PLATE 11

EXPLANATION OF FIGURES

18 A photomicrograph of a cross-section through the tibia-fibula of a 12 day experimental chick embryo. $t = \text{tibia}; f = \text{fibula}$. Black arrows indicate nerve bundles. Bodian preparation. Magnification X108

19 Same preparation as Figure 18. Magnification X268
PLATE 12

EXPLANATION OF FIGURES


21 Same preparation as Figure 20. White arrows indicate nerve bundle. White arrowhead = fine branches off nerve bundle entering periosteum.
PLATE 13

EXPLANATION OF FIGURES

22 A photomicrograph of a longitudinal section through the tibia-fibula of a 12 day control chick embryo. Black arrow indicates main nerve trunk. White arrow indicates bone tissue of diaphysis. Bodian preparation. Magnification X72

23 Same preparation as Figure 22. White arrowheads indicate periosteum. Magnification X135
PLATE 14

EXPLANATION OF FIGURES

24 A photomicrograph of a longitudinal section through the tibia-fibula of a 12 day control chick embryo. White n = nerve bundle traveling close to periosteal cells. White arrows indicate nerve fibers branching among periosteal cells. Bodian preparation. Magnification X1665

25 A photomicrograph of a longitudinal section through the tibia-fibula of a 12 day control chick embryo. White n = nerve bundle traveling close to periosteal cells. White arrows indicate nerve fibers branching among periosteal cells. Bodian preparation. Magnification X1665
PLATE 15

EXPLANATION OF FIGURES

26 An electron micrograph of nerve fiber (n) relationships to an endothelial cell (e) in the tibia-fibula of a 12 day control chick embryo. Stained with uranyl acetate and lead citrate. Magnification X9,576

27 An electron micrograph of nerve fiber (n) relationships to periosteal cells (p) in the tibia-fibula of a control chick embryo. Stained with uranyl acetate and lead citrate. Magnification X9,576
PLATE 16

EXPLANATION OF FIGURES

28 An electron micrograph of nerve fiber (n) relationships to a periosteal cell (p) in the tibia-fibula of a control chick embryo. Stained with uranyl acetate and lead citrate.
Magnification X20,520

29 An electron micrograph of nerve fiber (n) relationships to a periosteal cell (p) in the tibia-fibula of a control chick embryo. Stained with uranyl acetate and lead citrate.
Magnification X20,520
PLATE 17

EXPLANATION OF FIGURE

A graph showing the average number of movements per minute in the leg of control and experimental chick embryos from 6 through 12 days of incubation.
KEY:
- control
- exper.

MEAN FREQUENCY of MOVEMENTS per MINUTE

DAYS of INCUBATION
A photograph of tibia-fibulas from control (cn) and experimental (ce) embryos which have been stained via the Lundvall technique. The blue color of the skeleton indicates that this technique stains cartilage. Notice that no differences are discernable between the control (on the left) and experimental (on the right) limbs in each age group from 6 through 12 days of incubation.
PLATE 19

EXPLANATION OF FIGURE

32 A graph demonstrating the growth in length of the tibia in control and experimental embryos from 7 through 12 days of incubation. Measurements of the tibia are reported in centimeters.
PLATE 20

EXPLANATION OF FIGURE

A graph demonstrating the growth in width of the tibia in control and experimental embryos from 7 through 12 days of incubation. Measurements were taken at the mid-diaphysis and are reported in millimeters.
KEY:
- control
- exper.

WIDTH in mm. of Tibia

DAYS of INCUBATION
PLATE 21

EXPLANATION OF FIGURE

34 A graph demonstrating the wet weight of tibia-fibulas in control and experimental embryos from 7 through 12 days of incubation. Measurements are reported in milligrams.
KEY:

- control
- exper.
PLATE 22

EXPLANATION OF FIGURE

35 A graph demonstrating the dry weight of the tibia-fibulas in control and experimental embryos from 7 through 12 days of incubation. Measurements are reported in milligrams.
PLATE 23

EXPLANATION OF FIGURE

36 A graph demonstrating the concentration of calcium in mM found in the tibia-fibulas of control and experimental embryos from 7 through 12 days of incubation. The star (*) indicates that only one group of pooled specimens was analyzed. The thin black lines represent the standard deviation of the means.
PLATE 24

EXPLANATION OF FIGURE

37 A graph demonstrating the concentration of inorganic phosphate in mM found in the tibia-fibulas of control and experimental embryos from 9 through 12 days of incubation. The thin black lines represent the standard deviation of the means.
PLATE 25
EXPLANATION OF FIGURE

38 A graph demonstrating the concentration of magnesium in mM found in the tibia-fibulas of control and experimental embryos from 7 through 12 days of incubation. The star (*) indicates that only one group of pooled specimens was analyzed. The thin black lines represent the standard deviation of the means.
Magnesium levels for 7 to 12 days of incubation.

**KEY:**
- Control
- Exper.
A graph demonstrating the cross-sectional area in square centimeters of the flexor digitorum longus muscle and the tibialis anterior muscle of the tibia-fibula in control and experimental embryos from 7 through 12 days of incubation. Note that statistically significant differences are present between experimental and control groups on days 10, 11, and 12.
PLATE 27

EXPLANATION OF FIGURES

40 A phase photomicrograph of a cross-section through the tibia-fibula of a 6 day control embryo. T = developing anlage of the tibia; F = developing anlage of the fibula. Black arrow indicates the periphery of the tibial anlage. Von Kossa preparation. Magnification X234

41 Same preparation as Figure 40, except specimen represents a 6 day experimental embryo.
PLATE 28

EXPLANATION OF FIGURES

42 A phase photomicrograph of a cross-section through the tibia-fibula of a 7 day control embryo. T = developing anlage of tibia. Black arrow indicates periphery of the tibial anlage. Von Kossa preparation. Magnification X234

43 Same preparation as Figure 42, except specimen represents a 7 day experimental embryo.
PLATE 29

EXPLANATION OF FIGURES

44 A phase photomicrograph of a cross-section through the tibia-fibula of an 8 day control embryo. T = developing tibia. White arrowhead indicates an area of calcification (brown stain). Von Kossa preparation. Magnification X234

45 Same preparation as Figure 44, except specimen represents an 8 day experimental embryo.
PLATE 30

EXPLANATION OF FIGURES

46 A phase photomicrograph of a cross-section through the tibia-fibula of a 9 day control embryo. T = developing tibia. White arrow indicates an area of calcification (brown stain). Von Kossa preparation. Magnification X94

47 Same preparation as Figure 46, except specimen represents a 9 day experimental embryo.
PLATE 31
EXPLANATION OF FIGURES


49. Same preparation as Figure 48, except specimen represents a 9 day experimental embryo.
PLATE 32
EXPLANATION OF FIGURES

50  A phase photomicrograph of a cross-section through the tibia-fibula of a 10 day control embryo. T = developing tibia. White arrow indicates area of calcification (brown stain). Von Kossa preparation. Magnification X94

51  Same preparation as Figure 50, except specimen represents a 10 day experimental embryo.
PLATE 33

EXPLANATION OF FIGURES

52 A phase photomicrograph of a cross-section through the tibia-fibula of a 10 day control embryo. T = developing tibia. White arrowhead indicates area of calcification (brown stain). Von Kossa preparation. Magnification X234

53 Same preparation as Figure 52, except specimen represents a 10 day experimental embryo.
PLATE 34
EXPLANATION OF FIGURES

54 A phase photomicrograph of a cross-section through the tibia-fibula of an 11 day control embryo. T = developing tibia. White arrow indicates area of calcification (brown stain). Von Kossa preparation. Magnification X94

55 Same preparation as Figure 54, except specimen represents an 11 day experimental embryo.
PLATE 35

EXPLANATION OF FIGURES

56 A phase photomicrograph of a cross-section through the tibia-fibula of an 11 day control embryo. T = developing tibia. White arrowhead indicates area of calcification (brown stain). Von Kossa preparation. Magnification X234

57 Same preparation as Figure 56, except specimen represents an 11 day experimental embryo.
PLATE 36

EXPLANATION OF FIGURES

58 A phase photomicrograph of a cross-section through the tibia-fibula of a 12 day control embryo. T = developing tibia. White arrow indicates area of calcification (brown stain). Von Kossa preparation. Magnification X94

59 Same preparation as Figure 58, except specimen represents a 12 day experimental embryo.
PLATE 37

EXPLANATION OF FIGURES

60 A phase photomicrograph of a cross-section through the tibia-fibula of a 12 day control embryo. T = developing tibia. White arrowhead indicates area of calcification (brown stain). Von Kossa preparation. Magnification X234

61 Same preparation as Figure 60, except specimen represents a 12 day experimental embryo.
PLATE 38
EXPLANATION OF FIGURES

62 A photomicrograph of a cross-section through the tibia-fibula of a 6 day control embryo. t = anlage of tibia. Polychrome preparation for collagen. Magnification X250

63 Same preparation as Figure 62, except specimen represents a 6 day experimental embryo.
PLATE 39

EXPLANATION OF FIGURES

64 A photomicrograph of a cross-section through the tibia-fibula of a 7 day control embryo. t = developing tibia; f = developing fibula. Polychrome preparation for collagen. Magnification X115

65 Same preparation as Figure 64, except specimen represents a 7 day experimental embryo.
PLATE 40

EXPLANATION OF FIGURES

66 A photomicrograph of a cross-section through the tibia-fibula of an 8 day control embryo. t = developing tibia. f = developing fibula; c = cartilage of tibia. Polychrome preparation for collagen. Magnification X94

67 Same preparation as Figure 66, except specimen represents an 8 day experimental embryo.
PLATE 41

EXPLANATION OF FIGURES

68 A photomicrograph of a cross-section through the tibia-fibula of a 9 day control embryo. t = developing tibia; f = developing fibula; c = cartilage of tibia. White b = bone matrix of tibia. Polychrome preparation for collagen. Magnification X94

69 Same preparation as Figure 68, except specimen represents a 9 day experimental embryo.
PLATE 42

EXPLANATION OF FIGURES

70  A photomicrograph of a cross-section through the tibia-fibula of a 10 day control embryo. t = developing tibia; f = developing fibula; c = cartilage of tibia. White b = bone matrix of tibia. Polychrome preparation for collagen. Magnification X94

71  Same preparation as Figure 70, except specimen represents a 10 day experimental embryo.
PLATE 43
EXPLANATION OF FIGURES

72 A photomicrograph of a cross-section through the tibia-fibula of an 11 day control embryo. t = developing tibia; f = developing fibula; c = cartilage of tibia; white b = bone matrix of tibia. Polychrome preparation for collagen. Magnification X94

73 Same preparation as Figure 72, except specimen represents an 11 day experimental embryo.
74 A photomicrograph of a cross-section through the tibia-fibula of a 12 day control embryo.  
t = developing tibia;  
f = developing fibula;  
c = cartilage of tibia;  
white b = bone matrix of tibia.  
Polychrome preparation for collagen.  
Magnification X94

75 Same preparation as Figure 74, except specimen represents a 
12 day experimental embryo.
PLATE 45

EXPLANATION OF FIGURES

76 A phase photomicrograph of a cross-section through the tibia-fibula of a 6 day control embryo. t = developing anlage of tibia; Alcian blue preparation for acid mucopolysaccharides. Magnification X250

77 Same preparation as Figure 76, except specimen represents a 6 day experimental embryo.
PLATE 46

EXPLANATION OF FIGURES

78 A photomicrograph of a cross-section through the tibia-fibula of a 7 day control embryo. t = developing tibia; f = developing fibula. Alcian blue preparation for acid mucopolysaccharides. Note uniformity of blue stain. Magnification X115

79 Same preparation as Figure 78, except specimen represents a 7 day experimental embryo. White arrowheads indicate areas of decreased staining intensity.
PLATE 47
EXPLANATION OF FIGURES

80 A photomicrograph of a cross-section through the tibia-fibula of an 8 day control embryo. \( t = \text{developing tibia}; f = \text{developing fibula.} \) Note uniformity of blue stain. Alcian blue preparation for acid mucopolysaccharides. Magnification X94

81 Same preparation as Figure 80, except specimen represents an 8 day experimental embryo. White arrowheads indicate areas of decreased staining intensity.
PLATE 48

EXPLANATION OF FIGURES

82 A photomicrograph of a cross-section through the tibia–fibula of a 9 day control embryo. t = developing tibia; f = developing fibula; white c = cartilage of tibia; white b = bone matrix of tibia. Note uniformity of blue stain. Alcian blue preparation for mucopolysaccharides. Magnification X94

83 Same preparation as Figure 82, except specimen represents a 9 day experimental embryo. White arrowhead indicates areas of decreased staining intensity.
PLATE 49

EXPLANATION OF FIGURES

84  A photomicrograph of a cross-section through the tibia-fibula of a 10 day control embryo.  t = developing tibia;  f = developing fibula;  while c = cartilage of tibia;  white b = bone matrix of tibia.  Note uniformity of blue stain.  Alcian blue preparation for acid mucopolysaccharides.  Magnification X94

85  Same preparation as Figure 84, except specimen represents a 10 day experimental embryo.  White arrowhead indicates areas of decreased staining intensity.
PLATE 50
EXPLANATION OF FIGURES

86 A photomicrograph of a cross-section through the tibia-fibula of an 11 day control embryo. t = developing tibia; f = developing fibula; white c = cartilage of tibia; white b = bone matrix of tibia. Note uniformity of blue stain. Alcian blue preparation for acid mucopolysaccharides. Magnification X94

87 Same preparation as Figure 86, except specimen represents an 11 day experimental embryo. White arrowhead indicates area of decreased staining intensity.
PLATE 51

EXPLANATION OF FIGURES

88 A photomicrograph of a cross-section through the tibia-fibula of a 12 day control embryo. t = developing tibia; f = developing fibula; white c = cartilage of tibia; white b = bone matrix of tibia. Note uniformity of blue stain. Alcian blue preparation for acid mucopolysaccharides. Magnification X94

89 Same preparation as Figure 88, except specimen represents a 12 day experimental embryo. White arrowheads indicate areas of decreased staining intensity.
PLATE 52

EXPLANATION OF FIGURES

90  A fluorescent photomicrograph taken with U.V. illumination representing a cross-section through the tibia-fibula of a 6 day control embryo. Black arrow indicates control vascular channel between the anlage of the tibia and fibula. A Falck preparation. Magnification X108

91  Same preparation as Figure 90, except specimen represents a 6 day experimental embryo.
PLATE 53

EXPLANATION OF FIGURES

92 A fluorescent photomicrograph taken with U.V. illumination representing a cross-section through the tibia-fibula of a 12 day control embryo. \( t \) = developing tibia; \( f \) = developing fibula; white arrow indicates arteriole. Note that there is no fluorescence in the artery wall. A Falck preparation. Magnification X132

93 Same preparation as Figure 92, except magnification X332
PLATE 54

EXPLANATION OF FIGURES

94 A fluorescent photomicrograph taken with U.V. illumination representing a cross-section through the tibia-fibula of a 12 day experimental embryo. t = developing tibia; f = developing fibula. White arrow indicates artery. Note that there is no fluorescence in the artery wall. A Falck preparation. Magnification X108

95 Same preparation as Figure 94, except magnification X268
PLATE 55

EXPLANATION OF FIGURES

96  A fluorescent photomicrograph taken with U.V. illumination representing a cross-section through the tibia-fibula of a 2 day post-hatch chick. White arrow demonstrates an area of fluorescence in the wall of an artery. A Falck preparation. Magnification X132

97  A fluorescent photomicrograph taken with U.V. illumination representing a cross-section through the tibia-fibula of a 2 day post-hatch chick. White arrow demonstrates an area of fluorescence in the wall of an artery. Magnification X332
PLATE 56

EXPLANATION OF FIGURES

98 A photomicrograph of a cross-section through the tibia-fibula of a 12 day control embryo. t = developing tibia; f = developing fibula. White arrow indicates AChE activity in the periosteal cells of the tibia. AChE preparation. Magnification X240

99 Same preparation as Figure 98, except specimen represents a 12 day experimental embryo.
PLATE 57

EXPLANATION OF FIGURES

100  An electron micrograph of nerve fiber (n) relationships to a periosteal cell (p) in a 12 day control embryo. Arrows indicate AChE activity in the nuclear envelope of a periosteal cell (p). AChE preparation. Magnification X9,576

101  Same preparation as Figure 100, arrow indicates presence of AChE activity in juxtaposition to a nerve fiber.

102  Same preparation as Figure 100, arrow indicates presence of AChE activity in juxtaposition to a nerve fiber.
PLATE 58

EXPLANATION OF FIGURES

103  A photomicrograph of a longitudinal section through the tibia-fibula of a 6 day control embryo. Solid black arrow indicates anlage of tibia. Black outlined arrow indicates anlage of fibula. Black material represents areas of vascular channels. India ink injected preparation. Magnification X33

104  Same preparation as Figure 103, except specimen represents a 6 day experimental embryo.
PLATE 59

EXPLANATION OF FIGURES

105 A photograph demonstrating the vessels of the leg in control chick embryos at 7, 8, 10 and 12 days of incubation. India ink preparation on days 7, 8 and 10. Latex preparation on day 12.

106 A close-up photograph of injected lower limbs from a 7 and 8 day control chick embryo. India ink preparation.
DAYS of INCUBATION

7 8

10 12
PLATE 60

EXPLANATION OF FIGURES

107 A photograph demonstrating the equipment used to record blood flow and vessel diameters in the chick leg between 6 and 12 days of incubation. Black straight arrow (↓) indicates quartz rod. Black undulating arrow (§) indicates light source.

108 A photograph demonstrating the preparation of a chick embryo for recording the blood flow and vessel diameters in the leg. Black arrow indicates the quartz rod. Black outlined arrow indicates dripper tip used to bathe preparation with physiologic Ringer's solution.
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1) Awarded

   a. Narayanan, C. H. and Malloy, R. B.
      Angular Bone Growth and Development in Avian Embryos –
      A Quantitative Microscopic and Histochemical Analysis,
      Funded via Institutional Grant from LSU School of Dentistry,
      1971.

   b. Malloy, R. B., and Narayanan, C. H.
      Denervation of the Trigeminal Area on the Morphogenesis of
      the Temporo-Mandibular Joints in Avian Embryos, Funded via
      Institutional Grant from LSU School of Dentistry, 1972.

   c. Ruby, J., and Malloy, R. B.
      The Effect of SnF$_2$ and APF on Intact Enamel Surfaces: An
      In Vivo Study, Funded via Institutional Grant from LSU

2) Proposed

   a. Holmstedt, J. O. V., and Malloy
      Trophic Relationships of the Neural and Vascular Systems
      in Palatal Development. Submitted to The National Foundation
      (March of Dimes) in September, 1974.

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ABSTRACTS

(Papers Presented)


PUBLICATIONS


Malloy, R. B., Soni, N. N., Logan, D., Assessment of unilateral condylectomy in young guinea pigs: A triple fluorochrome study (Submitted to Anat. Rec.)