THE GROWTH OF BONES IN THE RAT AS INFLUENCED BY MAGNESIUM DEFICIENCY AND HORMONAL VARIATIONS

by

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SCOPE

In past studies, it has been demonstrated that the growth and development of cartilage and bone is modified in the magnesium-deficient animal. In a recent study by Hunt and Bélanger (1972), it was reported that there also occurs, in some rats, experimentally-produced generalized medullary bone growth (osteomyelosclerosis). This condition is similar to the physiological state produced in birds by estrogen stimulation during the egg-laying cycle. In addition, the investigators discovered the frequent occurrence of hyperplastic periosteal lesions at the femoral linea aspera. They noted that modification in the lesion composition depended on the presence or absence of the parathyroid gland.

The work reported in this thesis is a continuation of the Hunt and Bélanger studies. It attempts to assess the morphological effects of various hormones (parathormone, calcitonin, thyroxin, and estradiol) in the modification of bone growth and periosteal lesion production in the magnesium-deficient rat. In the parathyroid studies, a chemical analysis of magnesium, calcium, and phosphorus in bone is included. The study also includes morphological examination of renal tissue since renal defects, notably calcification, are commonly found in magnesium-deficient animals. Finally, to demonstrate the effects, retarding or otherwise, of magnesium deficiency on bone matrix formation, an autoradiographic study, with the use of a labeled protein precursor, was made.

CHAPTER 1

INTRODUCTION

The development, growth, maintenance, and aging of bone is influenced by such factors as diet and endocrine secretions. While many of the hormones act by controlling the rate of skeletal growth (Silberberg and Silberberg, 1971), an adequate supply of the nutritional components, including vitamins and minerals, is a pre-condition for optimal growth (Sissons, 1971). The action of hormones on bone growth in animals receiving a nutritionally adequate diet is fairly well documented. Not as thoroughly investigated is the effect of these hormones on bone in animals maintained on a diet deficient in an essential mineral component. The present study was undertaken, therefore, to assess the influence of the following hormones, parathormone, calcitonin, thyroxin, and estradiol, in modifying the bone structure in magnesium-deficient rats.

BONE GROWTH

In the young animal, the skeleton increases its size and attains its final shape by three processes: mesenchymal (membranous) ossification, osteochondral (endochondral) ossification, and bone remodeling. In the mature animal, when linear growth no longer

occurs, the normal bone structure is maintained by the process of internal bone remodeling, and thus depends on the balance between the opposed processes of bone deposition and bone resorption (by osteoclasia and/or osteocytic osteolysis). Each of these processes is described in an excellent chapter on the skeletal tissues (Bélanger, in press) and will not be discussed in detail here.

A. Effect of Hormones on Bone Growth

1. Anterior Pituitary and Thyroid

A large series of experiments carried out by Evans and his collaborators at the University of California during the first half of this century established the importance of anterior pituitary presence for continued optimal skeletal growth in animals (e.g. Asling et al, 1950; Becks et al, 1950; Ray et al, 1950). They observed that in the absence of the pituitary the cartilage cells of the epiphyseal plate of long bones ceased to proliferate and, as a result, the plate was narrower than normal. The chondrocytes present did not hypertrophy nor did the plate continue to be vascularized. At the same time, the metaphyseal trabeculae were reduced in number.

a) Thyroidectomy

In their studies on the effect of thyroid extirpation on bone growth, the same investigators observed, as did others (Silberberg and Silberberg, 1943) that the absence of the thyroid

gland in immature, growing animals retards the development of the skeleton and causes dwarfism. This retardation is mainly as a result of abnormal ossification of the junction cartilages of the long bones and the delay in the appearance of epiphyseal centers of ossification. In histological sections, they noted that the proliferation of cartilage cells was sometimes reduced, but that the maturation of the cartilage cells did not occur and vascularization was very much retarded.

b) Thyroid hormone administration

The California group, among others (e.g. Silberberg and Silberberg, 1943, 1946; Brush, 1945), demonstrated that the administration of thyroid hormone accelerated skeletal aging. It promoted to a minor degree growth and to a greater degree degeneration and resorption of cartilage. It stimulated, in particular, the phase during which resorption of cartilage and bone leads to epiphyseal-diaphyseal union. Enlarged capillaries are very prominent. From these studies and from an extensive series of experiments designed to study bone growth in hypophysectomized-thyroidectomized rats (reviewed by Simpson et al (1950) and Ray et al (1954)), Evans postulated that the main influence of thyroxin was to advance skeletal maturation while that of pituitary growth hormone was to stimulate skeletal growth without advancing skeletal maturation.

In addition to their effects on bone and their well-known effects on oxidative phosphorylation, the thyroid hormones (thyroxin and triiodothyronine) have been shown to raise the levels of plasma and urinary calcium (Engfeldt and Hjertquist, 1952; Krane et al, 1956; Nordin, 1965). In this respect, the action of thyroxin appears to be qualitatively similar to that of parathyroid hormone (see below). It has also been reported that thyroxin antagonizes the parathyroid hormone action at the level of calcium excretion through the kidney (Aubert et al, 1964).

2. Parathyroid

Von Reckinghausen first delimited osteitis fibrosa from other bone lesions (quoted by Krook, 1969). Proof of the parathyroid etiology of osteitis fibrosa was given forty years later by the animal experiments of Jaffe, Bodansky and Blair (Selye, 1947), who reproduced the characteristic bone lesions in animals by overdosage with parathyroid hormone.

a) Parathyroidectomy

In young rats, complete removal of the parathyroid glands causes pronounced inhibition of skeletal growth. Histologically, the most prominent feature is the inclusion of cartilage islands among the irregular trabeculae in the

subepiphyseal zone of the shaft (Selye, 1947). In older animals, the lesions are less characteristic.

b) Parathyroid hormone administration

A comprehensive review of the effect of excess parathyroid hormone in animals and man is included in an informative chapter on metabolic bone diseases of endocrine origin (Krook, 1969).

Parathyroid stimulation of young or old animals causes an almost immediate response in the trabeculae of bone. The mature osteocytes in the deeper portion of the bone respond initially (within a few hours) and become involved in the production of a "protease possibly linked to lysosome activity". Resorption of both the organic and inorganic components of bone thus occurs by increased osteocytic osteolysis (Bélanger et al, 1965). A description of the morphologic appearance of the cells is given by Bélanger (1971) as follows: "In the bones of normal animals and man, osteocytic resorption is characterized by the presence of large osteocytes, generally situated in the deeper portions of the tissue ... the more centrally located large osteocytes appear closer to one another than the small, peripheral cells. Counts of the relative number of cells per unit area of tissue have been made (Taylor and Bélanger, 1969) and used as a criterion of the intensity of osteolysis .... Alpharadiography has revealed that there is

a concomitant perilacunar loss of organic and mineral substance in osteocytic resorption. Also the enlarged and closely related lacunae often become confluent giving rise to resorption cavities."

"Osteolysis is the most important form of bone resorption both in normal remodeling and in the increased catabolic processes typical of, for example, generalized osteitis fibrosa. Osteoclasia is a late response" (Krook, 1969). Only after some period are osteoclasts seen eroding the surface of trabeculae. Bélanger et al (1965) concluded: "Osteoclasia appears as a specialized response to the presence of abnormal skeletal material either of general or local origin."

In the late 1940's it was believed that the action of parathyroid hormone in bone was dose dependent, since "very small doses of parathyroid hormone or large (but sublethal) doses given for a long time cause an inverse response with excessive proliferation of active osteoblasts" (Selye, 1947). C^{14}-glycine incorporation studies in bone (Vaes and Nichols, 1962), on the other hand, suggested that the action might be time-dependent. This was based on the observation that PTE at first reduced the glycine incorporation and the osteoblast population; after 48 hours, however, the C^{14}-glycine incorporation was increased to 50% above normal and the number of osteoblasts had returned to normal. When rats were injected

with 50 Units PTE for 20 days, increased bone formation resulted (Kalu, 1970). Kalu concluded from the H^3-proline incorporation studies of bone hydroxyproline in these rats that the "principal effect of parathyroid hormone on bone is anabolic". That this action is due to parathyroid hormone and not to calcitonin was substantiated by Kalu (1970) and by McGuire et al (1972) in thyroparathyroidectomized rats. The increase in osteoblastic activity may, nevertheless, be due to a compensatory mechanism.

c) Mode of action

Parathormone functions in at least four sites: blood serum, kidney, intestine, and bone. The solubility of calcium and phosphorus in serum is increased; renal excretion of phosphorus is increased; intestinal calcium absorption is promoted; osteoid mineralization is prevented and bone resorption is accelerated (Gies, 1966). Under the influence of parathyroid hormone, destruction of stable crystals of hydroxyapatite as well as of the organic matrix occurs. This mechanism makes available, for homeostatic regulation, an otherwise inaccessible source of calcium.

3. Calcitonin

Opposing the action of parathyroid hormone on bones is calcitonin. Its existence was first recognized by Copp and Davidson (1961) and attributed to be a secretion from the thyroid gland (Hirsch et al, 1964) or from the ultimobranchial gland (Copp, 1967). The hormone interferes with bone resorption and tends to lower the blood calcium.

Some investigators, studying either *in vitro* or *in vivo* systems, reported that the number of bone-resorbing osteoclasts is reduced following calcitonin administration, while others have found increased amounts of new bone in calcitonin-treated animals (see Copp, 1971). With regard to calcitonin, Goldhaber et al (1968) found no evidence to support the contention that the hormone stimulates new bone formation. There is, however, convincing alpharadiographic evidence that calcitonin decreases osteocytic osteolysis (Bélanger and Rasmussen, 1968; Bélanger and Copp, 1971).

4. Estrogen

In their very extensive reviews of the action of estrogens on bone, Silberberg and Silberberg (1956, 1971) traced the initial knowledge of the skeletal effects of the gonads to the ancients. Aristoteles (384-322 B.C.) is credited with recognizing that "if castrated at an early age, animals become taller and more delicate than the non-castrates; if they are, however, developed (at the time of castration), they do not add to their size " (quoted by Krook, 1969).

a) Ovariectomy

It is now well established that in the growing skeleton ovarian deficiency affects both endochondral (osteochondral) and intramembranous (mesenchymal) ossification, manifested histologically during the early stages by a hyperplasia and hypertrophy of the epiphyseal cartilage. In the rat, as in various other species, the bones have the following characteristics: they are delicate and thinner than usual, their breaking strength is decreased, callous formation is retarded, epiphyseal growth zones and cranial sutures close later than normally, and ossification centers are retarded in appearance (Silberberg and Silberberg, 1939). In young animals, body growth and the size of the tubular bones are increased but this is age and species dependent.

In the adult organism, ovarian deficiency appears to

exert no noticeable effect on cartilage. On the other hand, it appears to precipitate a state of osteoporosis, the bone deficit resulting from disturbed skeletal remodeling (Albright and Reifenstein, 1948). In these circumstances, the level of resorption is nearly always increased above that for age-matched normals, while bone formation appears to be largely within normal limits (Jowsey, 1968). Both osteoclasia and osteolytic osteolysis (Bélanger, 1969, 1971) appear to be actively involved (Jowsey, 1966). Although increased resorption may in part be due to the absence of the inhibiting effect of estrogenic hormone, other factors such as changes in serum calcium level and an increase in parathyroid hormone secretion may play a more prominent role (Jowsey, 1968).

b) Estrogen administration

Estrogens produce three major effects on the structure of the skeleton: inhibition of linear growth, acceleration of skeletal development and condensation of bone (Silberberg and Silberberg, 1971). However, the response of the bone to estrogen varies with age, sex, and strain (Gardner and Pfeiffer, 1943).

In growing rodents, estrogens cause a narrowing of the cartilage plate and inhibition of resorption of primary spongiosa. In the epiphysis, the maturation of chondrocytes is accelerated, the mucopolysaccharides are decreased

(Dziewiatkowski et al,1957; Priest and Koplitz, 1962), and there is an increase in the concentration of collagen in the matrix. In the primary spongiosa, osteoclasts are rare, and increased numbers of osteoblasts and preosteoblasts line the heavily calcified spicules. According to Simmons (1966) who used autoradiography and a mitotic labeling index, the increase in osteoblasts results from an induced conversion of precursor cells of connective tissue origin into bone forming cells. Newly formed matrix laid down by these osteoblasts calcifies to form bridges between spicules, thus giving rise to a network of interlacing, plate-like trabeculae. Whether or not the accelerated calcification of this matrix in some way causes an increased resistance to resorption by bone marrow elements is still speculative. The metaphyseal bone which is normally present between primary trabeculae is replaced by a poorly vascular fibrillar and hyalinized connective tissue (Silberberg and Silberberg, 1939). In the absence of resorption the trabeculae entend into the diaphysis and to the endosteum with the resultant thickening of the cortex (Urist, Budy, and McLean, 1948).

One of the most dramatic examples of an estrogen effect is the physiological osteomyelosclerosis produced in birds. In this instance, an entire new system of trabeculae of medullary bone is produced by outgrowth from the endosteal lining in the

estrogenic phase of the egg-laying cycle. (Priming doses of androgens are necessary - Bloom, McLean, and Bloom, 1942). A less dramatic osteosclerosis in the rat and mouse skeleton is produced by large doses of estrogens (Day and Follis, 1941; Gardner and Pfeiffer, 1943; Budy and McLean, 1948). In the mouse the osteosclerosis is a result of increased endosteal bone formation and inhibition of physiological bone resorption (Gardner and Pfeiffer, 1943; Urist, Budy, and McLean, 1948), whereas in the rat the osteosclerosis is merely a result of inhibition of physiological resorption of newly-formed bone (Urist, Budy, and McLean, 1948; Selye, 1953). Calcified structures, other than bone, do not seem to respond to estrogens (Stahl et al, 1950).

c) Mode of action

Although the effect of estrogen on bone may be partly mediated through the hypophysis (Silberberg and Silberberg, 1946), most of the effects are believed to be direct. That estrogens are taken up by bone has been shown by tracer studies in the mouse injected with estrone 16-C^{14} (Budy, 1960). Budy concluded that estrogen and/or its metabolites act on the bone marrow and cellular elements of bone. However, there is, as yet, no evidence for any estrogen receptor site in any cell in either bone or cartilage.

B. Effect of Nutrition on Bone Growth

Optimal dietary intake of nutrients, including vitamins and minerals are essential for normal growth of the body, including the skeleton. The lack or excess of any of these dietary factors leads to either bone growth cessation or retardation or to additional abnormalities. Many of the resulting conditions are described in a number of treatises (Bourne, 1971) and, except where they relate to magnesium deficiency, the pathology will not be reviewed here.

MAGNESIUM DEFICIENCY

A. General

Among the minerals essential for optimal growth of the rat is magnesium, the fourth most abundant cation in the body and the second most plentiful intracellularly. Bone contains about half the total body magnesium, the remainder being equally distributed between muscle and non-muscular soft tissues (Aikawa, 1963). In a rat bone, as well as in that of other species, this mineral normally constitutes 0.5 to 0.7% of the bone ash (Morgulis, 1931).

Under conditions of magnesium deprivation, the rat develops a syndrome (described by Orent, Kruse, and McCollum, 1932; Watchorn and McCance, 1937; Tufts and Greenberg, 1938; Bélanger, 1957; MacIntyre and Davidsson, 1958; Martindale and Heaton, 1963;

Whang and Welt, 1963; Heggtveit, Herman, and Mishra, 1964; Chutkow, 1965; Clark and Bélanger, 1967; Hunt, 1971; Hunt and Bélanger, 1972), the major features of which include initial hyperemia and edema of the extremities and the ears, followed by heightened neuromuscular irritability, hemorrhagic and necrotic skin lesions and generalized convulsions which often terminate in death. During this time there is generally growth retardation, hypomagnesemia, hypercalcemia and hypophosphatemia. Pathologic renal changes in the form of microliths in the ascending loops of Henle also occur. In addition, there is bone magnesium depetion and the bones become "brittle" (Cunningham, 1936; Watchorn and McCance, 1937; Duckworth, Godden and Warnock, 1940; McAleese and Forbes, 1961; Aikawa, Reardon and Harms, 1962; Chiemchaisri and Phillips, 1963; Smith and Field, 1963; Martindale and Heaton, 1964; Hunt, 1971; Hunt and Bélanger, 1972). In the young rat, as much as two-thirds of the bone magnesium may be lost during the period of deprivation. In the old mature rat, a much lesser portion of the bone magnesium appears to be available for exchange to restore diminished plasma magnesium concentration (Hunt, 1971). The mechanism by which this occurs is still uncertain although several extensive reviews on the distribution and metabolism of magnesium have appeared during recent years (Walser, 1967; Wacker and Parisi, 1968; Gitelman and Welt, 1969; Durlach, 1971).

The histologic changes in rat bone which occur as a result of magnesium deprivation have been described by Bélanger (1958), Bernick and Hungerford (1965) Clark and Bélanger (1967), Trowbridge and Seltzer (1967), Smith and Nisbet (1968), and Hunt and Bélanger (1972). These changes involve a general decrease in bone growth, a reduction in thickness of the epiphyseal plate and a reduced mucopolysaccharide and protein synthesis at the epiphyseal plate of the long bones. In the organic matrices of alveolar bone and dentin, there is an interference both with collagen formation and the sulfation of glycosaminoglycuronoglycans (Trowbridge, 1971). Very recently, Hunt and Bélanger (1972) also reported the occurrence of localized bone tumors as well as development of medullary trabeculae in the femurs of both young and mature (but still growing) magnesium-deprived rats.

B. Effect of Hormones

1. Estrogen

The presence of medullary bone as a physiologic phenomenon occurring in birds during the egg-laying cycle is well documented (Pfeiffer and Gardner, 1938; Bloom, McLean, and Bloom, 1942; Benoit and Clavert, 1945; Taylor and Bélanger, 1969; Silberberg and Silberberg, 1971). It serves as a reservoir for calcium which is drawn upon for egg shell formation. In the young mouse, the production of medullary bone of

endosteal origin was shown to be extensive following estrogen injections (Pfeiffer, 1938; Urist, Budy,and McLean, 1948). On the other hand, in the case of intact rats, although metaphyseal bone trabeculae are not resorbed following the administration of large estrogen doses, endosteal bone is not produced. Yet, by a simultaneous growth hormone and estrogen treatment, the endosteal surface could be stimulated to produce new bone (Selye, 1953). This situation raised the question as to whether estrogen administered to magnesium-deprived rats would augment medullary bone formation, increase tumor mass development, or modify other symptoms of the magnesium deficiency. That modification of symptoms might occur in rats was suggested by studies (Whitfield, 1968) in which the onset of clinical symptoms of magnesium deficiency was delayed by estradiol administration. One of the aims of the present study was to examine this question.

2. Parathormone

Magnesium deficiency induces in most species a loss of sensitivity of bone to parathyroid hormone. The rat is an exception (Larvor and Durlach, 1971).

In the magnesium deficiency experiments by Hunt and Bélanger (1972), the quality of the bone tumor mass could be modified by either parathyroidectomy or parathyroid hormone. In intact rats, the tumor mass consisted mainly of fibroblasts

interspersed with collagen fibers. In parathyroidectomized rats, in addition to fibrous tissue, the tumor mass also contained cartilage. No medullary bone was observed in parathyroidectomized rats. The administration of parathormone to these animals caused a regression of the cartilage component of the tumor in favor of the fibrous portion. Medullary bone was now present in these animals.

In the present investigation, these studies were extended to examine the effect of the separate or combined action of parathyroid hormone and of supplemental magnesium in rats that had been deprived of the mineral. The alkaline phosphatase distribution in the bone was detected histochemically and chemical analyses of calcium and magnesium in blood serum and bone were included.

3. Calcitonin

Calcitonin is generally without effect on magnesemia; however, a magnesium load seems able to induce its secretion (Larvor and Durlach, 1971). The effect of the hormone on bone during a magnesium deficiency has not yet been reported.

According to Bélanger (in press), "the intimate effects of calcitonin on bone cells is not yet fully understood. Bélanger and Rasmussen (1968) have established, however, that this substance when introduced into the organism at the same time as parathyroid hormone, inhibits the osteolytic activity of the osteocyte". This was further confirmed in chicks (Bélanger and Copp, 1971). Because of its opposing effects to parathormone, a further question as to whether calcitonin (or its absence) would modify the magnesium deficiency effects on bone was raised for examination in the present study.

4. Thyroid Hormone

It was mentioned earlier in this chapter that the thyroid hormones accelerate skeletal aging or maturation. Thyroxin also alters bone turnover by a direct action on the bone cells and by influencing calcium compartment sizes as well as flow to and from these compartments (Krane et al, 1956). Hyperthyroidism enhances magnesuria and may induce a magnesium deficit. The effect of thyroxin on bone growth during magnesium deprivation was, therefore, also included in the present investigation.

C. Effect on Renal Tissue

The close association of magnesium deficiency and renal calcinosis has been recognized since 1936 (Tufts and Greenberg, 1936). However, it was shown within recent years that the development of renal calcinosis in magnesium-deficient rats requires the presence of parathyroid hormone (Heaton and Anderson, 1965; Meyer and Forbes, 1968) and vitamin D (Lifschitz, Harrison and Harrison, 1967). On the other hand, thyroxin was found to prevent nephrocalcinosis (Forbes, 1965; Meyer and Forbes, 1968; Jacob and Forbes, 1970; Reeves and Forbes, 1971 and 1972) in magnesium-deficient rats and the morphologic lesions of kidney and bone induced by parathyroid extract overdosage (Gabbiani et al, 1967). Gabbiani et al (1968) subsequently reported that calcitonin also exerted a protective effect on the kidney and other soft tissue of rats that were administered parathyroid hormone.

One hormone which until now has not been studied for its effect on calcificiation of the kidney during the period of magnesium deficiency is estradiol. In the present study, a histologic examination of the kidneys was made to determine whether or not there was any correlation between the severity of bone lesions and extent of kidney calcification in rats of various hormonal states during a magnesium deprivation period.

D. Effect on Tooth Structure

The teeth of rats on a nutritionally adequate diet show characteristic lesions following parathyroidectomy (Erdheim, quoted by Selye, 1947). In parathyroidectomized rats, the newly formed dentin fails to calcify and the enamel is hypoplastic and irregular. If the rat is periodically treated with small doses of parathormone, rings of calcification, corresponding to each period of treatment, appear within the otherwise uncalcified dentin.

The teeth of rats on a magnesium-deficient diet also show characteristic lesions (first noted by Klein et al, 1935; Watchorn and McCance, 1937; Irving, 1940; and summarized by Trowbridge, 1971). In the odentoblasts, progressive atrophy is evident, and in some cases the cells fail to recede after laying down the predentin and thus become embedded in it. In the dentin that is formed, striations are very prominent. It has been suggested by Meyer and Forbes (1963) that magnesium deficiency increases parathyroid gland activity. Parathyroidectomy, however, does not significantly affect the formation of dentinal striations during a short term experimental period (Trowbridge, 1971).

It has been reported that the administration of estrogens do not seem to affect the hard structures (dentin, enamel, and cementum) of teeth, the pulp, or the gingiva of rats or mice, although large doses cause osteosclerosis of alveolar bone (Stahl et al, 1950). It has also been observed that the administration of

estradiol to ovariectomized-magnesium-deficient rats delays the onset of a number of the clinical magnesium-deficiency symptoms. It has, however, not been shown whether estrogens will delay the onset or in any other way modify the dental abnormalities caused by the magnesium deficiency. This question is examined here as an adjunct to the study on the effect of estrogens on bone in the magnesium deficient rat.

E. Effect of Protein Synthesis

1. Protein Synthesis in Normal Rat Bone

The extracellular components of mineralized bone tissue consists of inorganic crystals of calcium phosphate (hydroxy-apatite) oriented in a parallel fashion within the collagen fibrils of the organic matrix. The mineral portion constitutes approximately 70%, the matrix 25 - 30% (Eastoe, 1954; Eastoe and Eastoe, 1956). Collagen comprises the major portion of the organic matrix, about 90%, whereas the mucopolysaccharide-protein complex accounts for less than 2% and the other proteins for 6% (Eastoe and Eastoe, 1956). Glycine is the amino acid present in highest concentration (26%) in collagen (Eastoe, 1955). Since glycine does not contribute to the biosynthesis of mucopolysaccharides, keratosulfate, or chondroitin sulfate (Zambotti, 1958) but is incorporated into collagen (Slack, 1953) radioactive glycine has been used as a labeled precursor for new

protein synthesis in the organic bone matrix (Carneiro and Leblond, 1959; Vaes and Nichols, 1962 a and b; Young, 1962; Young and Greulich, 1963). Radioactive proline, which is more specific as a bone matrix precursor,has been used for the same purpose and with similar results (Ross and Benditt, 1962; Leblond, 1963). Using the radioautographic technique, Leblond demonstrated that thirty minutes after injection of H^3-glycine in mice, the cytoplasm of the osteoblasts in the growth zone of femura is strongly reactive while the prebone (osteoid) reacts moderately; at 4 hours it is outside these cells and within prebone; by 7 days the label is within calcified bone tissue (Leblond and Weinstock, 1971).

The synthesis of the mucopolysaccharide moiety of the matrix has also been investigated in long bones by autoradiography (Bélanger, 1954; Dziewiatkowski, 1954) with the use of S^{35} sulfate, inasmuch as the sulfate ion is incorporated into the sulfated mucopolysaccharides of the organic matrix (Bélanger, 1954, 1956). Other labeled matrix precursors of moieties have also been used (see Greulich and Leblond, 1953; Leblond and Weinstock, 1971).

2. Protein synthesis in magnesium-deficient rat bone

Magnesium ions are known to play an essential role in protein synthesis in that they activate many enzyme ractions. Almost fifteen years ago, Bélanger (1958) showed by autoradiography that, in rats fed a magnesium-deficient diet, the uptake

of $S^{35}O_4$ and S^{35}-methionine by the epiphyseal plate of long bones was inhibited as compared with controls. Bélanger concluded that "the decreased growth rate in magnesium deficiency is at least partly related to a deficient synthetic mechanism of the cartilage and bone cells affecting both proteins and sulfated mucopolysaccharides". In 1967, Trowbridge and Seltzer also used the $S^{35}O_4$ precursor but substituted H^3-proline for the collagen precursor. In their autoradiographic studies, they showed that organic matrix formation in alveolar bone was disturbed as evidenced by decreased H^3-proline uptake and by decreased S^{35} sulfate incorporation into glycosaminoglycuronoglycans. The long bones, however, were not investigated in their experiments.

In the present study in which the experiments of Bélanger served as a model, H^3-glycine is used as the collagen precursor, since glycine is sixty times more abundant than methionine in collagen (Eastoe, 1956). Furthermore, tritium, with a particle range of 18.5 keV, allows for much better autoradiographic resolution than does S^{35}, with a range of 167 keV.

CHAPTER II

MATERIALS AND METHODS

GENERAL

1. ANIMALS

Rats of the Charles River Caeserian Derived Strain (CRCD) were used in these experiments. Animals were obtained from the Charles River laboratories, Wilmington, Massachusetts, in groups of littermates of 70-90 grams body weight and were allowed to reacclimatize to the new surroundings for at least one day prior to any surgical treatment and for at least one week prior to any new dietary regimen. The animals were individually housed in metal cages under an ambient temperature of 74°F. Overhead fluorescent lamps provided lighting for ten continuous hours each day. Body weights were recorded three times weekly by means of a Mettler balance. Observations on the state of the animals were made daily and abnormalities were given a value according to a point system to quantitate the severity of each sympton or lesion.

2. DIETS USED

A. Standard:

The animals were maintained on Rockland chow pellets (a complete rat/mouse diet from Tecklad Laboratory, Monmouth, Ill.) and tap water before being placed on experimental diets and distilled water.

B. Experimental:

The experimental diets consisted of either a magnesium deficient diet (Mg-) or a magnesium deficient diet which was supplemented with magnesium (Mg+). Both diets were obtained from General Biochemicals, Chagrin Falls, Ohio. The formula for the magnesium deficient diet is given in the Appendix.

The magnesium content as determined by absorption spectrophotometry varied from 0.925 to 1.2 mg./100 gm dry weight (0.880 mg./100 gm wet weight) for the magnesium deficient diets and from 260 to 265 mg./100 gm dry weight (253 mg./100 gm wet weight) for the magnesium supplemented diets. The calcium content was 0.698 g./100 gm dry weight (0.660 g./100 gm wet weight) for the deficient and 0.657 g./100 gm dry weight (0.621 g./100 gm wet weight) for the supplemented diets.

When the rats were first placed on the experimental diets in exchange for the stock fare, the new regimen appeared to be accepted fully after two days. However, during these two days of inanition, most animals lost weight. During the remainder of the experimental period, the animals appeared to tolerate the diets reasonably well, although the rats on the deficient diet after one week consumed somewhat less per day than did the controls on the supplemented diet.

3. SURGICAL PROCEDURES

A. Ovariectomy

Post-weanling female rats were bilaterally ovariectomized under sodium pentabartital anesthesia (0.1 ml. of a 3% solution/100 gm body weight intraperitoneally) via two small longitudinal dorsolateral incisions at the level of the kidneys. With a pair of forceps, each ovary was isolated and the follopian tubes were tied with surgical thread 2 mm. below each ovary. All the tissue above the surgical thread was removed with a sharp scalpel. The muscle incision was closed by a thread suture and the skin wound either by a thread or by metal clips. That all ovarian tissue had been removed successfully was confirmed at autopsy.

B. Parathyroidectomy

Male rats of approximately 150 grams were parathyroidectomized by electrocautery at the Charles River laboratories four days before they were received in Ottawa. To prevent tetany, calcium chloride was added to the rats' drinking water during the four days.

C. Thyroidectomy

The parathyroid glands were allowed to remain intact in female rats (70-80 grams) from which the thyroid glands were surgically removed at the Charles River laboratories. The presence of parathyroid tissue was confirmed in rats that survived five weeks on water not supplemented with calcium chloride, whereas the completeness of thyroidectomy was ascertained by the fact that the animals had gained no weight after one week (Eartly and Leblond, 1954), and that after autopsy no thyroid tissue was found in serial sections of trachea.

4. AUTOPSY

The animals were sacrificed either by an overdose of ether or chloroform or by severence of the spinal cord and resection of the jugular vein. The exanguinated blood (approximately 4 ml.) was collected for magnesium determinations (see section 6 of this chapter). A complete gross autopsy was performed on each animal.

5. TECHNIQUES FOR MORPHOLOGICAL STUDIES

A. Introduction

Four main morphological techniques were used. The first, histology, included the preparation of histological sections stained by routine procedures. The second, microroentgenography, made use of a fine-grained photographic emulsion to record the extent of penetration of soft X-rays projected through microscopic portions of bone tissue, thereby providing an indication of the relative mass concentration in cells and tissues within the microscopic unit (Engstrom et al, 1955). A variant of this technique, alpharadiography, made use of α-particles from a source such as polonium^{210} (radium F, half-life = 140 days) instead of X-rays. Under standardized operational conditions, differences of alpha particle absorption by a soft tissue (or one of low mineralization) as recorded by a fine grained photographic emulsion is indicative of mass variations in different parts of the sample (Bélanger and Bélanger, 1959). This technique was especially useful to study density variations of demineralized sections.

The third, autoradiography, also made use of the photographic emulsion to record radioactive emissions, this time from a source originating from within the bone itself (Bélanger and Leblond, 1946).

When a photographic emulsion (silver bromide crystals embedded as a lattice in a gelatin matrix) is placed in intimate contact with a histological tissue section containing a radioactive element, the rays emitted by the section initiate in the emulsion a reduction of silver bromide to metallic silver. Upon photographic development of the emulsion, the metallic silver appears as black grains immediately over the area of tissue which was the source of the radioactive element. This technique was used to locate the sites of incorporation of the radioactive amino acid, glycine-H^3 (as a precursor of collagen), into bone.

The fourth technique involved the histochemical detection of alkaline phosphatase. The site of alkaline phosphatase activity in femura and tibiae were localized by the method of Gomori (1952) which revealed the activity as black deposits of cobalt sulfide, and by the method of Burstone (1958) which marked the site of enzyme activity by azo dye deposits. The Gomori method is based on the ability of alkaline phosphatase to split glycerophosphate (substrate) and thus release phosphate ions. The latter combine with calcium ions (in the substrate) to form insoluble calcium phosphates which precipitate. These precipitates are then converted to cobalt phosphate and finally to cobalt sulfide, which is seen as the black deposits. The azo dye method of Bustone makes use of 1) substrates such as naphthols (e.g. Naptol AS-BI phosphate) which are hydrolyzable by alkaline phosphatase and 2) diazonium salts (e.g. Fast Red Violet LB) which couple with the product of enzymatic hydrolysis. The alcoholic part of the substrate is coupled with the diazonium salt to form a colored azo dye at the sites of alkaline phosphatase activity.

A detailed description of these four techniques is given later in this section.

B. Fixation

During the course of the complete autopsy performed on each animal, the kidney, one femur, one tibia and one jaw were placed in AFA fixative (15 parts 95% ethanol:4 parts formaldehyde:1 part acetic acid). These tissues, thus fixed for 24 hours, were prepared for histological examination. In some experiments the second femura of some animals were frozen for subsequent chemical magnesium determinations (see 6A), whereas the tibiae were fixed for 48 hours in 10% neutral formalin for subsequent microroentgenographic preparations. (Formalin neutralized with an excess of magnesium carbonate was used to avoid any decalcification of the bones to be studied by microroentgenography.) In all other animals, both the second femura and tibiae were fixed in 10% neutral formalin.

C. Preparation of kidneys and bones for histological examination:

Following fixation, the kidneys were dehydrated in graded ethanols (30%, 50%, 70%, 95%, 100%), cleared in three changes of toluene, infiltrated and embedded in paraffin. Five-micron-thick sections, cut on a Spencer AO microtome, were deparaffinized in three changes of xylol, hydrated with descending graded ethanols, and stained with hematoxylin-phloxine-Orange G or toluidine blue (see Appendix 2).

After a 24-hour fixation in AFA, the bones were placed in individual loose cheese cloth bags and were transferred to a solution of (saturated) 10% disodium ethylenediaminetetraacetate (EDTA) for

decalcification. By the use of the "constant replacement" technique[1] of Bélanger et al (1965), complete decalcification was achieved in three weeks. This was followed by a 30-minute rinse in running tap water to remove all trace of EDTA. The bones could now be cut easily with a razor blade. Sections of bone taken for histology included a cross section of the midshaft of the long bones and the distal end of the femur and the proximal end of the tibia split longitudinally. The remainder of the bone was examined before being discarded. The jaw was cut so that a cross section included the first molar tooth. The bones and teeth were dehydrated in an ascending series of graded ethanols over a period of two days, cleared in three changes of toluene the following day, infiltrated (six changes over eight hours) and embedded in melted paraffin the next day. Five-micron-thick sections were stained with diluted Wright stain (Bélanger, 1957; see Appendix 2).

D. Preparation of bones for microroentgenography:

The bones that were fixed in neutral formalin were transferred after 48 hours to 80% ethanol. With the use of a dental drill fitted with a burr, 10 mm. cross sections of the mid portion of the shaft of the bones were cut. These were dehydrated progressively in 95% ethanol, in three changes of absolute ethanol the following day, and in two changes of acetone the third day. The bone samples were then infiltrated with increasing concentrations of Epon resin according to the

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[1] The advantage of the "constant replacement" technique over others lies in its gentleness to tissue and to its efficiency in chelating calcium. The rapid saturation of EDTA with calcium, however, necessitates that the solution be renewed constantly. This is achieved by placing the bones in a flask to which one drop per second of fresh EDTA from a reservoir is constantly added to replace a drop that is lost from the flask by overflow (Bélanger et al, 1965).

following schedule (Luft, 1961). During the first hour, the bones in test tubes containing Epon mixture 1[1] were centrifuged at 1800 rpm and the stoppered tubes were stores in a desiccator overnight. In the morning the bones were again centrifuged, the first hour with mixture 2[2], the second hour with mixture 3[3]. Finally, the bones were infiltrated under vacuum (18 mm. Hg) for four hours with mixture 4[4]. Each bone sample was then embedded in an acrylic capsule (a piece of round tubing, 12 mm. in diameter, affixed to a square acrylic base) partially filled with fresh mixture 4[4] and the capsules were stored for thirty minutes under vacuum at room temperature. During the following thirty-six hours the Epon resin was allowed to polymerize in an oven for an equal length of time at each of three consecutive temperatures, 35°, 45°, and 60°.

When the Epon was completely polymerized, the acrylic capsule and its contents were trimmed with a band saw and cemented on to a thin plate of similar material fitting the cutting table of the Gillings diamond-wheel cutting machine (Gillings and Buonocore, 1959). Sections of 100 ± 10 microns were obtained. The uniformity of the section

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[1]Mixture 1: Solution A: Solution B: Acetone 1:1:6

[2]Mixture 2: Solution A: Solution B: Acetone 1:1:2

[3]Mixture 3: Solution A: Solution B: Acetone 3:3:2 to which is added 3% DMP accelerator (2,4,6 trimethyl amino ethyl phenol)

[4]Mixture 4: Solution A : Solution B 1:1 to which is added 3% DMP

Solution A: 62 ml. Shell Epon 812
100 ml. DDSA (Dodecenylsuccinic anhydride)

Solution B: 100 ml. Shell Epon 812
89 ml. NMA (Nadic methyl anhydride)

All reagents were obtained from Fisher Scientific Co., Fair Lawn, N.J.

thickness was always verified with a bench micrometer gauge (Gillings et al, 1959) which measured the thickness at a number of individual locations within the section. Only those sections with thickness variations of no more than 10% were picked up by a 2-inch strip of Scotch band adhesive tape. This was then affixed to a glass slide so that during storage each section was sandwiched between the tape and the slide (Bélanger et al, 1963).

E. Microroentgenography (microradiography)

Materials: The emulsions were supplied by the Canadian Kodak Sales Co., Toronto 15, Canada, on 1 x 3 inch glass plates coated with 10 micron thick layer of NTA nuclear emulsion or EK 649.0 spectroscopic emulsion. All the micrographs were prepared with the use of a microradiograph unit built by Garth Westenskow Co. and the Jackson Scientific Machine Co. of Salt Lake City, Utah.

Methods: Before use, the nuclear emulsion plates were coated with a thin layer of celloidin by dipping them once in a 1% solution of celloidin in ethanol-ether, and the plates were dried for at least twenty-four hours.

In the darkroom, the bone section adhering to the tape (see section D, above) was lifted from the storage glass slide and transferred to the emulsion covered plate so that the bone section now was sandwiched between the tape and the photographic emulsion. Intimate contact between bone and emulsion was essential to insure high resolution. The bone sample now was ready to be microradiographed.

1. Exposure

The micrograph unit consists of a high voltage power supply, a line voltage regulator, a control panel, X-ray tube and a camera to position the film and specimens to be X-rayed. The emulsion plate and adhering section were positioned in the camera in a light-tight environment. The camera tube was then placed part way into the X-ray beam tube and the metal plate on the camera was rotated 180° to a position where the emulsion and section were directly exposed to the X-ray tube. The apparatus allowed two separate samples to be exposed simultaneously to X-rays generated at a regulator voltage of 115 volts, an X-ray voltage of 1000 volts, and an X-ray current of 10 milliamperes. Each set of samples was exposed to the X-rays for 25 minutes. This exposure time was found to be optimal under the above operating conditions where the geometry of the apparatus was fixed, the sensitivity of the photographic emulsion remained constant, and the thickness of section of long bone studied was uniform. Only the extent of calcification of the bone varied.

2. Development and Fixation

In the darkroom the exposed emulsion plate and adhering section and tape were removed from the camera, and the adhesive tape bearing the bone section was trasnferred again to a clean glass slide for storage. The protective coat of celloidin covering the emulsion plate was dissolved in a bath of acetone followed by a series of graded ethanols down to water. The photographic plate was then developed for five minutes in Eastman Kodak D-19

developer (15°C), briefly rinsed in distilled water, and fixed for fifteen minutes in Kodak acid fixer with hardener. It was then washed in cold running water for thirty minutes, dried in an air stream, and mounted with a coverslip with a synthetic resin, such as Permount.

F. Alpharadiography

Materials: In these experiments, the polonium source was in the form of a deposit of polonium electroplated as a rectangle on a 1 x 3 inch gold plate manufactured by U.S. Radium (Bélanger et al, 1967) and Nuclear Radiation Developments, Grand Island, N.Y. (Bélanger, 1971). The plate was stored in a plastic slide box. The fine grained emulsions, NTA nuclear emulsion and 649.0 spectroscopic plates, and D-19 developer and acid fixer were obtained from Canadian Kodak Sales Co., Toronto, Canada.

Methods:

1. Exposure

The emulsion plates were coated with celloidin as in section 5E. In the darkroom 10-micron paraffin sections of demineralized bone were floated on distilled water, picked up and centered on the emulsion coated plates. The excess water was allowed to drain before the plate and section were sandwiched between several layers of filter paper. Light pressure was applied over the filter paper to flatten the paraffin section on the plate, and each plate was allowed to dry overnight in a slide box containing desiccant. Immediately prior to exposure, the paraffin from the section was removed in two successive baths of toluene

and two quick rinses in absolute ethanol. When the emulsion and section were dry, the plate was positioned in the slide box at a distance of 2.7 cm from the polonium source.[1] The length of exposure in the sealed box varied from two hours to four days depending on the intensity of the polonium and the type of photographic emulsion used. Spectroscopic plates require four times longer exposure than do NTA plates but give a higher resolution (Bélanger and Bélanger, 1959).

2. Development and Fixation

The celloidin protective coat was removed from the emulsion plate with two baths of acetone and a descending series of concentrations of ethanol to water. In addition to removing the celloidin, this procedure also separated the tissue from the emulsion. The emulsion plate was allowed to dry and was then developed in D-19, rinsed in water, fixed in acid fixer and processed as described in section 5E.

G. Autoradiography

Materials: 2-glycine-H^3, with a specific activity of 10.7 Ci/mM. was obtained from New England Nuclear Corp., Boston, Mass. For injection the glycine-H^3, which was supplied dissolved in a small volume of 0.1N HCl, was further diluted with distilled water to a concentration of 1 mCi/0.2 ml.

NTB 2 bulk emulsion, microdol X developer, and acid fixer were all purchased from Canadian Kodak Sales Ltd., Toronto 15, Canada.

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[1] Alpha particles of Po^{210} do not penetrate more than 3.84 cm. of air at 15°C. (Yagoda, H. Radioactive measurements with nuclear emulsion, New York, John Wiley and Sons, 1949)

Methods: The femura and tibiae of rats that had been injected with glycine-H^3 twenty-four hours earlier (see Specific Procedures, Experiment 5) were fixed in AFA and processed according to the procedure outlined for bone in section 5C. Unstained five-micron paraffin sections on slides were deparaffinized in three changes of xylol, transferred to ethanol, and hydrated in decreasing concentrations of ethanol in water. The excess water was drained from the slides.

The autoradiographic procedure used was a modification (Messier and Leblond, 1957, Bogoroch, 1972) of the original "coating" method devised by Bélanger and Leblond (1946).

1. "Coating"of sections

In the darkroom 25 ml. of Eastman Kodak NTB 2 nuclear bulk emulsion was melted in a beaker kept in a 40°C constant temperature water bath. After 20 minutes the melted emulsion was diluted with an equal volume of a warm (40°C) solution of 1% glycerine in distilled water, according to the procedure described in the Appendix 3, page 79. Thirty ml. of the diluted emulsion was transferred to a container calibrated at the 30 ml. level. The remainder served as a reservoir from which the depleted emulsion was periodically replaced. In this way the level of emulsion in the calibrated container remained fairly constant. Each of the ten slides from each experimental group was dipped vertically into the diluted emulsion, withdrawn at constant speed, hung to dry while the excess emulsion was allowed to drain on to moistened filter paper. When the emulsion had gelled, the slides were stored in numbered plastic slide boxes for exposure at 4°C.

2. Development and Fixation

The period of optimal exposure was dependent on the concentration of isotope within the bone, the particle energy (18 Kev) and half-life of tritium (12.3 years), and the sensitivity and thickness of the emulsion. However, since the concentration within the bone was not uniform, several exposure times varying from two to eight months were used. For each series of preparations that was developed at any one time, one slide from each experimental group was included.

The slides were developed in undiluted microdol X (16°C) for five minutes, rinsed in distilled water, fixed in acid fixer, and washed in four 5-minute changes of distilled water. The series of slides were left unstained to serve as controls for the effect of poststaining on the autoradiographic image. These slides were dehydrated in two changes of 95% ethanol, two changes of absolute ethanol, followed by a four-hour change in a 1:1 mixture of ethanol-cedarwood oil. A final overnight infiltration in cedarwood oil was helpful in reducing tissue artifacts. For permanent preservation, the preparations were mounted in Permount under a coverslip.

3. Poststaining

The Kingsley component of the Bélanger stain (1961) was used to poststain the autoradiographs (see Appendix 3, p.84, for formulae and details). The slides were rinsed in phthalate buffer (pH 6.9), stained for two minutes in Kingsley stain, rinsed in buffer to remove excess stain, and dehydrated in 95% ethanol (one

2-minute change) and absolute ethanol (two 2-minute changes). They were further processed in cedarwood oil as described above for unstained autoradiographs.

4. Quantitation of grains

Comparable areas in the metaphyseal, endosteal, and periosteal bones from control and magnesium deficient rats were selected for grain counting. The developed grains in four fields, each 50 um. square, were counted for each of the selected areas in each slide. Comparison between groups was made for autoradiographs which had been exposed for four months and for those exposed for six months.

H. Histochemistry

1. Alkaline phosphatase

Materials: All chemicals were obtained from Fisher Scientific Co., Fair Lawn, N.J. Reagent grade sodium B-glycerophosphate. H_2O was used.

Methods: The bones were fixed at 4°C in 2.5% glutaraldehyde in 0.1% cacodylate buffer (pH 7.3) for two hours, then washed several times with cold sucrose (0.2M)-containing cacodylate buffer to remove all trace of glutaraldehyde. The small bone samples were decalcified in two daily changes of 10% EDTA in tris-maleate buffer (Füllmer and Link, 1964) over a period from two to five days. After decalcification, the EDTA was completely removed by several changes of sucrose-cacodylate buffer, and the bones were kept in buffer until sectioned with a cryostat (International Equipment Co.). Ten-micron sections were mounted directly on slides by pressing each slide against a frozen section still on a knife.

a. Gomori method

The slides were then incubated for one hour in a glycerophosphate medium (3% sodium B-glycerophosphate; 2% sodium diethyl barbiturate; 2% calcium chloride; and 10% magnesium chloride in the ratio of 10:10:20:1 in a 50 ml. final solution at pH 9.2). After a wash in running water, the slides were transferred for two minutes to a 2.5% yellow ammonium sulfide solution in water and washed once more with four changes of double distilled water. Some of the sections were counterstained for five minutes in dilute (0.0125%) basic fuchsin; others were dried completely under a fan. The sections were then cleared in three changes of xylol and mounted under a coverslip with Permount. Alkaline phosphatase activity was demonstrable as black deposits.

b. Burstone method

The slides were incubated for one hour in naphthol AS-BI phosphate-diazonium salt medium (a filtered solution of 2.5 mg. naphthol AS-BI phosphate dissolved in 1 ml. N, N-dimethylformamide to which was added 50 ml. 0.05M tris-maleate buffer (pH 8.3) and 40 mg. Fast Red Violet LB). The sections were then rinsed several times in distilled water. Some were counterstained with haematoxylin. All slides were mounted in glycerin jelly. Alkaline phosphatase activity was seen as a vivid red azo dye deposit.

2. Toluidine blue metachromasia

Paraffin sections of bones and kidneys were deparaffinized, hydrated, and stained for 30-60 seconds in 1:1000 aqueous solution of toluidine blue, dehydrated with acetone and cleared in xylol. The

nuclei of cells stained deep blue, the cytoplasm light blue, whereas cartilage matrix and mast cell granules stained deep violet. The preosseus layer of bone stained orthochromatically and became metachromatic when it calcified (Vincent, 1957).

6. TECHNIQUES FOR CHEMICAL STUDIES

A. Estimation of magnesium

The concentration of magnesium in the diet, in blood serum, and in bone was estimated by atomic absorption spectrophotometry according to the method of Hunt (1969).

1. In blood

The collected blood was allowed to clot for one hour, and centrifuged at 1200 rpm for 20 minutes. The separated serum was stored frozen (at -20°C) in polyethylene test tubes until magnesium determinations were carried out with the use of the EEL 140 Atomic Absorption Flame Photometer. At that time, an aliquot of 0.1 ml. of serum was diluted with top quality water to a volume of 10 ml. and read against a standard curve of magnesium from 0.2 to 0.8 ppm (0.2 to 0.8 ug/ml) in water.

2. In bone

The frozen bones were allowed to thaw before being placed in boiling distilled water for 10 minutes. Each bone was then scraped completely free of the softened muscle, periosteum, and ligaments with a sharp scalpel. To obtain accurate weights on the bone, each bone in a platinum crucible was dried to constant weight in an oven which was not allowed to exceed 100° . In selected

cases, the bone was split into three fragments consisting of shaft and the two condyles of the bone. All samples were ashed in a muffle furnace for 16 hours at 800°C. After the samples were cooled, 2 ml. of 5N redistilled HCl was added to each crucible which was then placed in a bath of boiling water for 30 minutes. The dissolved ash was quantitiatively transferred to a 10 ml. flask and made up to volume with top quality distilled water. The sample was further diluted with double distilled water and lanthanum chloride so that the final dilution (2000 times) contained 0.2 to 0.8 ppm magnesium, 0.5% $LaCl_3$, and 0.005N HCl. ($LaCl_3$ was added to suppress the enhanced absorption of magnesium seen in the presence of potassium, sodium, and phosphate in bone.) The sample was read against fresh reagent blanks and a standard curve prepared with 0.2 to 0.8 ppm magnesium, 0.5% $LaCl_3$ and 0.005N HCl.

SPECIFIC PROCEDURES

Experiment 1:

Effect of Magnesium Deficiency in young adult male Rats

Thirteen young adult (170-174 gms) male rats were divided into two groups. Seven were placed on the magnesium deficient diet (Mg-) for 20 days while the remaining six rats, to serve as controls, were given the supplemented magnesium (Mg+) diet. In this experiment, the diets and distilled water were supplied ad libitum. After 20 days all animals were sacrificed under chloroform anesthesia.

Experiment 2:

Effect of Magnesium Deficiency and Parathyroidectomy in male Rats.

A total of 142 male rats were used in this experiment. One hundred and twenty-six of these were parathyroidectomized when they weighed 100 gms. One week later all animals were divided into groups which received a magnesium deficient or a magnesium supplemented diet. On days 18 and 19 on the diet, one group of animals received twice daily intraperitoneal injection of 150 units of parathyroid extract (PTE). Twenty days after the start of the new dietary regimen some of the animals, including all the animals which had received PTE, were sacrificed; others were allowed to continue on the same diet for either 2, 6, 8, or 12 days. Some of the animals which had been fed the magnesium deficient diet for 20 days were now given a magnesium supplemented diet for the additional 2, 6, or 8 days. In addition, some of the rats which were allowed to survive beyond the standard 20 days were also injected intraperitoneally with 50 units of PTE each day beginning at day 21 until sacrifice. A summary of the treatment each group of animals received is given on the following page.

At the time of sacrifice on day 20 to 32, blood for magnesium and calcium determinations was collected after resection of the abdominal aorta. Some of the femura and tibiae were prepared for detection of the presence of alkaline phosphatase activity. The second femura and tibiae were subjected to chemical analytical magnesium, calcium, and phosphorus determinations.

Group	Number of Rats per Group	20-Day Diet	Number of Additional Days on Diet		IP. PTE[1] Injection
Intact					
I	9	Mg-	0		0
II	8	Mg+	0		0
Parathyroidectomized					
III	8	Mg-	0		0
IV	8	Mg+	0		0
V	7	Mg-	0		150U 2X daily for 2 days
VI	7	Mg+	0		150U 2X daily for 2 days
VII	6	Mg-	2	Mg-	0
VIII	8	Mg-	2	Mg+	0
IX	7	Mg-	2	Mg-	50U/day for 2 days
X	7	Mg-	2	Mg+	50U/day for 2 days
XI	7	Mg-	6	Mg-	0
XII	7	Mg-	6	Mg+	0
XIII	7	Mg-	6	Mg-	50U/day for 6 days
XIV	7	Mg-	6	Mg+	50U/day for 6 days
XV	7	Mg-	8	Mg-	0
XVI	7	Mg-	8	Mg+	0
XVII	7	Mg-	8	Mg-	50U/day for 8 days
XVIII	7	Mg-	8	Mg+	50U/day for 8 days
XIX	5	Mg-	12	Mg-	0
XX	6	Mg-	12	Mg-	50U/day for 12 days

[1]PTE was obtained from Eli Lilly and Co. (Canada) Ltd., Toronto, Ont.

Experiment 3:

Effect of Magnesium Deficiency and Thyroidectomy in young adult female Rats

A total of 57 female rats with an initial weight of 70-80 grams were used. Two weeks before transfer to an experimental diet, forty of the animals were thyroidectomized and the parathyroid glands were transplanted. The remaining seventeen rats were divided into two groups (I and II). As in Experiment 1, Group I (9 rats) received a magnesium deficient diet and Group II (6 rats) were fed the magnesium supplemented diet for 20 days. These served as unoperated control animals.

The thyroidectomized rats were also divided into groups which received either the deficient or the supplemented magnesium diet. In addition, some of the rats received replacement maintenance doses of either L-thyroxine[1] (Swanson, 1957; Grad and Hoffman, 1955) or DL-triiodothyronine[1] (Tata and Pitt-Rivers, 1959), or, as a control, the saline vehicle only. The replacement therapy was begun at the time when the animals were placed on the experimental diets. The treatment each group of animals received is given in the following table.

[1] L-thyroxin (T_4) and DL-triiodothyronine (T_3) were obtained as the sodium salts from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Group	Number of Rats per Group	Diet	0.1 ml Daily IP Injection
III	9	Mg-	0
IV	3	Mg-	0.9% NaCl
V	7	Mg-	3 ug L-thyroxin in alkaline 0.9% NaCl
VI	5	Mg-	20 ug DL-triiodothyronine in alkaline 0.9% NaCl
VII	5	Mg-	0
VIII	3	Mg-	0.9% NaCl
IX	4	Mg-	3 ug L-Thyroxin in alkaline 0.9% NaCl
X	4	Mg-	20 ug DL-triiodothyronine in alkaline 0.9% NaCl

The diets and distilled water were given ad libitum and the daily consumption of food and water by each animal was periodically recorded. After either 20 or 25 days on the experimental diet, those animals that survived to the end of the experimental period were sacrificed by severance of the spinal cord and resection of the jugular vein. The kidney and long bones were prepared for histological examination and serial sections were made of tracheas to assess the completeness of thyroidectomy.

Experiment 4:

Effect of Magnesium Deficiency and Ovariectomy in adult Rats

As a pilot experiment to assess the effect of ovariectomy on bone morphology, 2 rats (70 gms) were ovariectomized while their littermate served as an unoperated control. All rats received the standard

laboratory diet and tap water during the six weeks of the experiment. They were sacrificed under chloroform anesthesia. The long bones were prepared for microradiographic and histological examination.

A total of 49 rats were used in the ovariectomy-magnesium deficiency experiment. Thirty-four of the rats were ovariectomized when they weighed between 90 and 100 grams. The remaining fifteen served as unoperated controls. The unoperated animals were further divided into Groups I and II, the ovariectomized rats into Groups III to VIII. Six weeks later all animals were placed on the experimental diets. At that time, some of the ovariectomized rats began to receive large daily doses of 17-B-estradiol[1] (Singhal et al, 1967) in corn oil, while others were injected intramuscularly with the corn oil alone. The treatment each group of animals received is given in the table on the following page.

The diets and distilled water were given ad libitum. All animals were sacrificed on the 20th day of the dietary regimen. The uteri were examined to establish completeness of ovariectomy. The kidneys and the long bones were prepared for histological sections. In addition, alpharadiographs were prepared of the long bones.

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[1] 17-B-estradiol was obtained from Sigma Chemical Co., St. Louis, Mo. To 12.5 mg. estradiol was added 2 ml. absolute ethanol and 25 ml. Mazola corn oil. When the estradiol was completely dissolved, the ethanol was boiled off and another 25 ml. of corn oil was added.

Group	Number of Rats per Group	Diet	0.1 ml Daily IM Injection
Intact			
I	9	Mg-	0
II	6	Mg+	0
Ovariectomized			
III	5	Mg-	0
IV	6	Mg-	Mazola corn oil
V	11	Mg-	25 ug 17-B-estradiol in Mazola corn oil
VI	3	Mg+	0
VII	3	Mg+	Mazola corn oil
VIII	6	Mg+	25 ug 17-B-estradiol in Mazola corn oil

Experiment 5:

Effect of Magnesium Deficiency on Protein Synthesis in pair-fed young adult male Rats

Ten young adult (165-185 gms) male rats were divided into five pairs of comparable weights. One of each pair was fed a magnesium deficient (Mg-) diet ad libitum. The second of the pair was fed each day a magnesium supplemented (Mg+) diet in the quantity its pair on the deficient diet had consumed the previous day. On day 21 of this regimen all ten rats were each injected intraperitoneally with 2 ml. of an aqueous solution of 2-glycine-H^3. Each 2 ml. dose contained 1 mCi of labelled amino acid. Twenty-four hours later all rats were sacrificed under chloroform anesthesia. In addition to routine histological sections, autoradiographs were prepared of the long bones.

Addendum to Chemical Studies, page 41.

Calcium and phosphorus in blood serum and calcium in bone were determined according to the method of Hunt.

CHAPTER III

RESULTS

GENERAL

1. ANIMAL GROWTH RATES

The growth rates of animals during the periods of dietary regimentation is shown in graph form in Charts 1 - 4. In all experimental groups, the final weights of unoperated animals that received the magnesium deficient diet were significantly less than those of rats that received the supplemented diet. This was evident whether the animals were given diet ad libitum or were pair fed (Chart 1A and 1B). Under either condition, however, any divergence from the normal rate of weight gain was discernible in rats only after seven to ten days on the deficient diet. At this time, the slope of the growth curve became divergent from the normal and the rate of growth, although constant and continuous, decreased relative to the control.

The effects of surgical removal of various glandular tissues on the growth rate of rats on either the deficient or supplemented diet are shown in Charts 2 - 4. The effect of

each procedure on the growth rate will be recorded in the section under Specific Procedures.

2. CLINICAL SYMPTOMS

Preceding or coinciding with the beginning of impaired growth, other symptoms started to become evident in intact rats fed the magnesium deficient diet. The earliest symptom was a generalized erythema which was most pronounced in the pinnae of the ears. As the deficiency progressed, the ears became more edematous and stasis of the blood was common. An ataxis, aggravated by swollen erythematous paws, was observed in many of the animals. In addition, hyperemia of the proximal portion of the tail and desquamation of the surrounding skin was regularly noted. Occasionally, many of the symptoms became less intense or disappeared after 11 to 14 days. In some rats the symptoms returned after a time. Ruffled fur, accompanied in many instances by underlying skin lesions, or focal alopaciae were further common manifestations of the deficiency at this time. Most erythematous desquamated areas occurred close to the scruff of the neck, around the eyes, above the whiskers on either side of the nostrils, and on the undersurface of the jaw.

A feature common to all deficient intact rats was an increase in irritability and aggressiveness. The rats startled easily and were acutely sensitive to any disturbance, and most

particularly to noise. Occasional cardiac arrhythmias were also noted. In these experiments, intact rats were never seen to convulse. Late in the course of the deficiency, however, some of the rats in the parathyroidectomized group and in the thyroidectomized group that received hormone replacement reacted with convulsive seizures which culminated in death.

At autopsy, many organs appeared to have been affected by the deficient diet. Although the presence and the severity of the pathology differed from one animal to another, many of the rats exhibited one or another of the following changes in a decreasing order of frequency: enlarged kidneys with nephrocalcinosis; hemorrhages in intestines and thymus; enlarged thymus, heart, and spleen; white deposits on the surface of the spleen; and stippling of the surface of the liver. These changes were most frequent and most pronounced the younger the rats were when placed on the deficient diet (e.g. compare Experiment 3 with Experiment 4). This age-dependent-frequency relationship also held true for the bone changes to be described in the following sections.

SPECIFIC PROCEDURES

Experiment 1:

Effect of Magnesium Deficiency in Young Male Rats

A. Kidneys

The kidneys of the rats on the Mg(-) diet were generally larger than those from rats on the Mg(+) diet and contained variable amounts of calcareous deposits especially in the areas of cortico-medullary junction (Plates 1 and 2). The number of calculi were far in excess of those which occurred spontaneously in untreated animals on an adequate diet. Even at the gross level the presence of nephroliths was evidenced by the gritty surface of the sliced kidneys. Accompanying hemorrhages were common. A further common feature of these kidneys was an increase in the number of mast cells in the interstitium of the outer cortex and to a lesser extent the inner cortex of the kidney (Plate 3, Fig. B and D. Compare with Fig. A and C). The mast cells appeared to be most prominent around capillaries surrounding distal convoluted tubules but were not restricted to those areas. However, no mast cells were seen in the area of the cortico-medullary junction where nephroliths were found. The granules of the mast cells stained metrachromatically with toluidine blue and in many instances appeared dispersed in the cytoplasm of cells which were degenerating (Plate 4).

An additional effect of the Mg(-) diet on the kidney was an apparent increase in size of the cells of the macula densa and the juxtaglomerular cells of the juxtaglomerular apparatus. This is illustrated in Plate 5.

Occasionally, infiltration was seen mainly in the cortex, distending the spaces between convoluted tubules (Plate 6, Fig. A). Amorphous acidophilic casts were often present in many dilated tubules which showed no calcification (Plate 6, Fig. B).

B. Bones

In rats fed a Mg(-) diet for 20 days, the following changes from normal bone histology were noted in the long bones. In the epiphyseal plate, the rows of both small and large chondrocytes were decreased in number with the resultant reduction in thickness of the plate (Plate 7. Compare Figs. A and B). Although not consistently so, the small chondrocytes tended to be smaller than normal. The cartilage matrix was much less azurophilic suggestive of a depletion of the sulfated mucopolysaccharides. Generally, no apparent difference was noted in the number of hypertrophied cells, but there was a decrease in the calcifying matrix in the zone of provisional calcification. New subepiphyseal spicules were few in number and contained osteocytes and osteoblasts that were smaller than normal and elongated, and surrounding blood

vessels were markedly dilated. The trabeculae extending into the diaphysis were short, wide, and irregular compared with those in normal bones in which trabeculae were long, slender and parallel.

In the diaphysis, which generally was thinner in magnesium deficient rats, the matrix was more fibrillar than in the control rat bone and the osteocytes close to the periosteum were smaller and elongated when compared with the larger osteocytes of the bone from control rats. In addition, osteocytic resorption was generally decreased as demonstrated by alpha-radiography (Plate 7, Figs. C and D), although this could not be established with certainty because of the wide variations found in different areas of the bones of the rat.

Medullary bone of the trabecular type was present in the marrow cavity of some of the femura and tibiae of the Mg(-)-fed rats but was absent from the cavity of the bones of the rats on the Mg(+) diet. In addition, in 3 of the 7 animals on the Mg(-) diet, a periosteal desmoid defect, a hyperplasia consisting of fibroblasts interspersed with collagen fibers, was also present, and was localized mainly to the linea aspera of the femur (Plate 8). Many mitotic figures were observed. The center of the mass consisted of young bone of a type more primitive than that found in other regions of the diaphysis.

Experiment 2:

Effect of Magnesium Deficiency and Parathyroidectomy in Male Rats

A. Growth Rates

Throughout the experimental period, the rate of growth of parathyroidectomized rats was identical to that of intact animals (Charts 1A and 2A). As in experiment 1, growth was significantly reduced in all the animals fed the Mg(-) diet. When this deficient diet was replaced by a supplemented Mg(+) diet, the rats immediately began to gain weight at an accelerated rate (Fig. 2B), that is, at the same rate as animals on the control diet. The additional daily administration of 50 Units of parathyroid hormone (PTE) during the last days of the experiment had no effect on the weight gain pattern in either the magnesium deficient or supplemented rats (Chart 2B). In contrast, when the total amount of PTE (600 Units) was given in doses of 150 Units, twice daily, all rats on either diet lost weight.

B. Magnesium, Calcium and Phosphorus Concentration in Blood Serum and Bone

The effect of parathyroidectomy and magnesium deficiency on the magnesium and calcium concentrations in serum is shown in Table 1; the effect on magnesium, calcium and phosphorus concentration in bone is shown in Tables 2 - 4.

1. Serum magnesium

The serum magnesium concentration was approximately 2.2 meq/L in intact rats fed an adequate magnesium diet but fell to 0.5 meq/L in rats on a deficient diet for 20 days. These values did not appear to be altered significantly by the absence of the parathyroid glands. On the other hand, the replacement of the deficient diet with one containing adequate magnesium caused the serum magnesium concentration to approach its normal value within two days.

2. Serum calcium

Unlike the changes observed in serum magnesium, the serum calcium concentration control value of 5.54 meq/L remained unchanged in rats fed a deficient magnesium diet. As expected, parathyroidectomy caused a statistically significant fall in serum calcium concentration to 5.34 meq/L in rats fed an adequate magnesium diet. However, in rats fed a magnesium deficient diet, no significant fall was observed. On the other hand, when the deficient diet was replaced by one containing adequate magnesium, the serum calcium concentration in the parathyroidectomized animals again fell (5.19 meq/L) to below the control value (5.5 meq/L).

The administration of parathormone to parathyroidectomized rats significantly raised the serum calcium concentration to above normal values (to an average of

6.0 meq/L) in all animals whether on an adequate or deficient magnesium diet, but only when a large dose of 300 Units per day was given. The smaller dose of 50 Units per day appeared to have minimal effect.

During magnesium deprivation, when the serum magnesium fell to low levels, the serum calcium appeared to be slightly elevated above its normal values. With the replacement of adequate magnesium in the diet, the reverse occurred. The serum magnesium rose and the serum calcium fell to its control level.

3. Bone magnesium

In intact growing animals on an adequate magnesium diet, the magnesium concentration of femurs averaged 927 mg/100 gm ash weight (shaft, 925 mg; head, 890 mg; condyle, 967 mg - Table 2). These values did not change in rats that had been parathyroidectomized, but were lowered to 894 mg/100 gm ash weight (shaft, 887; head, 865; condyle, 926 mg/100 gm) in parathyroidectomized rats that received 300 Units parathormone.

In intact animals on a diet deficient in magnesium for 20 days, the magnesium concentration in femurs (average of all regions, Table 2) fell to 310 mg/100 gm ash weight, one-third of that found in rats on an adequate diet. In the absence of the parathyroid glands the value was only

somewhat higher (368 mg/100 gm ash), and did not alter appreciably in parathyroidectomized rats that received 300 Units parathormone.

The longer the rats remained on the deficient diet, the lower was the concentration of magnesium in the femurs, (e.g. after 26 days, 284 mg/100 gm ash; after 32 days, 246 mg/100 gm ash in parathyroidectomized rats - Table 3). However, soon after the replacement of the deficient diet with one containing adequate magnesium, the magnesium concentration in the femur began to increase to a value of 419 mg/100 gm ash after 2 days and up to a value of 505 mg/100 gm ash after 8 days. Parathormone administration increased these concentrations only slightly.

4. Bone calcium

In intact growing animals on an adequate magnesium diet, the calcium concentration of femurs averaged 39.7 g/100 gm bone ash (Table 2). Parathyroidectomy caused no appreciable change in total femur calcium concentration although there was a small decrease in calcium in the head of the femur. On the other hand, the administration of parathormone produced the expected fall in bone calcium in all regions of the femur. The average value after parathormone was 37.6 g/100 gm bone ash.

In intact animals, acute magnesium deficiency was accompanied by an apparent increase to 42.4 g/100 gm bone ash in bone calcium concentration. A lesser significant increase was observed in parathyroidectomized animals. Although the administration of parathormone to magnesium-deficient-parathyroidectomized rats led to a lowered bone calcium concentration (i.e., 38.5 g/100 gm bone ash), the values were greater than those found in the femurs of rats on an adequate diet and parathormone treatment (37.6 g/100 gm bone ash).

The replacement of the magnesium deficient diet by one adequate in magnesium did not affect the bone calcium concentrations during at least 6 - 8 days of replenishment (Table 3). Furthermore, during this time, 50 Units of parathormone per day also appeared to have no effect.

5. Bone phosphorus

In these studies, magnesium deficiency had no effect on the phosphorus concentrations in the femurs of parathyroidectomized animals (Table 4).

C. Kidney Weights

On an absolute weight basis, there was no significant difference between the weights of kidneys from control or magnesium deficient animals (Table 5). However, when the weights

were expressed as mg/kg body weight, the kidneys of rats on the magnesium deficient diet always weighed more than those on the supplemented diet. The combination of parathyroidectomy and magnesium deficiency produced the heaviest kidneys. Replacement doses of 600 Units PTE during the last days of the experiment neither increased nor reduced kidney weights. In contrast, the substitution of a magnesium supplemented diet for the magnesium deficient diet significantly reduced the kidney weights.

D. Gross Appearance of Bones

The effect of parathyroidectomy and magnesium deficiency on the growth in length of rat femura is shown in Table 6 and in Plates 9 - 14. During the interval investigated, parathyroidectomy had no effect on the growth in length of the long bones but may have slightly reduced bone growth at the linea aspera, the prominent longitudinal ridge on the middle third of the bone. On the other hand, in both intact and parathyroidectomized rats, the length of the bones of animals on the Mg(-) diet was always significantly shorter than of those on the supplemented diet. Furthermore, when the deficient diet was replaced by a Mg(+) diet, the bones of the animals on the replenished diet after eight days showed a greater increase in length than did those of the rats that remained on the deficient diet.

As in experiment 1, hyperplastic areas of bone, especially along the linea aspera of femura, were apparent in rats maintained on a Mg(-) diet (Plate 9, Figs.C and D). These areas were more pronounced and extensive in rats that were parathyroidectomized prior to being placed on the diet (Plate 10, Figs. C and D). The longer the animals were on the Mg(-) diet, the more dramatic was the hyperplasia (28 days, Plate 12, Figs. A and B; 32 days, Plate 14). The fragility of the bones was increased and the tumor-like areas were even more evident in the deficient animals which were administered parathormone (Plate 11, Figs. C and D; Plate 12, Figs. C and D).

When the deficient diet was replaced by a Mg(+) diet, the tumor-like areas in the bones of the rats on the replenished diet dramatically began to disappear. After eight days some bones showed much reduced signs of hyperplasia (Plate 13, Figs. A and B). However, the administration of parathormone during the period of replenishment somewhat retarded the gross disappearance of the hyperplasia (Plate 13, Figs. C and D).

E. Histology of Bones

1. Epiphysis

The epiphyseal plate in normally fed animals was unaltered by parathyroidectomy. However, following the administration of parathyroid extract, the epiphyseal plate in both magnesium deficient rats and controls became

enlarged. The large mucopolysaccharide secreting cells which had become small and less active during magnesium deficiency now appeared to have regained their activity. There was also an increase in the number of small cells arranged in irregular columns. On the underlying surface of the plate, the number and size of the bony spicules appeared to increase.

2. Diaphysis

Except for a possible greater accumulation of cells in the cambium of the long bones of parathyroidectomized animals, no significant changes in bone histology of diaphyses were noted between parathyroidectomized and intact rats on the diet adequate in magnesium. The administration of parathyroid extract to the parathyroidectomized animals on this diet produced only the expected increased resorption.

3. Linea Aspera

In the bones of parathyroidectomized rats on the Mg(-) diet, the hyperplastic areas of the subperiosteum consisted from outside in of subsequent layers of fibrous tissue, cartilage, and bone. The administration of parathormone to these rats appeared to alter the composition of the tumor-like masses by considerably reducing the cartilage while increasing the fibrous component (Plate 15).

Membrane bone of the trabecular type was also present in the cavities of the long bones of deficient animals administered parathormone but was absent from the cavities of parathyroidectomized animals.

F. Alkaline Phosphatase in Bones

The Gomori-Takamatsu and the Burstone methods for the detection of alkaline phosphatase activity gave essentially similar results (Plates 16 and 17), although false positive reactions for the enzyme was common with the Gomori method. In all groups studied, alkaline phosphatase activity was detected in the linings of the Haversian canals and in the endosteum and inner periosteum of long bone diaphyses. The maximal enzyme reaction occurred at the linea aspera over osteoblasts, adjacent fibroblasts, collagen fibers and osteocytes in the formative phase.

1. Magnesium Deficiency

In the presence of magnesium deficiency, the hyperplastic subperiosteal tissue components all stained positively for alkaline phosphatase.

2. Parathyroidectomy

In parathyroidectomized, magnesium-deficient rats where cartilage was a prominent component of the hyperplasia, the enzyme was also detected in both the precartilage

fibroblasts and in the large chondrocytes (Plate 17, Figs. A and C) as well as in the Havarsian canals, endosteum and periosteum (Plates 18 and 19).

3. Parathormone

Administration of parathormone to these parathyroidectomized, magnesium-deficient rats led to an expected increase in osteolytic osteolysis. Some of the osteocytes in these osteolytic areas were observed to give a positive reaction, indicating the presence of the enzyme (Plate 20, Fig. D).

4. Replacement of Magnesium in the Diet

Following the addition of magnesium to the diet of magnesium-deficient rats, the subperiosteal hyperplasia began to diminish (Plate 15, Fig. D). Although the alkaline phosphatase distribution in these areas remained the same, the intensity of the reaction appeared to decrease. After 6 days on this magnesium-replenished diet, the histology and pattern of alkaline phosphate distribution in the bones approached normal.

Experiment 3:

Effect of Magnesium Deficiency and Thyroidectomy in Female Rats

A. Growth Rate

A comparison of the weight gain of thyroidectomized rats on a Mg(+) and on a Mg(-) diet is shown in Chart 3. As expected, following thyroidectomy, the young rats gained significantly less weight than did the unoperated animals. After two weeks, due to temporarily adverse environmental conditions, the thyroidectomized animals suffered a transitory weight loss. This loss was recovered shortly in rats on a Mg(+) diet, although no additional weight gain occurred after this time. In rats fed a Mg(-) diet, however, the loss in weight was only minimally recovered so that the final weights of these rats were significantly lower than those on the Mg(+) diet. Further, the presence of magnesium in the diet allowed thyroidectomized animals, which had ceased growing, to begin to gain weight when administered daily replacement doses of either L-thyroxin or triiodothyronine. The slope of the new growth curve was the same as that of the curve for unoperated animals. On the other hand, the daily administration of replacement thyroxin or triiodothyronine in the absence of magnesium in the diet initially caused a weight loss in thyroidectomized rats. This was followed by only a slight increase in weight. However,

the slope of this curve was not unsimilar to that of the curve for unoperated animals on the Mg(-) diet.

Unlike intact rats which survived throughout the experimental period, some thyroidectomized rats succumbed during adverse environmental conditions (room temperature fell to below 70°C). Among the 7 that died, 3 had been on the Mg(+) diet, the remaining 4 on the Mg(-) diet. Some of the casualties in each group had also been administered either thyroxin or triiodothyronine.

B. Clinical Symptoms

1. Thyroidectomy

In addition to lack of weight gain, the thyroidectomized animals on a Mg(+) diet (Groups VII and VIII) exhibited the classical symptoms of thyroidectomy--hypothermia, intolerance to cold, rough fur, and lethargy. The surface blood vessels appeared to be constricted. The ears were pale. All these symtoms were absent in the thyroidectomized rats of Groups IX and X which were administered daily doses of thyroid hormone. These rats appeared completely normal.

2. Magnesium deficiency

Unlike unoperated animals on a Mg(-) diet, thyroidectomized animals on a deficient diet rarely showed the classical symptom of erythema. On the contrary, the ears

of these animals often appeared paler than those of thyroidectomized animals on the Mg(+) diet. However, other magnesium deficiency symptoms, such as rough fur and skin lesions, were common although decidedly less pronounced than in the unoperated animals. Furthermore, thyroidectomized animals on the deficient diet appeared more irritable than did the rats on the Mg(+) diet, but less so than the unoperated rats on the deficient diet.

C. Kidney Morphology

As expected, thyroidectomized rats on a Mg(+) diet had considerably smaller kidneys than did intact rats, and, as in intact animals, the kidneys of thyroidectomized rats on a Mg(-) diet were generally larger than those of rats on a Mg(+) diet. However, in the absence of the thyroid gland, the kidneys of rats on the Mg(-) diet did not contain the large number of granulated mast cells seen in intact animals.

The effect of both thyroid hormone (Tx) and calcitonin (CT) deficiencies on the incidence and severity of magnesium-deficiency induced nephrocalcinosis is shown in Table 7. As in experiment 1, all the kidneys of unoperated rats on the Mg(-) diet contained nephroliths. In these animals, the nephrocalcinosis was extensive (Plate 21, Fig. B). In the animals that had been thyroidectomized before being fed the Mg(-) diet the nephrocalcinosis was considerably less extensive

(Plate 21, Fig. C). The number and size of nephroliths was significantly reduced although the same cortico-medullary areas were involved. In contrast, in those thyroidectomized animals in which thyroid hormone replacement was given together with the Mg(-) diet, no nephroliths were found (Plate 21, Fig. D). Congestion of the venae rectae, however, was not uncommon in this group.

D. Bone Morphology

1. Thyroidectomy

Thyroidectomy resulted in the expected decrease in skeletal growth of rats on either an adequate or magnesium-deficient diet. In the epiphyses of the long bones of thyroidectomized rats on an adequate magnesium-supplemented diet, the cartilage plate appeared somewhat narrower than the normal (compare Plate 22, Fig. A with Plate 23, Fig. A). The transverse diameter was smaller, and calcification was irregular. The zone of provisional calcification was markedly reduced. Vascularization of this abnormal cartilage was very much retarded. In general, the "maturation" of the bone was retarded.

In thyroidectomized rats to which 3 ug L-thyroxin or 20 ug DL-triiodothyronine was administered daily (Groups IX and X), the long bones did not achieve the length of those of unoperated rats (Group II), but they were

significantly longer than in thyroidectomized untreated rats (Groups VII and VIII). The epiphyses of these hormone-treated thyroidectomized rats (Plate 23, Fig. C; Plate 24, Fig. C) appeared similar to those of normal rats (Plate 22, Fig. A; Plate 24, Fig. A); although fewer and smaller osteoblasts lined the trabeculae. The primary and secondary spongiosa were well developed and spicules were long and regular. Vascularization had returned to normal.

2. Magnesium Deficiency--Epiphysis

As in intact animals, magnesium deficiency was manifested in thyroidectomized rats (Groups III and IV) by narrowing of the epiphyseal growth zone (Plate 23, compare Figs. A and B). The result, therefore, was a plate with fewer proliferating and hypertrophic chondrocytes (as in intact deficient animals), very few degenerating cartilage cells and calcifying cartilage, and minimal new bony spicule formation. Much of the cartilage matrix was unresorbed. The longer the rats were maintained on the Mg(-) diet (e.g. 25 days), the more sparse was both the primary and secondary spongiosa. In some cases, very little secondary spongiosa was present in the axial portion of the bone, the medulla of the bone being filled mainly with rather sparse bone marrow elements surrounding occasional wide,

plate-like spicules. The area of primary spongiosa was extremely narrow, made up of a few thick but very short spicules. Osteoblasts lining the spicules were few, thin, and elongated. Surrounding blood capillaries were generally dilated.

In magnesium-deficient, thyroidectomized rats to which thyroxin or triiodothyronine was administered daily (Groups V and VI), the epiphyseal plate of the long bones generally resembled that from an intact rat on a magnesium-deficient diet, although osseous spicules were longer and more numerous (Plate 23, Fig. D; Plate 24, Fig. D). Considerably more primary and secondary spongiosa was present than in bones of untreated thyroidectomized magnesium-deficient rats (compare Figs. B and D, Plate 23).

3. Magnesium Deficiency--Diaphysis

In intact rats on an Mg(-) diet, the diaphyses often were narrower than in rats fed the control diet (Plate 25, Figs. A and B), and not much wider than those of thyroidectomized rats fed a control diet (Plate 25, Fig. C).

In intact rats on the Mg(-) diet, 8 of the 11 rats showed periosteal hyperplasia mainly of the fibrous type. Medullary bone was present in 2 of the 8 rats. And, as in experiment 1, although difficult to quantitate, there was a subjective impression that osteolytic osteolysis was reduced in 7 of the rats.

In thyroidectomized rats on a Mg(-) diet, 6 of 12 rats showed some periosteal hyperplasia while 2 of the 12 showed medullary bone formation.

In thyroidectomized rats administered thyroxin or triiodothyronine, 7 of the 11 rats contained "tumors" similar to those seen above in the thyroidectomized untreated rats. Again, 2 of the 11 rats contained some medullary bone in the femura and tibiae.

Experiment 4:

Effect of Magnesium Deficiency and Ovariectomy in young adult Rats

A. Growth Rate

The effect of ovariectomy and magnesium deficiency on the weight of rats is shown in Chart 4. When the rats were fed a normal adequate stock diet (day 1 - 32), ovariectomized rats continued to gain significantly more weight than did unoperated animals. When the normal diet was replaced by a Mg(-) diet (day 33 - 52), unoperated rats continued to gain weight (curve 6), but at a slower rate than their controls on a Mg(+) diet (curve 5). In contrast, ovariectomized animals on a Mg(-) diet continued an accelerated growth rate for 10 days, then gained no further weight (curve 2), while the ovariectomized control rats on the Mg(+) diet continued the accelerated growth

rate (curve 1). The administration of large doses of estradiol to rats (day 33 - 52) caused a prompt loss in weight in the ovariectomized groups of rats on either diet. Paradoxically, the rats on the Mg(-) diet (curve 3) lost less weight than did those on the Mg(+) diet (curve 4). Although all weight loss was subsequently regained, the daily increment of weight gain was small in the animals on the Mg(-) diet and only slightly greater in the animals on the Mg(+) diet.

B. Clinical Symptoms

1. Ovariectomy

Except for the obvious weight increase, ovariectomized animals appeared normal.

2. Magnesium deficiency

All the ovariectomized animals on the Mg(-) diet (Groups III and IV) exhibited all the classic symptoms of magnesium deficiency. The symptoms appeared earlier and were uniformly more pronounced in the ovariectomized than in the intact animals on the same diet. In contrast, the magnesium-deprived ovariectomized rats which were injected daily with large doses of estrogen (Group V) showed no external symptoms whatsoever, whereas rats which were injected daily with the oil vehicle only all developed symptoms.

C. Kidney Morphology

Ovariectomy did not appear to alter kidney morphology to any extent. However, following the administration of large doses of estradiol to the ovariectomized rats, the blood vessels of the cortex and medulla became engorged. Atrophy and focal areas of degeneration were also present in some tubules (Plate 26, Figs. B and D).

As in the previous experiments, the Mg(-) diet induced enlargement and calcinosis of the kidneys of intact rats. In ovariectomized rats, half the number of animals on the Mg(-) diet contained calcareous deposits. However, the extent of calcification was less than in intact animals (compare Plate 27, Fig. C with Plate 1, Fig. B). Occasional hemorrhages and focal necrotic tubules were also seen. Following the administration of large doses of estradiol to ovariectomized rats on a Mg(-) diet, the blood vessels of the cortex and medulla were greatly distended with blood (Plate 27, Fig. B) and occasional cysts were scattered throughout the cortex into the medulla. Except in two rats, the cortico-medullary areas and the outer medulla of all kidneys contained large numbers of calcareous deposits, many of which completely obliterated the lumen and destroyed the epithelium of the tubules (Plate 27, Fig. D). Although the ascending limb of Henle seemed to be the main site of these calcifications, the descending limb was to a lesser degree also affected.

D. Bone Morphology

1. Ovariectomy

The long bones of young growing rats that had been ovariectomized for 2 months were longer and more delicate, but their diaphyseal width was narrower, than the long bones of intact control rats. The epiphyseal growth zone was wider, that is, their epiphyseal cartilages were thicker, containing an increased number of chondrocytes, but there was often a decreased number of osseous spicules in the metaphyses of femurae and proximal tibiae (compare Figs. A and C, Plate 28). These tended to be irregular.

In ovariectomized rats that received daily estradiol injections (25 ugms) during the second month post-surgery (Group VIII), the long bones were similar in length to the control intact rats at time of sacrifice. However, the epiphyseal cartilages were significantly narrower (about 50% of control thickness, Fig. E, Plate 28) than those of both the intact control and the untreated ovariectomized rats. The intracartilagenous matrix was increased in amount. It appeared more dense and often showed evidence of sclerosis. In some of the rats in this Group VIII, there was also an increase in the number of coarse interlacing osseous spicules in the metaphyses. In all these animals, regardless of

treatment, unresorbed cartilage was present in the metaphyses and extended into the diaphyseal bone (Plate 29). This, however, was most pronounced in the rats that were administered estradiol. Many of the spicules were confluent, sometimes forming a continuous band parallel to the plate.

In all animals on the magnesium supplement diet, the plate stained fairly intensely for metachromasia with toluidine blue.

2. Magnesium deficiency

As in previous experiments, the epiphyseal plates of all rats fed the Mg(-) diet were generally reduced to about 50% of the control thickness (Plate 28, Figs. B, D, and F; Plate 30, Fig. B), although the plates of ovariectomized rats were sometimes wider and those of estradiol-administered rats were often even narrower than those of intact animals. Metachromatic staining of the plates was markedly reduced.

Within the magnesium deficiency groups, the number, size, and shape of spicules in both intact and ovariectomized rats was fairly comparable. However, in rats administered estradiol, a greater number of unresorbed spicules was present, although there was a degree of variation in this group. Osteoclasts were relatively scarce. Alpharadiographs showed that metaphyseal trabeculae had a greater matrix

content than did the spicules closer to the plate.

In the diaphyses of all these animals, large areas of unresorbed cartilage was evident.

Endosteal bone formation was not a common feature in this experiment. Whereas 5 of the 8 intact rats on the Mg(-) diet contained endosteal bone, only one ovariectomized rat on the Mg(-) diet showed any evidence of its formation (Plate 31). Also, only one-quarter of the ovariectomized animals showed some periosteal hyperplasia.

E. Tooth Morphology

The teeth of rats on the magnesium deficient diet occasionally were loose and the surface of the enamel was rough. The alveolar bone was considerably more fragile than normal and was easily fractured.

1. Enamel organ

In these experiments no striking effects of the magnesium deficiency were noted in the enamel organ although in some animals ameloblasts tended to be lower columnar than normal. In estradiol treated rats there was a tendency for the ameloblasts to appear shrunken.

2. Dentin

Magnesium deficiency had the most profound effects on dentin formation. Many odontoblasts appeared to be

degenerating as evidenced by pycnotic nuclei and shrunken cytoplasm. The odontoblast layer became irregular and occasionally individual odontoblasts apparently failed to recede after laying down matrix. This was most common in areas of dentin adjacent to enamel. The width of the predentin was usually increased and the matrix did not always become calcifiable. The presence of "alternate bands" (Yamane and Singer, 1953) or striations was noted in the erupting teeth of most of the ovariectomized rats on the magnesium deficient diet (Groups III and IV, Plate 32, Fig. B), but not in the teeth of rats in any other group including the ovariectomized-estradiol-supplemented magnesium-deficient rats (Plate 32, Fig. D). Alpharadiographs of the sections of the teeth revealed that these alternating bands varied in density (Plate 33), suggesting alternate areas of greater and lesser dentinal matrix content.

In the hormone supplemented animals, very little new dentin formation was apparent. The odontoblasts were very irregular and were mostly degenerating.

In all rats on the Mg(-) diet the blood vessels of the pulp were greatly dilated.

Experiment 5:

Effect of Magnesium Deficiency on Protein Synthesis in the Bones of Young Intact Male Rats

A. Growth Rate

The curves for the growth rates of rats on the Mg(-) diet and for their pair-fed controls on the Mg(+) diet are shown in Chart 1, Fig. B. Although the quantity of food ingested by both groups was identical, the rats on the Mg(-) diet gained significantly less weight than those on the supplemented diet. The final weight of the rats on the deficient diet was 236 gms.; that of the pair-fed controls was 284 gms.

B. Clinical and Histologic Findings

As in the previous experiments, the rats on the Mg(-) diet exhibited all the symptoms of the deficiency, and periosteal hyperplasia was evident in the bones. The length of the femura and tibiae was less in the deficient rats than in the controls, and the epiphyseal plates were narrower.

C. Autoradiography of Bones

1. Cartilage

Twenty-four hours after the administration of H^3-glycine as a marker for new protein synthesis, the label was found to be diffusely concentrated throughout the cartilage matrix of the proliferating zone of epiphyseal

cartilage in bone of the normal rats on the Mg(+) diet. The greatest, although not sole, accumulation appeared in association with the azurophilic staining component of the cartilage and the capsular matrix (Plate 34, Fig. A). The chondrocytes were minimally labeled.

In the rat fed the Mg(-) diet, both the large and small chondrocytes were reduced in number and in size, and the matrix stained poorly with the azure component of the stain (Plate 34, Fig. B). As in the control, the chondrocytes did not contain significant amounts of glycine-H^3 label. However, the cartilage matrix did incorporate the labeled amino acid, although generally to a lesser extent (as evidenced by the number of black stains) than did the matrix of the control.

2. Bone

Twenty-four hours after the administration of H^3-glycine, the prebone (osteoid) layer of some subepiphyseal spicules of bone were labeled in the rats on the magnesium-supplemented diet (Plate 35, Fig. A). In some areas the reaction band was found in the bone matrix itself at a distance from the osteoblasts. In the rat on the magnesium-deficient diet, no discernible autoradiographic reaction band was noted in spicules in preparations which were exposed and developed at the same

time as the section of the magnesium-supplemented control (Plate 35, Fig. B).

In the conical portion of the shaft of bone from rats on a magnesium-supplemented diet, an autoradiographic reaction band was prominent in the prebone layer close to osteocytes lining the endosteum (Plate 36, Figs. A and C). In magnesium-deficient rats, there were areas of this region where osteocytes appeared small and inactive. These areas (e.g. Plate 36, Figs. B and D) gave no autoradiographic reaction.

In the diaphyses of the rats on either the Mg(-) or Mg(-) diet, the periosteal surface of the bone was more heavily labeled than was the endosteal. In all cases, the prebone close to the osteoblasts showed an intense band of label, while the osteoblasts all contained lesser uniform numbers of grains. No striking differences in the labeling were seen between the bones of the magnesium-deficient and the control rats.

In the magnesium-deficient rat, the hyperplastic periosteal area was also labeled, but to a lesser extent than the prebone. The osteoblasts, fibroblasts, and the collagen fibers contained the most label, while cartilage components contained the least.

CHAPTER IV

DISCUSSION

Protein Synthesis

The results of these studies confirm the previously reported observations on the skeletal effects of magnesium deficiency (Duckworth et al, 1940; Bélanger, 1958; Bernick and Hungerford, 1965; Clark and Bélanger, 1967; Smith and Nisbet, 1968; Hunt, 1971; Hunt and Bélanger, 1972). In the long bones of all growing animals, the most important effect relating to growth was the decreased size of the epiphyseal plate. The diminution appeared to be a result of a decreased mitotic rate in chondrocytes of the proliferative zone. Accompanying this mitotic depression was an apparent decreased secretion of mucopolysaccharides (evidenced by decreased azurophilia) and a decreased secretion of protein (demonstrated by the decreased autoradiographic reaction obtained with the protein precursor, H^3-glycine, Plate 34. Localization of glycine was similar to that reported by Carneiro and Leblond, 1959). The decreased ability of the magnesium-deficient rat to incorporate the labeled glycine into bone protein was presumably even greater than illustrated in Plates 34 - 36 for the following reason. In

experiment 5 as in other experiments, during the 21-day dietary regimen, intact magnesium-deficient growing rats had gained approximately 20% less weight than did their pair-fed controls. Regardless of weight, however, each rat had been injected with 1 mCi H^3-glycine. In effect, magnesium-deficient animals received 20% more labeled glycine per kilogram body weight than did control rats. Even with this increased administered dose, magnesium-deficient rats incorporated less of the labeled glycine into bone protein than did the control animals. This held true mainly in the epiphyseal and metaphyseal regions (Plates 35 and 36).

These results are compatible with the decreased H^3-proline incorporation seen by Trowbridge and Seltzer (1967) in alveolar bone of magnesium deficient animals.

Magnesium is known to activate the enzymatic reaction between amino acids and ATP to yield aminoacyl adenylates and pyrophosphate (Lehninger, 1950) and to contribute to the stability of ribosomes (quoted by Trowbridge and Seltzer, 1967). Its absence, therefore, could result in an inhibition of protein synthesis. Magnesium may also be involved in synthetic reactions relating to mucopolysaccharides (glycosaminoglycuronoglycans). In this regard, studies with $S^{35}O_4$ (Bélanger, 1958; Trowbridge and Seltzer, 1967) and with metachromatic staining procedures (Bernick and Hungerford, 1965; Clark and Bélanger, 1967; Trowbridge and Seltzer, 1967) have established that chondroitin sulfate production is decreased

in the bones of magnesium deficient rats. It appears, therefore, that a magnesium deficiency suppresses the metabolic activities of cells capable of synthesizing a calcifiable matrix. Any change in local concentration of calcium and phosphate ions may be of secondary importance.

Clinical Symptoms

In agreement with many previous studies (see reviews by Walser, 1967; Wacker and Parisi, 1968; Trowbridge and Jenks, 1968; and Gitleman and Welt, 1969), all growing intact magnesium-deficient rats showed the characteristic signs of magnesium deficiency (i.e., hyperemia, edema of the extremities, neuromuscular irritability and skin lesions). And as demonstrated by Watchorn and McCance (1937), Smith and Field (1963), and Hunt (1971), the onset and severity of the symptoms were age dependent, the young, rapidly growing rats (experiment 3) consistently developing hyperemia 3 - 4 days earlier than the more mature animals (experiment 4). However, the absence, presence, or excess of various hormones greatly modified the response to the magnesium deficiency. For example, in thyroidectomized rats (experiment 3), no hyperemia, edema, or skin lesions were evident throughout the duration of the experiment, whereas in ovariectomized rats (experiment 4), symptoms were produced earlier than normal. In this regard, it should be noted that thyroidectomized animals ceased to grow and gained little

or no weight (Chart 3), while ovariectomized rats gained weight at a faster rate than normal (Chart 4). Since "clinical symptoms can only be produced in the rapidly growing animal" (Widdowson and Dickerson, 1964), the absence of symptoms in thyroidectomized animals can be explained. It is also noteworthy that ovariectomized rats administered large doses of estrogen were symptom-free for the duration of the experiment. These animals, too, gained very little weight during the 20 days on the magnesium-deficient diet (Chart 4). (A reduced rate of growth in animals receiving estrogen is expected (Bernick and Ershoff, 1963; Ferguson and Hartles (1970).) Symptoms in intact rats were severe, but in ovariectomized-estrogen-treated rats, absent. In a recent follow-up experiment, in collaboration with Bélanger and Guertin (unpublished), it was observed that the administration of large doses of estrogen to intact rats delayed, but did not completely prevent, the onset of clinical symptoms of magnesium deficiency. These observations are in agreement with those of Whitfield and Tidball (1968) with ovariectomized rats. In these two sets of experiments the rats, when placed on the dificient diet, were considerably younger (100 gms) than were the rats in the present experiment 4 (200 gms).

It is unfortunate that serum magnesium concentration was not determined in these animals. Although serum magnesium concentration is not a reliable index of the extent of magnesium deficiency in most species (Walser, 1967), in the rat, hypomagnesemia appears to vary directly with the dietary intake (Hunt, 1971).

If the rats were, in fact, truly magnesium deficient, one possible explanation for the apparent absence or delay of hyperemia in estrogen-treated, magnesium deficient rats may derive from the sparing action of estrogens on mast cells. Normally, following injury, mast cells degranulate, releasing histamine and phosphatase into the perivascular ground substance. The result is increased permeability and vasodilation. Conjugated estrogens inhibit the breakdown of mast cells (Schiff and Burn, 1961), decrease the permeability of the ground substance (Asboe-Hansen, 1966), and thus increase capillary strength. In magnesium-deficient animals, there is an increased early breakdown of mast cells (Bélanger et al, 1957; Bois et al, 1960; Bois, 1968). Estrogens may delay the breakdown.

Renal Studies

The characteristic renal lesions associated with magnesium deficiency (Schneeberger and Morrison, 1965) were observed in all deficient intact rats (Plates 1 and 2). In these, the extent of calcification usually varied with the length of duration of the deficiency. However, as noted by others, hormonal conditions

superimposed on the deficiency altered the extent of the lesion when it was present, but not its site. It is also noteworthy that the extent of calcification bore no relation to the severity of clinical symptoms. For example, the symptom-free, estrogen-treated ovariectomized rats consistently had more extensive lesions than did the symptom-ridden, untreated animals on the same deficient diet. It should be pointed out that whereas in this instance estradiol alone caused renal abnormalities, it did not stimulate the production of calcifications described by Geary and Cousins (1970).

In experiment 3 (Table 7), the finding that thyroxin prevented the renal deposition of calcium resulting from magnesium deficiency is in agreement with the reports by Forbes (1965); Meyer and Forbes (1968); Jacob and Forbes (1970); Reeves and Forbes (1971), 1972). In the same experiment is the paradoxical finding that calcitonin appears to increase the incidence of kidney calcification. According to Table 7, in the presence of parathormone, calcitonin, and thyroxin, the magnesium deficiency was accompanied by extensive calcification of the kidney. In the absence of calcitonin and thyroxin, fewer, less extensive lesions were present, whereas in the absence of calcitonin only, no lesions occurred.

It is well known that the renal mineral accumulation in magnesium deficient rats requires the presence of the parathyroid

gland, and that parathyroidectomy prevents the increased concentration of calcium, phosphorus, and to a lesser extent, magnesium, in the kidney. Thus, the incidence of calcification of the kidney is decreased after parathyroidectomy. In experiment 3, the animals were thyroidectomized so as to allow the parathyroids to remain. Since the serum calcium was not monitored in these animals, the possibility exists that the parathyroid tissue was removed by the surgery or had atrophied in some animals. This might account for the fewer animals with lesions. Against this possibility is the fact that these animals showed no signs of parathyroid hormone insufficiency even though they were allowed to drink only distilled water.

Calcitonin has been shown to have a phosphaturic and calciuric effect (Aldred, 1970, quoted by Copp, 1971) and may act in the same direction as parathyroid hormone in producing kidney calcification. At present, the observations of Gabbiani et al (1967, 1968) that calcitonin in fact inhibits the morphologic lesions caused by parathyroid extract overdosage, refutes this view. Because of this discrepancy, further study, to assess the renal effects of large doses of pure calcitonin administered to magnesium-deficient rats, is indicated.

Teeth

In these studies, as in those reported by Trowbridge et al (1971) and others, the dental hard structures were severely affected by the absence of magnesium in the diet. The most striking feature was the presence of a type of dentinal striation which appeared to be unique to this condition. The alpharadiographic results of this study and the histochemical data of Trowbridge et al (1971) suggest that faulty matrix is produced which is subsequently irregularly calcified. And, as in the studies by Irving (1940) and by Trowbridge (1971), trapped odontoblasts within the synthesized matrix was observed.

The absence of these striations in the estrogen-treated, magnesium-deficient animals in experiment 4 was unexpected. One possible explanation for this phenomenon is that the animals were not in a rapid state of growth, and were, therefore, not as severely magnesium deficient (Widdowson and Dickerson, 1964). The epiphyseal plate of the long bones of these animals could not be used as a reliable index of the extent of the deficiency since both magnesium deficiency and estrogen administration causes a reduction in the thickness of the plate, which in this case was markedly reduced. It appeared from the histological studies, however, that dentin formation was greatly retarded in these rats and that little new matrix was produced. To establish that the absence of the striations is due to complete lack of growth of

dentin and not to a protective effect of estrogen, further studies are indicated. Fluorochromes, such as used by Krook et al (1970) and Trowbridge (1971), administered during the experimental period, would help shed light on the problem.

Hyperplastic Subperiosteal Lesions

The results of these studies also confirm the reported observations of Hunt and Bélanger (1972) and of Bélanger, Hunt, and Narbaitz (1972) on the presence of subperiosteal hyperplastic lesions at the femural linea aspera of magnesium deficient rats. The parathyroid hormone appears to influence the quality and extent of the lesion (Plates 9 - 15) more than any of the other hormones examined. In these studies, no significant effects of thyroxin, calcitonin, or estradiol on lesion production could be established with certainty. Unpublished studies by Bélanger and Hunt have shown that the incidence and size of the tumoral mass greatly increases the longer the animals are allowed to remain on the magnesium deficient diet. It is suggested, therefore, that by extending the experimental period to well beyond the 20 days of this experiment, more significant data as to the effects of the various hormones on the lesion might be obtained in future experiments.

Medullary Bone Growth

The administration of estrogen did not produce a significant increase in medullary bone formation in the present experiments. In these and especially in the unpublished recent experiments in collaboration with Bélanger and Guertain, it appeared that untreated magnesium-deficient rats contained more medullary bone than did the estrogen treated animals. At this time no explanation for this finding can be offered.

In the metaphysis, the length of the unresorbed spongy bone depends on the dose of estrogen, the period over which it is administered, and the rate of growth of the animal (Lindquist et al, 1960). In experiment 4, the animals already weighed over 200 grams before any estrogen was administered. In any case, in the absence of estrogens during a magnesium deficiency more medullary bone appeared to have been produced than in the presence of estrogens. But, as has been suggested earlier, the estrogen-treated animals may not have been effectively magnesium-deficient. The problem, therefore, needs further investigation.

Conclusion

Hormones influence the growth of bones and teeth under normal conditions and modify their growth during magnesium deficiency. Parathyroid hormone aggravates and increases the effects of the

deficiency by inducing osteolysis with the subsequent release of bone magnesium stores. Since magnesium is an important enzyme activator, its lack is probably responsible for enzymatic malfunction in many synthetic reactions occurring in bone. Other hormones also modify bone growth during magnesium deficiency, but the mechanism by which this occurs is largely unknown.

CHAPTER V

SUMMARY

1. The literature on the influence of various hormones on the growth of bones and teeth in normal magnesium-deficient animals was reviewed. Special reference was made to parathormone, thyroxin, calcitonin and estradiol. Also included in the review was the subject of renal calcinosis as it relates to magnesium deficiency and hormonal status.

2. Five groups of experiments were performed on a total of 328 growing rats of various ages and hormonal status, as follows: a) male and female intact rats (experiments 1 - 5); b) male parathyroidectomized rats (experiment 2); c) female thyroidectomized rats (experiment 3); and, d) femal ovariectomized rats (experiment 4).

3. Experimental rats in each group were fed a magnesium-deficient diet containing 5 - 12 ppm magnesium while their controls received a diet supplemented by 2,500 ppm magnesium. Beginning with day 1 of the dietary regimen, some animals in each subgroup (c and d) were administered daily doses of hormone. In experiment 3, rats received a replacement dose of 3 ug L-thyroxin; while rats in experiment 4 were given large doses of estradiol (25 ugms).

On the 18th and 19th day of the regimen or beginning with the 21st day, parathyroidectomized rats in experiment 2 were injected with parathyroid extract. Most animals were sacrificed after 20 days on the diet, the remaining rats, after 22, 25, 26, 28, or 32 days. Sections of the long bones, jaws, and kidneys were stained with hematoxylin-phloxine-Orange G, Wright's stain, and toluidine blue. Alpharadiographs were prepared of the demineralized hard structures in all but the parathyroidectomized rats. In these animals, cross sections of the diaphyses were incubated and stained for alkaline phosphatase activity. In experiment 5, rats on a magnesium deficient diet for 20 days and their pair-fed controls were injected with 1 mCi glycine-H^3 twenty-four hours before they were sacrificed. Autoradiographs were prepared of the long bones.

4. Clinical findings, clinical pathology of bones and kidneys, and chemical measurements of magnesium, calcium and phosphorus on the bones from normal and magnesium deficient rats were correlated with findings as reported in the literature.

5. The onset and severity of the characteristic signs of magnesium deficiency (hyperemia, edema of the extremities, neuromuscular irritability and skin lesions) varied with age. The symptoms appeared earlier and with greater severity in rapidly growing intact young rats. In contrast, magnesium-deficient animals that had ceased to grow, for example, thyroidectomized rats, showed none of the typical clinical signs of magnesium deficiency. Similarly, ovariectomized rats treated with large doses of estradiol showed no weight gain and subsequently no clinical magnesium-deficiency symptoms. However, magnesium-deprived ovariectomized rats, in the absence of estradiol, developed severe symptoms earlier than control animals.

6. Autoradiographs of the long bones of magnesium-deficient rats injected with H^3-glycine demonstrated the incorporation of less label into the cartilage plate and into spicules of epiphyses and metaphyses than did normal rats, thus indicating reduced protein (collagen) synthesis in magnesium-deficient rat bone.

7. In all experiments, regardless of hormonal treatment, the epiphyseal plate was reduced in size as a

result of decreased mitotic rate in chondrocytes of the proliferative zone and decreased secretion of mucopolysaccharides and protein. This resulted in a decrease in the growth of bone. Generally, there appeared to be a decrease in osteocytic osteolysis.

8. Subperiosteal hyperplastic lesions occurred in some rats in each of the five experiments. The highest incidences and largest lesions occurred in the parathormone-treated rats. The quality of the lesion was modified in parathyroidectomized rats, as shown by Hunt and Bélanger. Neither thyroxin, calcitonin, nor estradiol seemed to have any significant effect on the incidence or quality of the lesion, at least during the 20-day period of the experiment.

9. Medullary bone of the trabecular type was produced in intact magnesium deficient rats and in parathormone treated rats but not in parathyroidectomized animals. Ovariectomy did not alter the incidence of medullary bone formation whereas large doses of estrogens to ovariectomized rats appeared to delay the occurrence.

10. Characteristic striations in the dentin of teeth occurred in magnesium deficient rats. Such striations

were not present in magnesium-deficient rats treated with large doses of estradiol. The absence of these striations may possibly have been due to either lack of formation of any new dentin or to a protective effect of estrogen.

11. The characteristic calcified renal lesions associated with magnesium deficiency were observed in all deficient intact rats, but the extent of calcification bore no relation to the severity of clinical symptoms. Estrogen-treated ovariectomized rats had more extensive lesions than untreated animals on the same magnesium deficient diet. Thyroxin prevented nephrocalcinosis, but the absence of calcitonin also appeared to inhibit calcifications. This finding warrents further investigation.

12. It is concluded that hormones influence the growth of bones and teeth under normal conditions and modify their growth during magnesium deficiency.

CHARTS

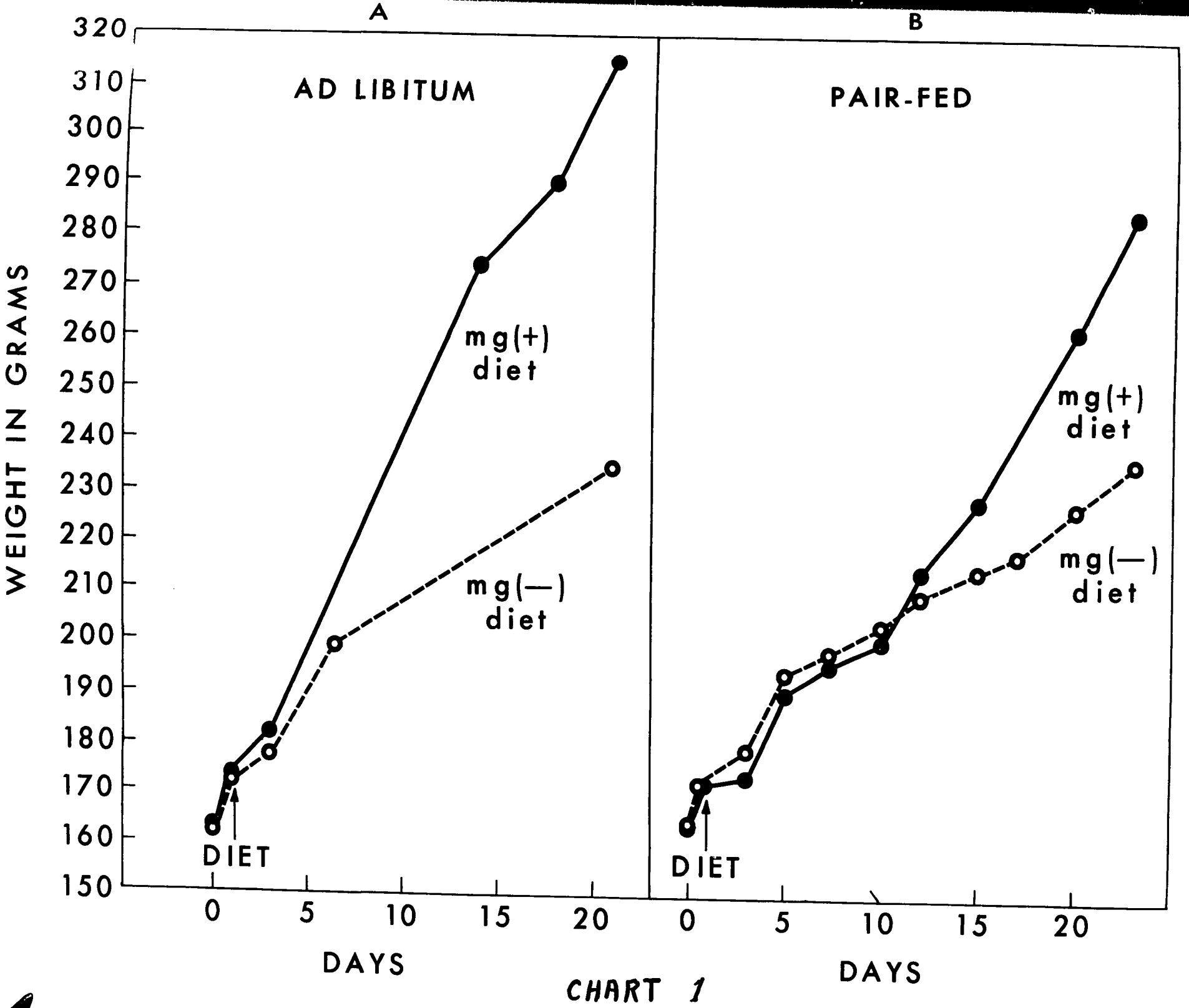

A
B
AD LIBITUM
PAIR-FED
WEIGHT IN GRAMS
320
310
300
290
280
270
260
250
240
230
220
210
200
190
180
170
160
150
mg(+) diet
mg(—) diet
DIET
0
5
10
15
20
DAYS

CHART 1

EFFECT OF PARATHYROIDECTOMY AND MAGNESIUM DEFICIENCY ON THE WEIGHT OF RATS

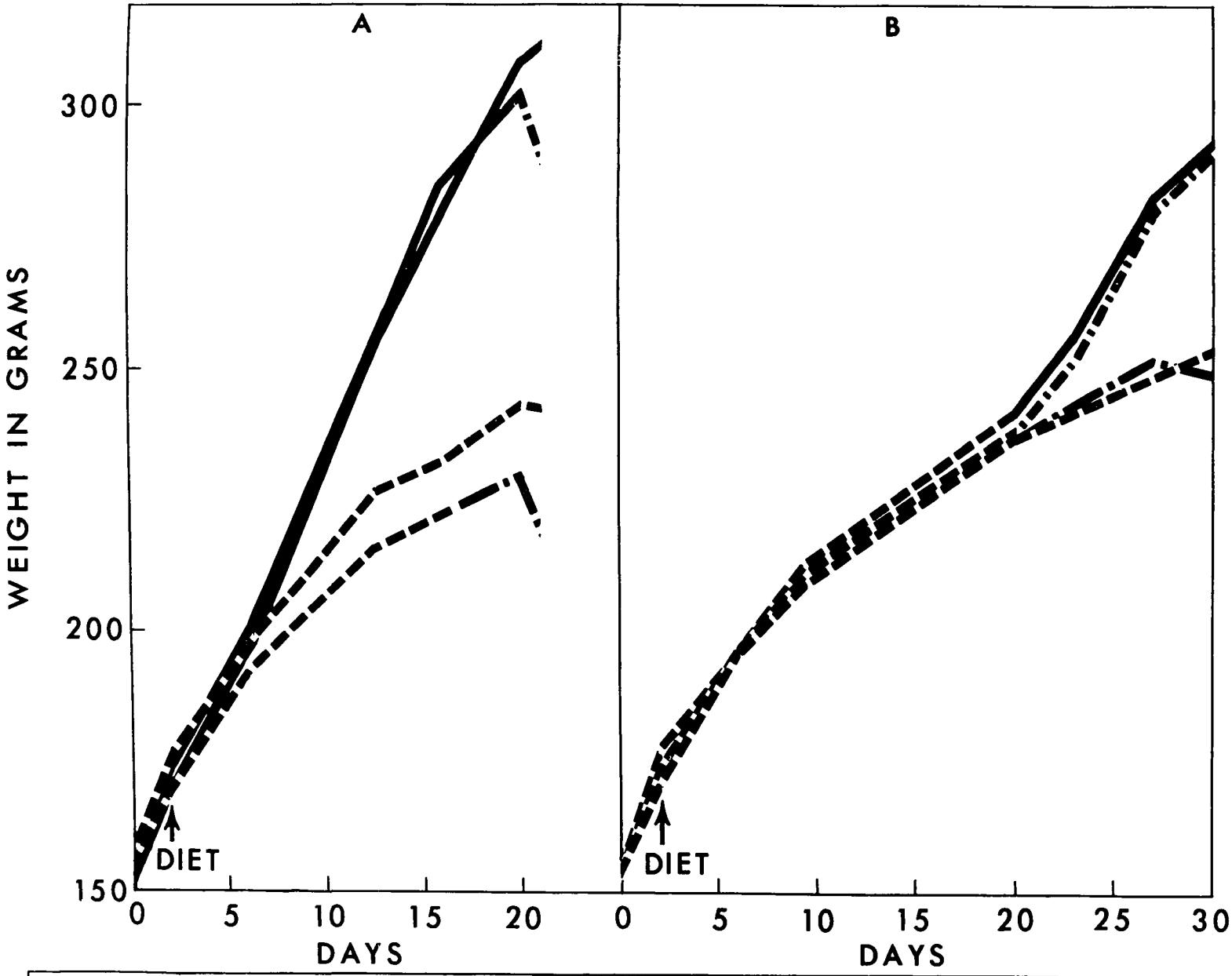


CHART 2.

EFFECT OF THYROIDECTOMY AND MAGNESIUM DEFICIENCY ON THE WEIGHT OF RATS

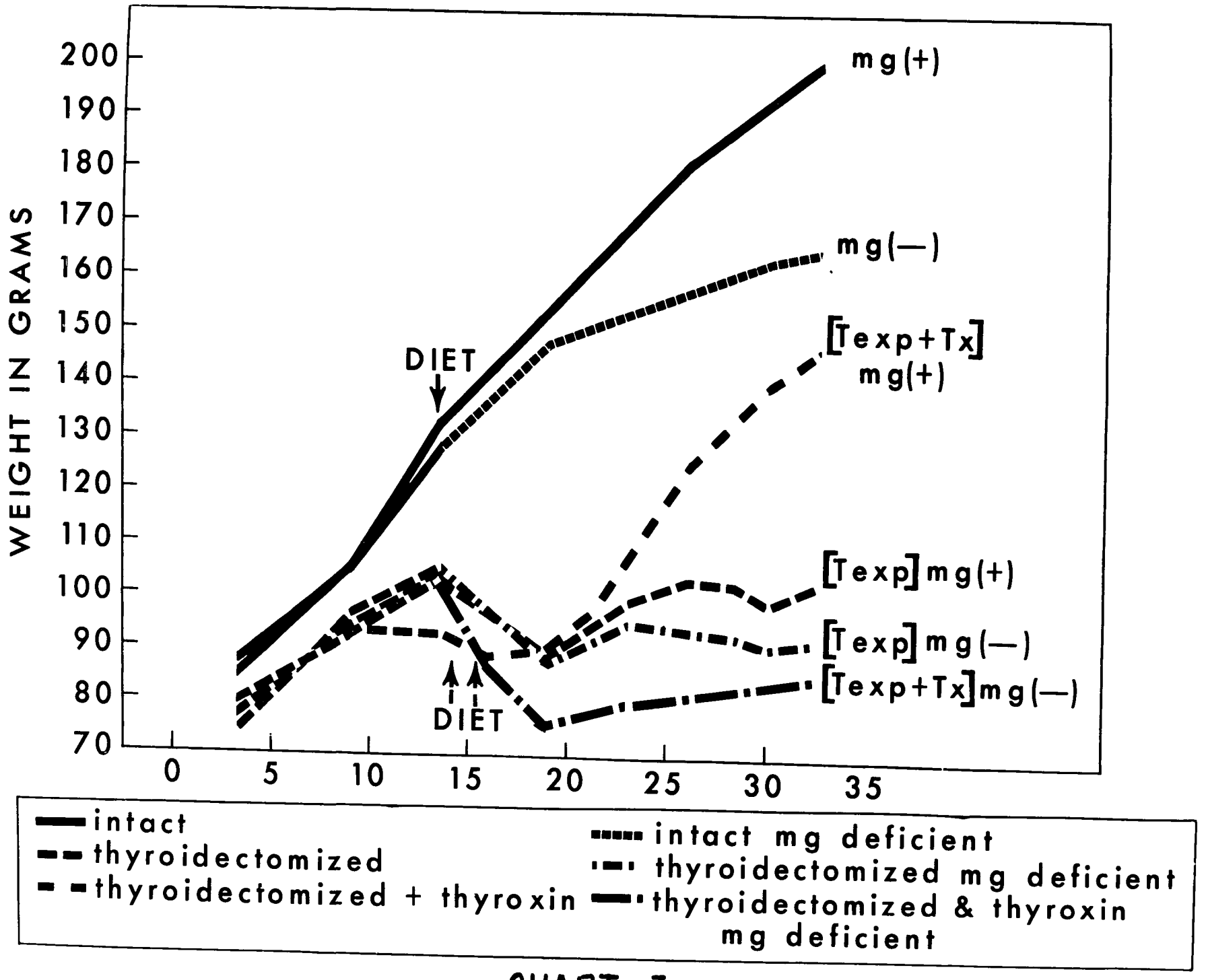


CHART 3.

EFFECT OF OVARIECTOMY AND MAGNESIUM DEFICIENCY ON THE WEIGHT OF RATS

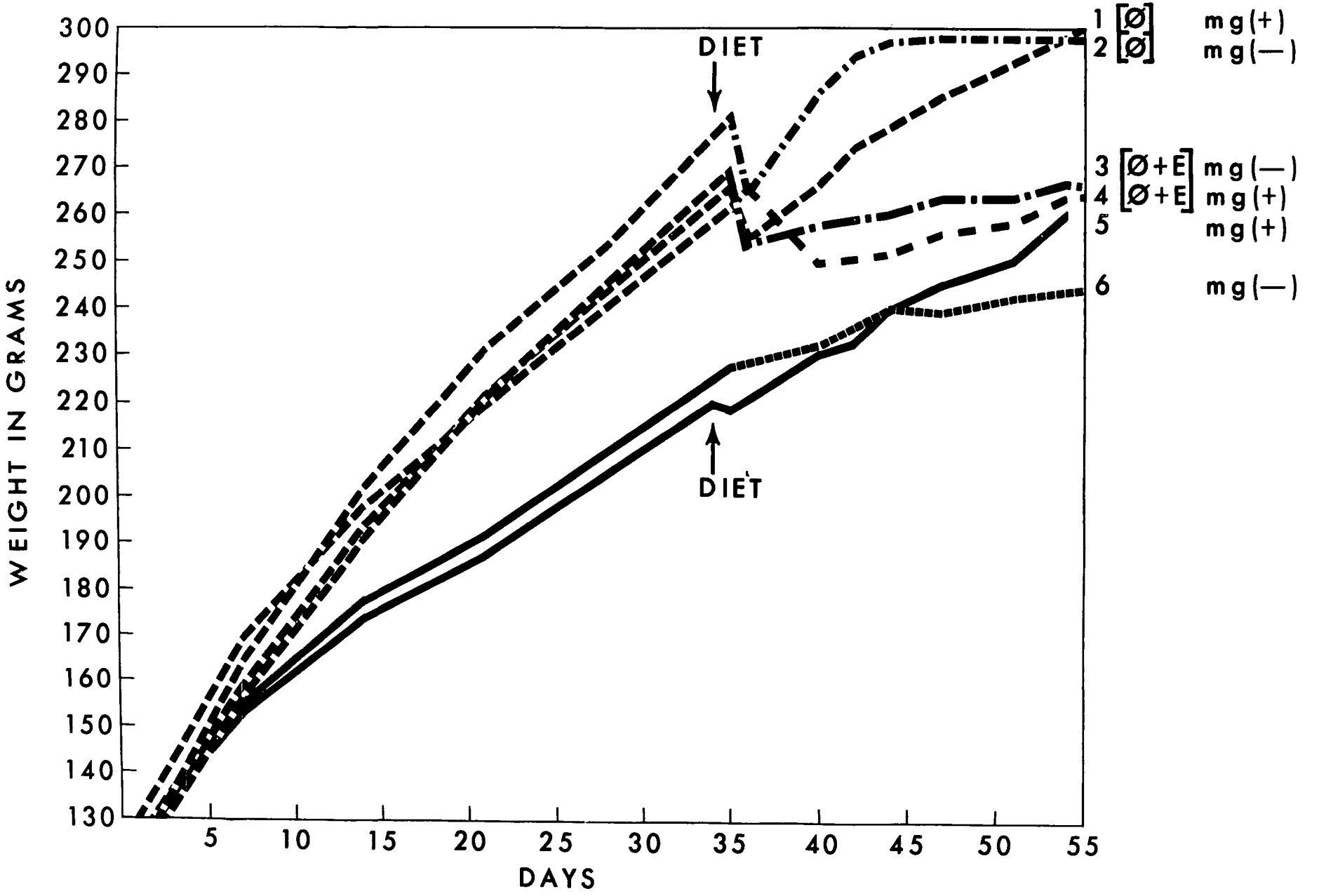


CHART 4

TABLE 1

THE EFFECT OF PARATHYROIDECTOMY, MAGNESIUM DEFICIENCY,

AND REPLACEMENT THERAPY ON THE MAGNESIUM AND CALCIUM CONCENTRATIONS[1] IN SERUM

Diet	Duration Days	Intact Rats		Parathyroidectomized Rats Untreated		Parathyroidectomized Rats +PTE	
		Mg	Ca	Mg	Ca	Mg	Ca
Mg(-)	20	0.50	5.50	0.54	5.43	0.81	5.90[2]
Mg(+)	20	2.18	5.54	2.02	5.34	2.28	6.12[2]
Mg(-)	22			0.65	5.60	0.63	5.67[3]
Mg(-) replaced by Mg(+)	20 2			1.90	5.19	1.96	5.21[3]
Mg(-)	28			0.64	5.51	0.65	5.55[3]
Mg(-) replaced by Mg(+)	20 8			2.39	5.19	2.18	5.21[3]

[1] Expressed as milliequivalents of Mg or Ca per liter of blood serum.

[2] PTE administered in a daily dose of 300 Units for 2 days.

[3] PTE administered in a daily dose of 50 Units.

TABLE 2

THE EFFECT OF PARATHYROIDECTOMY, MAGNESIUM DEFICIENCY, AND REPLACEMENT THERAPY ON THE MAGNESIUM AND CALCIUM CONCENTRATION[1] IN REGIONAL PORTIONS OF FEMURS

Bone Sample	Diet 20 days	Intact Rats		Parathyroidectomized Rats Untreated		Parathyroidectomized Rats +PTE	
		Mg	Ca	Mg	Ca	Mg	Ca
Condyle	Mg(-)	198 ± 6[2]	43.2 ± 0.5[2]	279 ± 4[2]	40.0 ± 0.3[2]	289 ± 5[2]	39.1 ± 0.5[2]
	Mg(+)	966 ± 13	38.5 ± 0.5	965 ± 8	38.7 ± 0.3	926 ± 12	37.5 ± 0.3
Head	Mg(-)	340 ± 6	43.0 ± 0.3	362 ± 5	39.3 ± 0.1	386 ± 6	38.4 ± 0.2
	Mg(+)	890 ± 8	41.8 ± 0.3	885 ± 7	39.2 ± 0.1	865 ± 7	37.9 ± 0.2
Shaft	Mg(-)	391 ± 11	41.0 ± 0.4	463 ± 9	38.8 ± 0.2	446 ± 11	38.1 ± 0.2
	Mg(+)	924 ± 4	38.9 ± 0.3	928 ± 7	38.0 ± 0.1	888 ± 10	37.6 ± 0.1

[1] Magnesium expressed as mg/100 gm ash weight and calcium as g/100 gm

[2] Standard error

TABLE 3

THE EFFECT OF PARATHYROIDECTOMY, MAGNESIUM DEFICIENCY,

AND REPLACEMENT THERAPY ON THE MAGNESIUM AND CALCIUM CONCENTRATION[1]

IN WHOLE FEMURS

Diet	Duration Days	Parathyroidectomized Rats — Untreated			Parathyroidectomized Rats — +PTE		
		Mg	Ca		Mg	Ca	
Mg(-)	22	311 ± 6[2]	39.7 ± 0.3[2]	n.s.	314 ± 6[2]	38.3 ± 0.1[2]	n.s.
Mg(-), replaced by Mg(+)	20 2	419 ± 10	39.3 ± 0.2		419 ± 8	38.4 ± 0.4	
Mg(-)	26	283 ± 8	38.7 ± 0.4	n.s.	261 ± 8	38.6 ± 0.1	n.s.
Mg(-), replaced by Mg(+)	20 6	487 ± 6	38.8 ± 0.1		503 ± 19	38.3 ± 0.3	
Mg(-)	28	270 ± 9	38.7 ± 0.1	2.88	259 ± 5	38.7 ± 0.2	2.78
Mg(-), replaced by Mg(+)	20 8	504 ± 8	38.1 ± 0.2		514 ± 8	38.0 ± 0.1	
Mg(-)	32	245 ± 8	38.8 ± 0.3		214 ± 4	38.7 ± 0.2	

[1] Magnesium expressed as mg/100 gm ash weight and calcium as g/100 gm

[2] Standard error

TABLE 4

THE EFFECT OF PARATHYROIDECTOMY, MAGNESIUM DEFICIENCY, AND REPLACEMENT THERAPY ON THE PHOSPHORUS CONCENTRATION[1] IN WHOLE FEMURS

Diet	Duration Days	Parathyroidectomized Rats	
		Untreated	+PTE
Mg(-)	22	18.9 ± 0.36[2]	18.9 ± 0.29[2]
Mg(-), replaced by Mg(+)	20 2	18.8 ± 0.15	18.7 ± 0.17
Mg(-)	26	18.8 ± 0.20	18.8 ± 0.15
Mg(-), replaced by Mg(+)	20 6	19.6 ± 0.62	19.0 ± 0.36
Mg(-)	28	18.8 ± 0.20	18.9 ± 0.26
Mg(-), replaced by Mg(+)	20 8	19.3 ± 0.32	19.5 ± 0.20
Mg(-)	32	19.0 ± 0.20	18.6 ± 0.51

[1] Expressed as g/100 gm ash weight

[2] Standard error

TABLE 5

THE EFFECT OF PARATHYROIDECTOMY, MAGNESIUM DEFICIENCY,

AND REPLACEMENT THERAPY ON THE WEIGHT OF KIDNEYS

Diet	Duration Days	Intact Rats		Parathyroidectomized Rats Untreated		Parathyroidectomized Rats +PTE	
		a	b	a	b	a	b
Mg(-)	20	1.20 ± 0.04[c]	4.6	1.21 ± 0.05[c]	5.0	1.11 ± 0.03[c]	5.0
Mg(-)	20	1.31 ± 0.04	4.1	1.23 ± 0.03	4.0	1.27 ± 0.05	4.4
Mg(-)	28			1.26 ± 0.05	5.0	1.25 ± 0.05	5.0
Mg(-), replaced by Mg(+)	20 8			1.22 ± 0.05	4.2	1.33 ± 0.02	4.6

[a] Total weight of kidney in grams

[b] Expressed as mg./Kgm body weight

[c] Standard error

TABLE 6

THE EFFECT OF PARATHYROIDECTOMY, MAGNESIUM DEFICIENCY,

AND REPLACEMENT THERAPY ON THE LENGTH (IN MM.) OF RAT FEMURA

Diet	Duration Days	Intact Rats	Parathyroidectomized Rats Untreated	Parathyroidectomized Rats +PTE
Mg(-)	20	25.38 ± 0.16[c]	25.55 ± 0.21[c]	25.41 ± 0.20[c]
Mg(+)	20	26.82 ± 0.08 [a]	26.70 ± 0.16 [a]	27.01 ± 0.21 [a]
Mg(-)	28		26.22 ± 0.25	26.31 ± 0.18
Mg(-), replaced by Mg(+)	20 8		[b] 26.90 ± 0.21	[a] 27.43 ± 0.19

[c] Standard error

[a] $P < 0.001$

[b] $P < 0.01$

TABLE 7

THE EFFECT OF HORMONES AND MAGNESIUM DEFICIENCY

ON THE OCCURRENCE OF NEPHROLITHS

TREATMENT		NUMBER OF ANIMALS	HORMONES PRESENT			NEPHROLITHS		
						Number of Animals		
Diet	Hormone		PTH	CT	Tx	Extensive	Medium	None
Intact Animals								
Mg(+)		6	+	+	+	0	0	0
Mg(-)		11	+	+	+	11	0	0
Thyroidectomized Animals Parathyroids Intact								
Mg(+)		8	+	-	-	0	0	0
Mg(+)	T4	5	+	-	+	0	0	0
Mg(+)	T3	3	+	-	+	0	0	0
Mg(-)		12	+	-	-	1	8	3
Mg(-)	T4	7	+	-	+	0	0	0
Mg(-)	T3	5	+	-	+	0	0	0

PLATE 1

Figure A. Left. The cortico-medullary area of the kidney of an intact rat fed a magnesium-supplemented diet for 20 days. No microliths are present in the tubules.

Figure B. Right. The cortex and outer medulla of the kidney of an intact rat fed a magnesium deficient diet for 20 days. The lumena of many of the loops of Henle in the cortico-medullary area are filled with calcareous deposits.

Hematoxylin-phloxine-eosin (HPO) X40

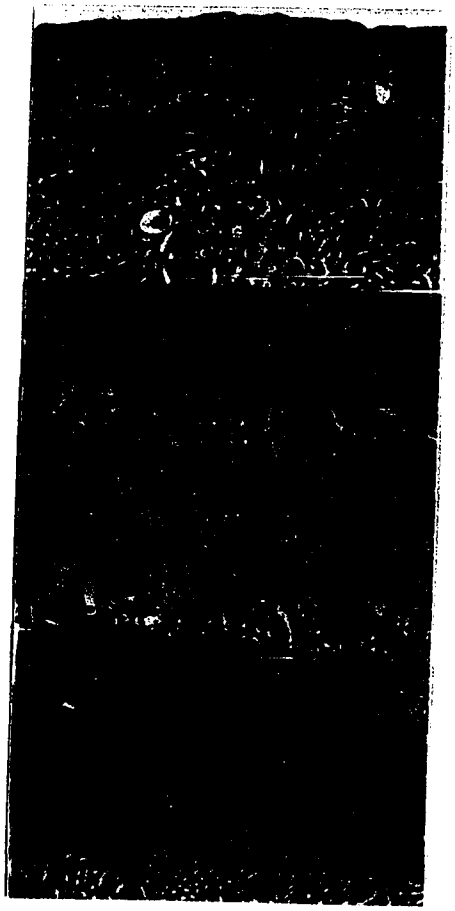


PLATE 2

Figure A. Upper. The cortico-medullary portion of the kidney from a rat fed a magnesium-deficient diet for 20 days. Calcareous deposits fill the lumen of the loops of Henle. The ascending loops are more generally affected.

Figure B. Lower. The cortico-medullary portion of the kidney from a magnesium-deficient rat. The calculi appear to be composed of deposits arranged in a concentric pattern.

HPO stain x500

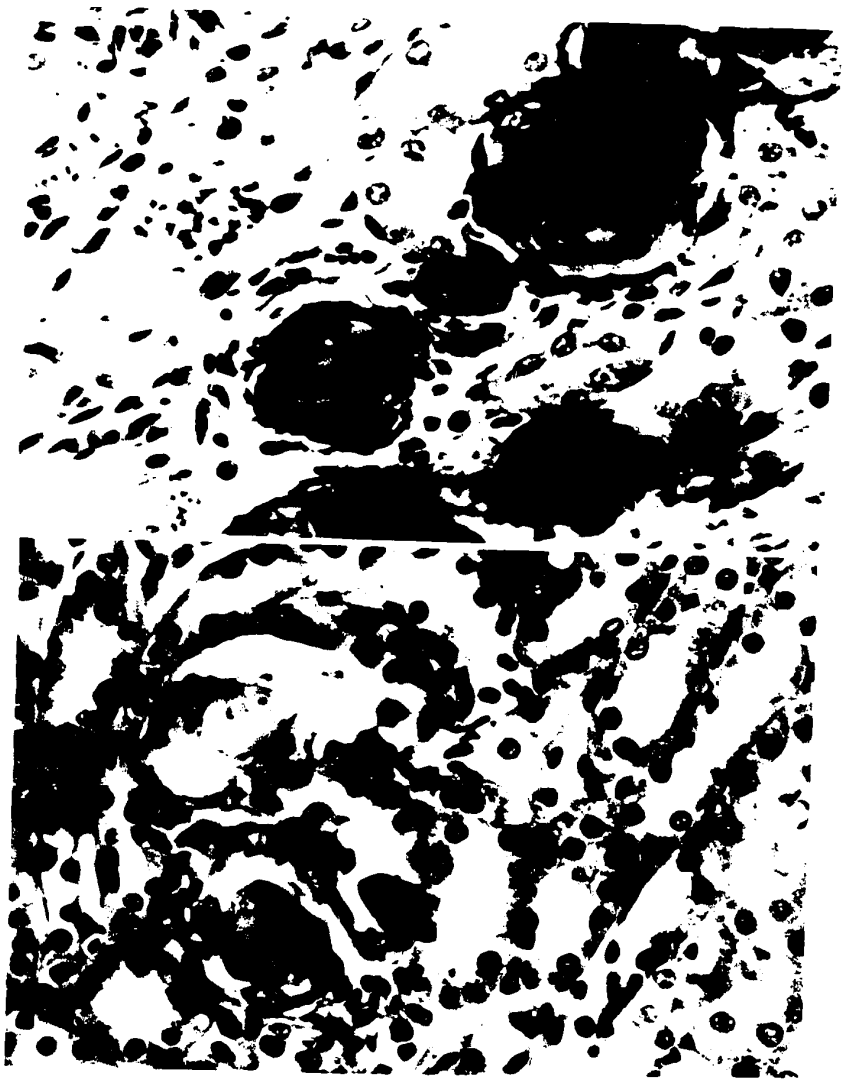


PLATE 3

Figure A. Upper left. The outer cortex of a kidney from a normal intact rat fed an adequate magnesium-supplemented diet for 20 days. Note the paucity of mast cells.

Figure B. Upper right. The outer cortex of a kidney from an intact rat fed a magnesium-deficient diet for 20 days. Note the increased number of mast cells (arrows). Compare with Figure A.

Figure C. Lower left. The inner cortex of the kidney from the rat in Figure A.

Figure D. Lower right. The inner cortex of the kidney from the rat in Figure B. Compare with Figure C.

HPO stain x500

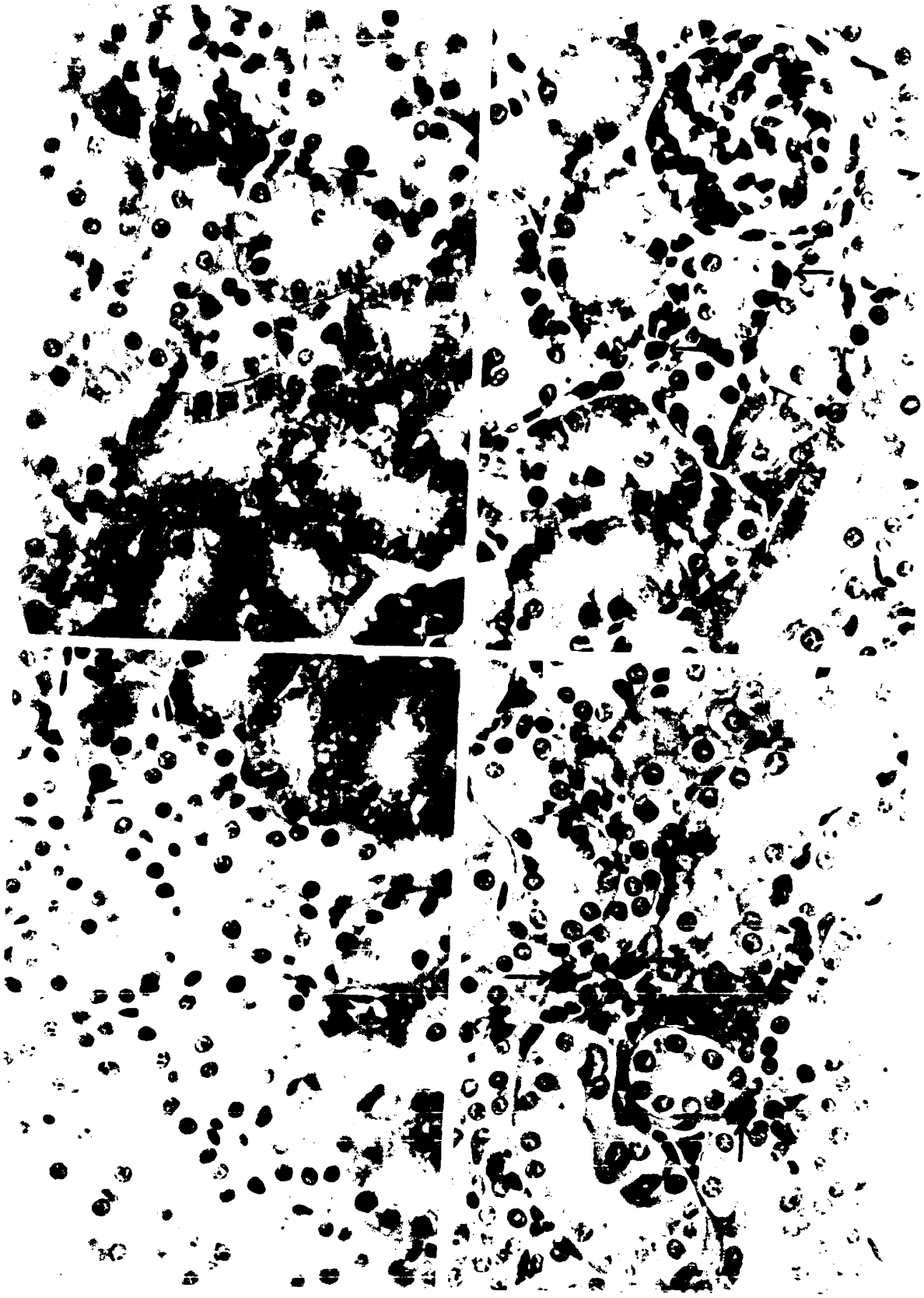


PLATE 4

The inner cortex of a kidney from an intact rat fed a magnesium-deficient diet for 20 days. Arrows point to mast cells.

HPO stain x500

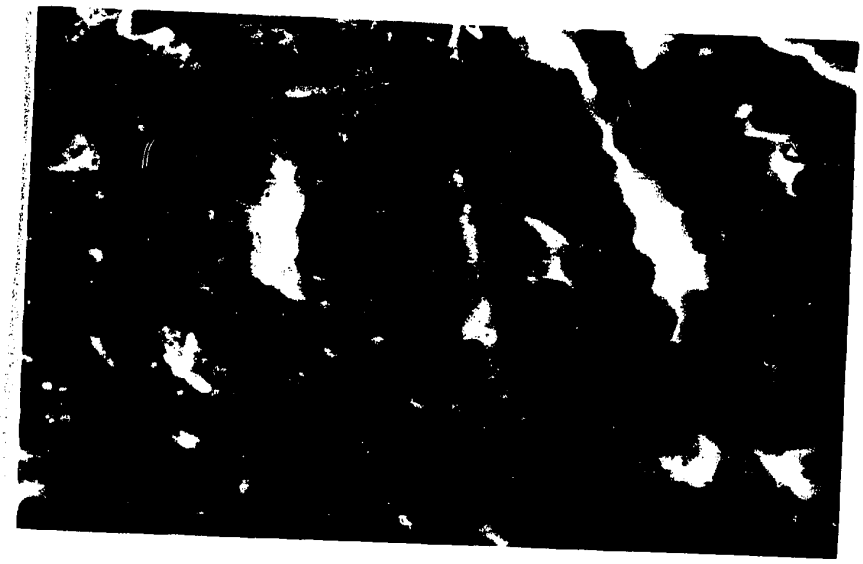


PLATE 5

Figure A. Upper left. The region of the juxtaglomerular apparatus of a kidney from a rat fed an adequate magnesium-supplemented diet for 20 days.

Figure B. Upper right. The region of the juxtaglomerular apparatus of a kidney from a rat fed a magnesium-deficient diet for 20 days. Note the increased size of the juxtaglomerular cells. Compare with Figure A.

Figure C. Lower left. As in Figure A.

Figure D. Lower right. As in Figure B.

HPO stain x500

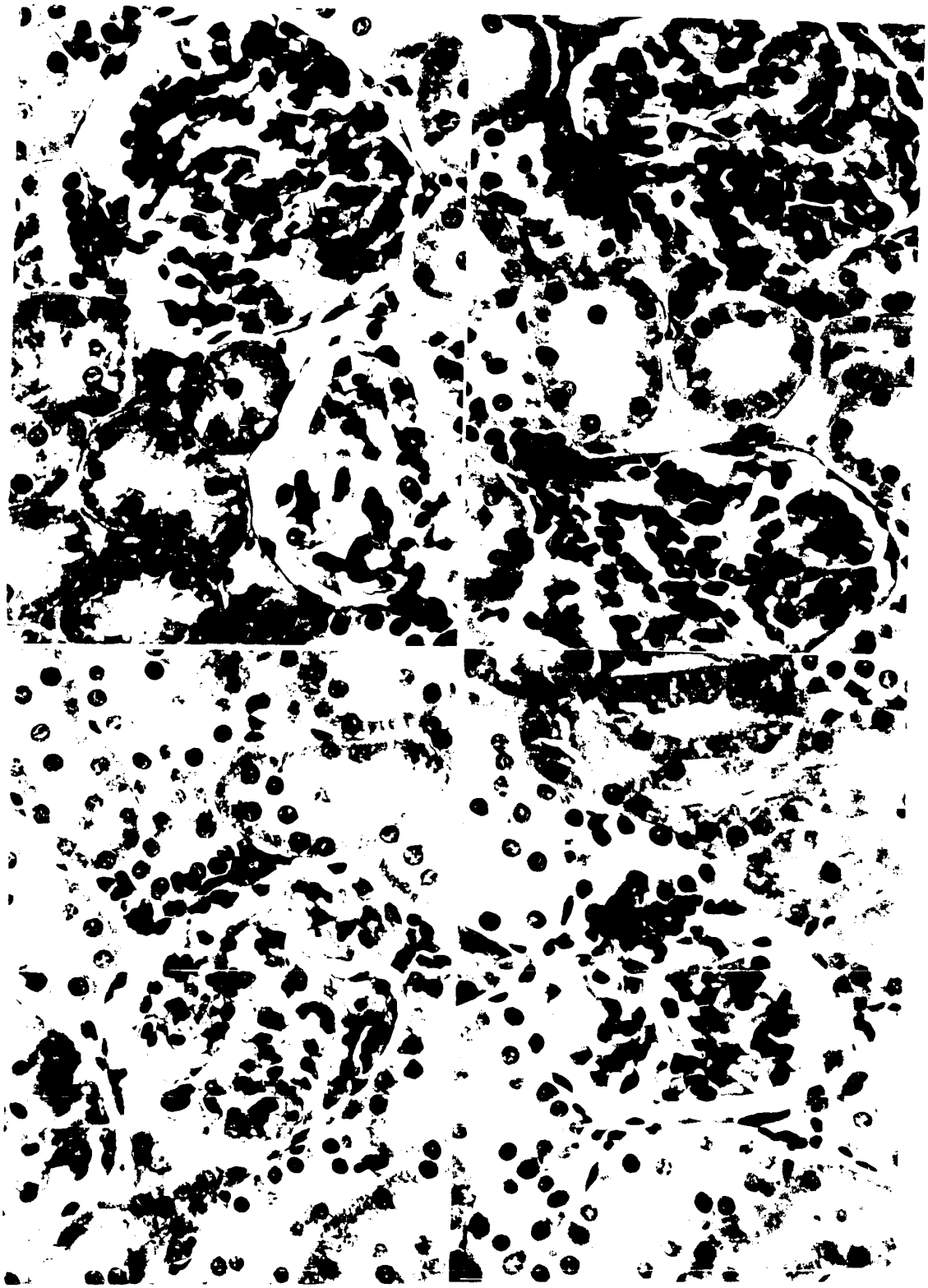


PLATE 6

Figure A. Upper. Section of kidney from a rat fed a magnesium-deficient diet for 20 days. Blood vessels are dilated and there are focal areas of white cell infiltration.

Figure B. Lower. Section through the cortico-medullary portion of the kidney from a rat fed a magnesium-deficient diet for 20 days. Dark staining proteinaceous casts are present in tubules, especially in collecting ducts. These casts do not necessarily correspond to the sites where calcification occurs.

HPO stain x500

PLATE 7

Alpharadiographs of longitudinal sections through the epiphysis (A and B) and diaphysis close to the metaphysis (C and D) of the femurs of intact rats.
x200

Figure A. Upper left. From a rat fed an adequate magnesium-supplemented diet for 20 days.

Figure B. Upper right. From a rat fed a magnesium-deficient diet for 20 days. Compare with Figure A. Note the reduction in the size of the cartilage plate and in the number of small and large chondrocytes.

Figure C. Lower left. From a rat fed an adequate magnesium-supplemented diet for 20 days.

Figure D. Lower right. From a rat fed a magnesium-deficient diet for 20 days. Compare with Figure C. Osteocytic lacunae appear to be smaller and there appears to be less osteocytic osteolysis.

PLATE 8

Figure A. Upper left. Cross section of the diaphysis of a rat fed a control diet. HPO x10

Figure B. Upper right. Cross section of the diaphysis of an intact rat fed a magnesium deficient diet for 20 days. Note large subperiosteal desmoid tumor. HPO x10

Figure C. Lower. Alpharadiograph of a small segment of the cross section of the diaphysis of a rat as in Fig. B. The demoid tumoral mass is less dense than the bone. x80

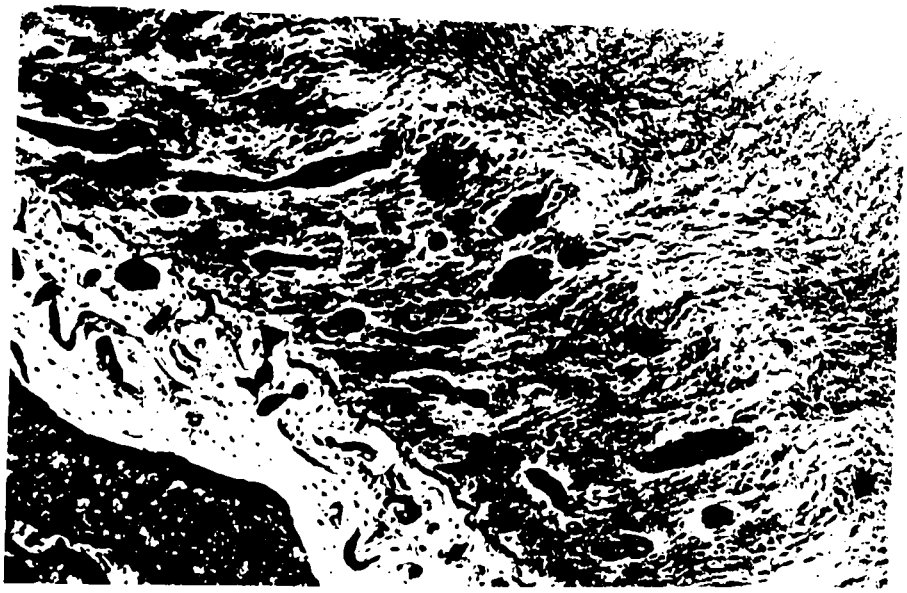


PLATE 9

Figure A. Upper left. Anterior aspects of femura of intact young male rats fed a magnesium-supplemented diet for 20 days. Note smooth contours of distal portion of shafts.

Figure B. Upper right. Posterior aspect of bones of Figure A.

Figure C. Lower left. Anterior aspects of femura of intact young male rats fed a magnesium-deficient diet for 20 days. Note the irregular contours and increased width of portions of the shaft (e.g. Bone 887). Compare with Figure A.

Figure D. Lower right. Posterior aspect of bones of Figure C.

x1.6

PLATE 10

Figure A. Upper left. Anterior aspect of femura of young parathyroidectomized male rats fed a magnesium-supplemented diet for 20 days. (Sacrifice was 5 weeks after parathyroidectomy.)

Figure B. Upper right. Posterior aspect of bones of Figure A.

Figure C. Lower left. Anterior aspect of femura of young parathyroidectomized male rats fed a magnesium-deficient diet for 20 days. Note large tumor-like mass in distal shaft area.

Figure D. Lower right. Posterior aspect of bones of Figure C. The tumor-like areas are more extensive than in Plate 9, Figure D. Compare also with Figure C.

xl.6

141
142
143
144
145
146

PLATE 11

Figure A. Upper left. Anterior aspect of femura of young parathyroidectomized male rats fed a magnesium-supplemented diet for 20 days and injected with a total of 600 Units parathormone during days 18 and 19.

Figure B. Upper right. Posterior aspect of bones of Figure A.

Figure C. Lower left. Anterior aspect of femura of young parathyroidectomized male rats fed a magnesium deficient diet for 20 days and injected with a total of 600 Units parathormone during days 18 and 19. Note tumor-like areas as in Plate 10.

Figure D. Lower right. Posterior aspect of bones of Figure C. Note that bones were easily broken.

x1.6

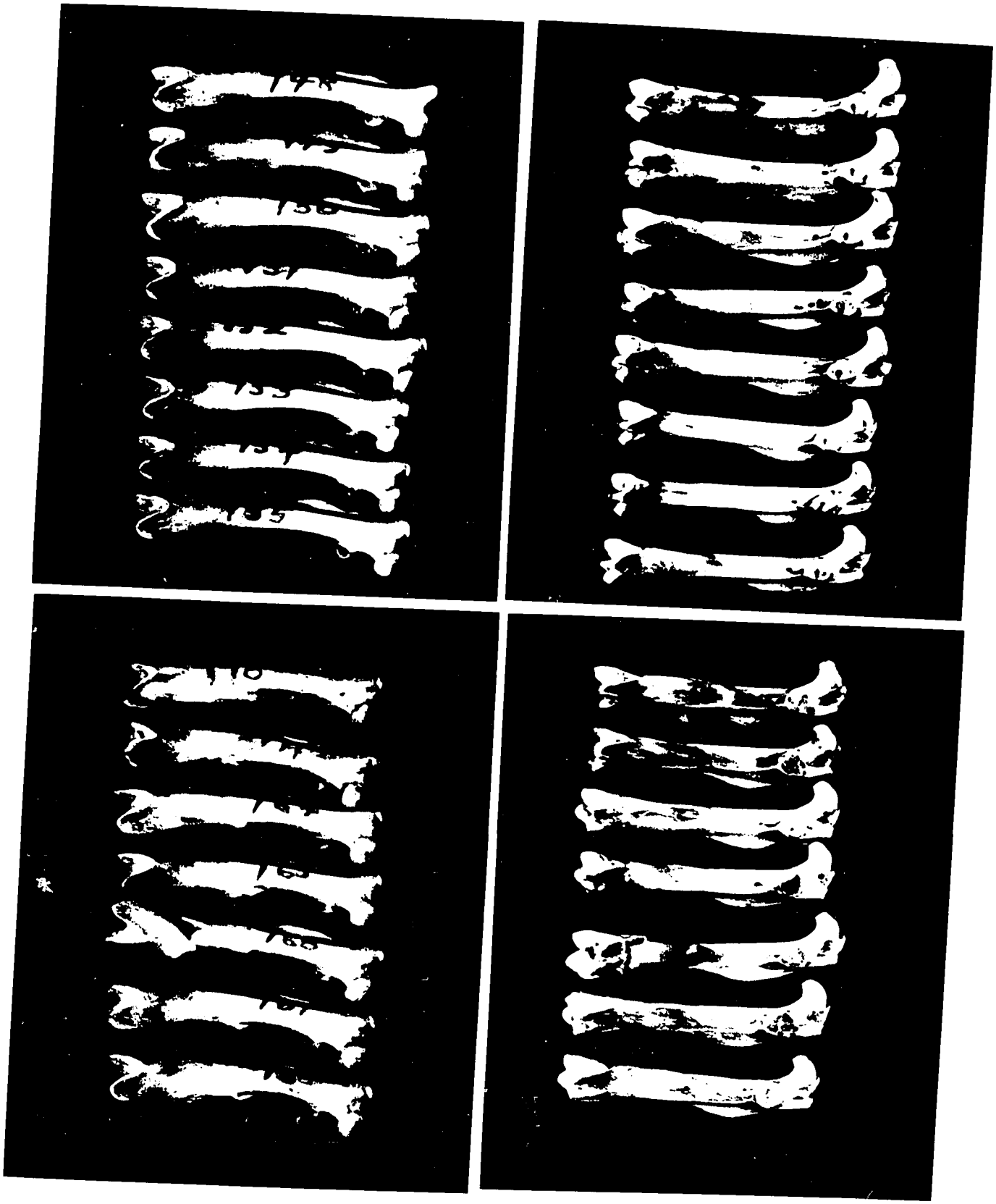


PLATE 12

Figure A. Upper left. Anterior aspect of femura of young parathyroidectomized male rats fed a magnesium-deficient diet for 28 days. Turmor-like areas are extensive.

Figure B. Upper right. Posterior aspect of bones of Figure A.

Figure C. Lower left. Anterior aspect of femura of young parathyroidectomized male rats fed a magnesium-deficient diet for 28 days and during the last 8 days injected daily with 50 Units parathormone. Compare with Figure A.

Figure D. Lower right. Posterior aspect of Figure C. Note that the most extensive hyperplasia occurred in this group of bones.

x1.6

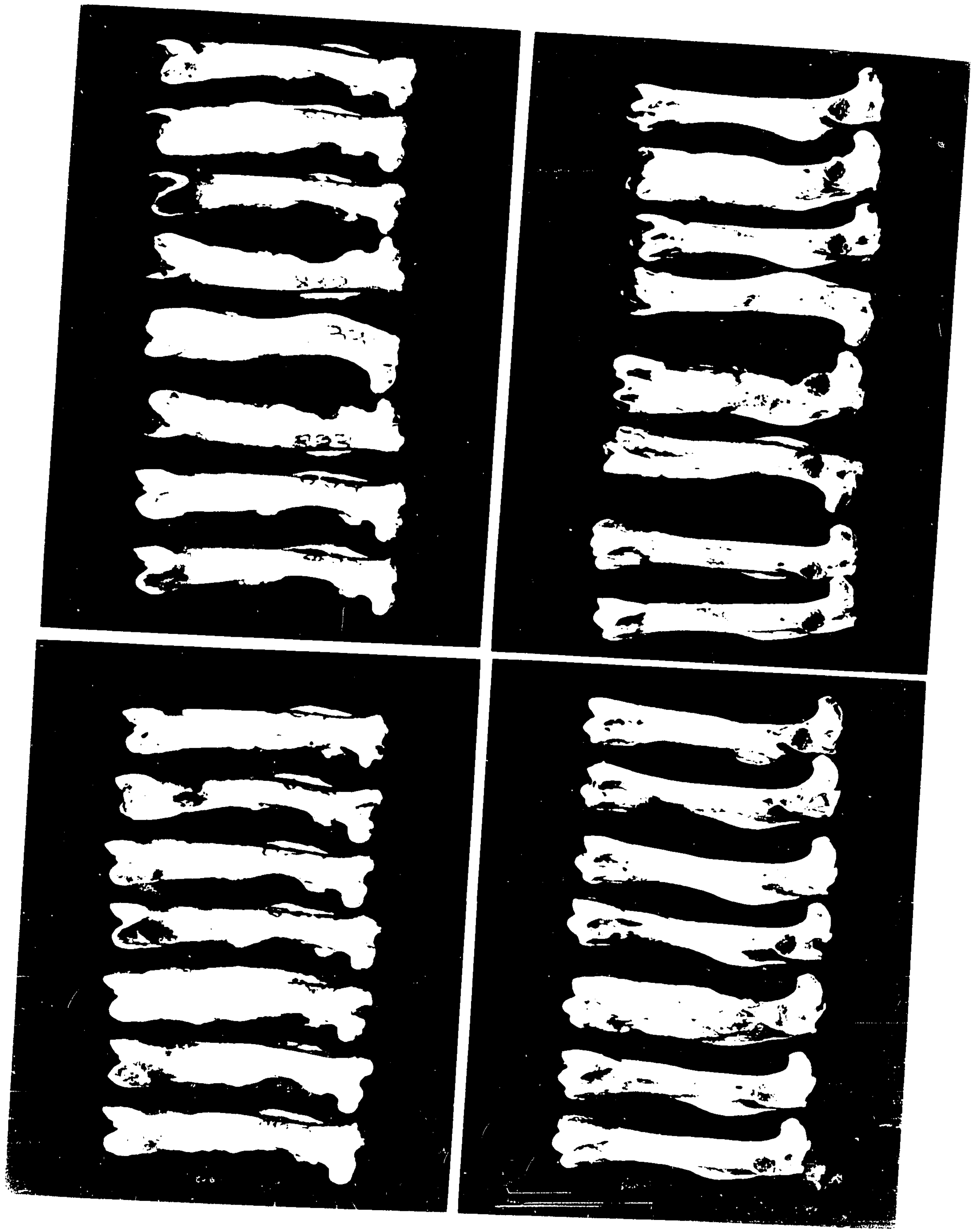


PLATE 12

Figure A. Upper left. Anterior aspect of femura of young parathyroidectomized male rats fed a magnesium-deficient diet for 20 days followed by a magnesium-supplemented diet for 8 days.

Figure B. Upper right. Posterior aspect of bones of Figure A. Note that three of the bones are approaching the appearance of those in Plate 10, Figure B.

Figure C. Lower left. Anterior aspect of femura of young parathyroidectomized male rats fed a magnesium-deficient diet for 20 days followed by a magnesium-supplemented diet for 8 days and during these last 8 days injected daily with 50 Units parathormone.

Figure D. Lower right. Posterior aspect of bones of Figure C. Note that the tumor-like masses are reduced in size and number compared to Plate 11, Figure D, but are still more numerous than in Figure B, this plate.

x1.6

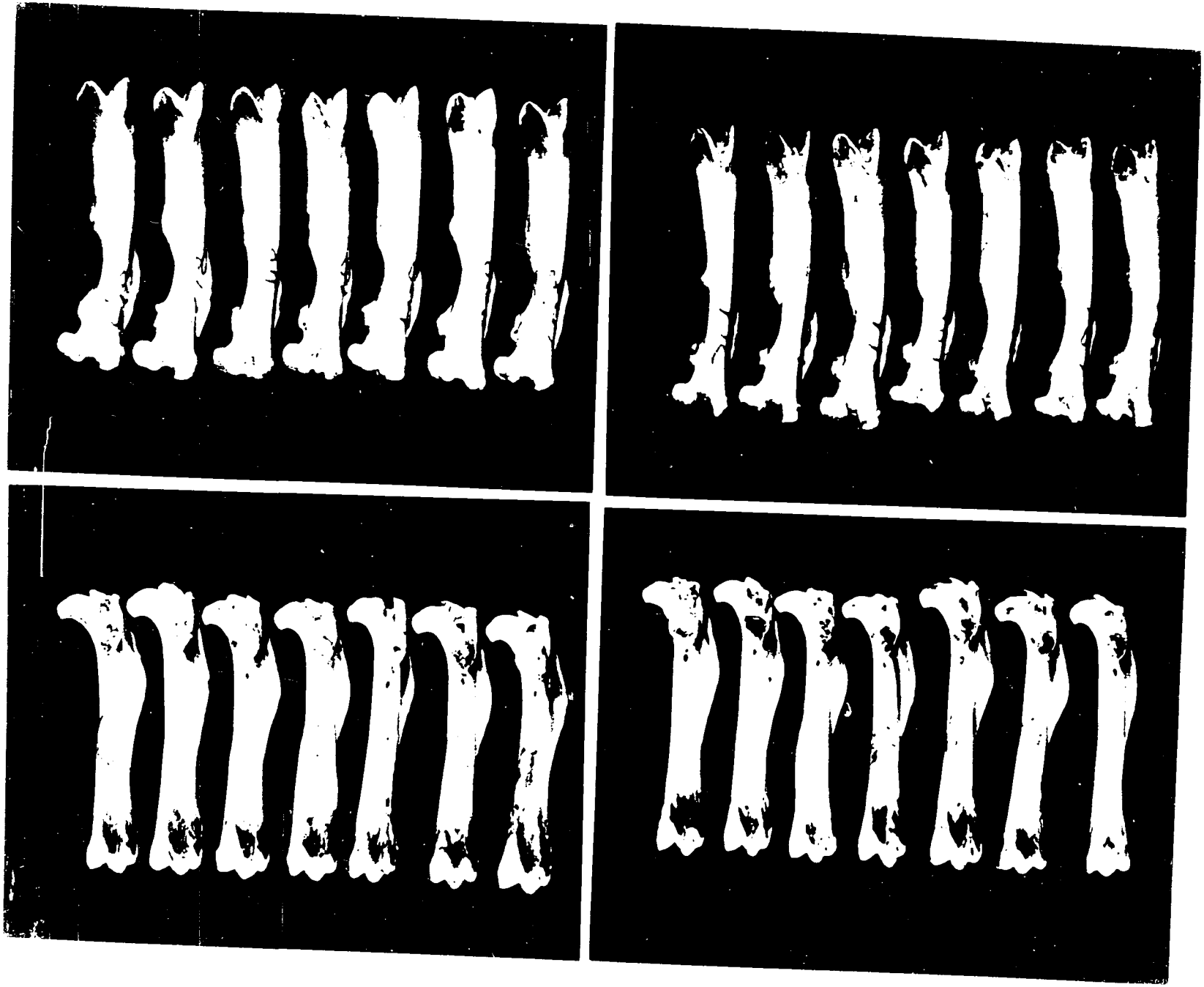


PLATE 14

Anterior aspect of femura of young parathyroidectomized male rats fed a magnesium-deficient diet for 32 days. Note that the longer the rats were on the deficient diet, the more extensive was the hyperplasia of the shafts.

x1.6

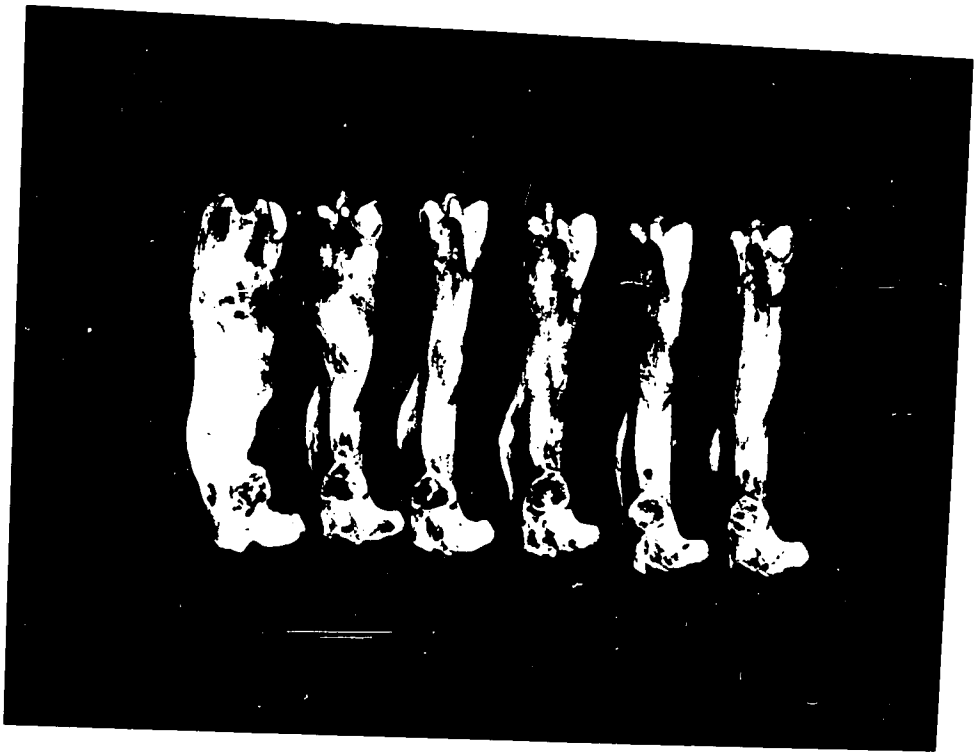


PLATE 15

Cross sections at the level of the linea aspera of femoral diaphyses of parathyroidectomized rats.

Gomori and Dilute Wright stain x37.5

Figure A. Upper left. Rat was fed a supplemented magnesium diet for 20 days. No hyperplasia is present.

Figure B. Upper right. Rat was fed a magnesium-deficient diet for 20 days. A large hyperplastic mass is present in the lower center of the field. Under higher magnification (see Plate 17) the mass is shown to consist of cartilage, fibroblasts and interspersed collagen fibers.

Figure C. Lower left. Rat was fed a magnesium-deficient diet for 20 days and injected with a total of 100 Units parathormone during the last 2 days. There is a reduction in the central cartilage component of the hyperplastic mass.

Figure D. Lower right. Rat was fed a magnesium-deficient diet for 20 days followed by a magnesium-repleted diet for 2 days. The mass has begun to decrease in size.

Under all conditions, the mass stains positively for alkaline phosphatase.

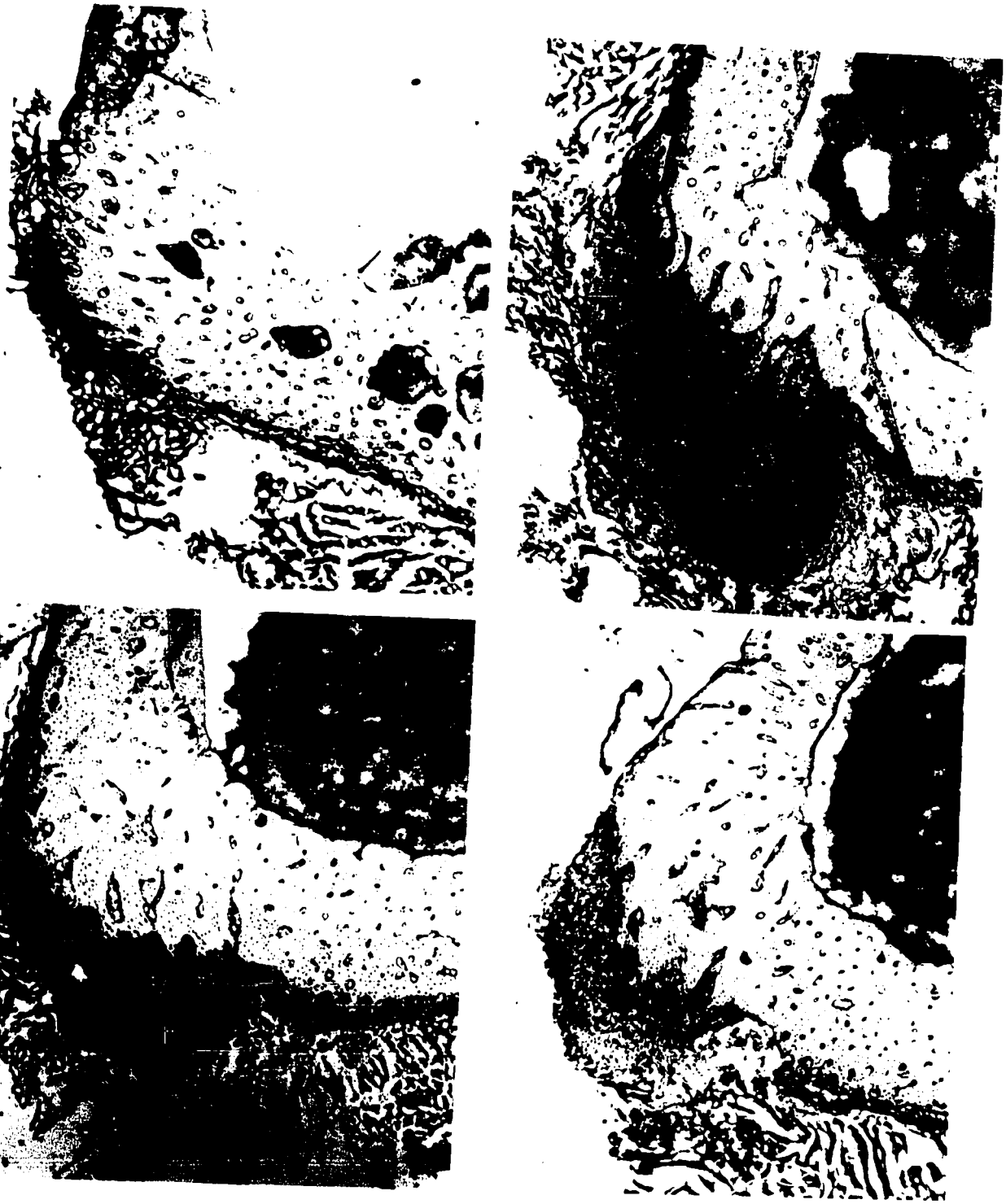


PLATE 16

Alkaline phosphatase reactions in Haversian canal and osteocytic lacunae seen in cross section of femoral diaphyses. Parathyroidectomized animals were fed a magnesium deficient diet for 22 days and injected with a total of 100 Units parathormone during the last 2 days.
x500

Figure A. Left. Stained by the Gomori-Takamatsu procedure. The lining of the Haversian canal and the lacunae and some osteocytes in the immediate vicinty of the canal show a positive enzyme reaction.

Figure B. Right. Stained by the Burstone procedure. Same structures as in Figure A are stained by this technique. Note in this section that the endosteum also gives a positive reaction.

PLATE 17

Alkaline phosphatase reaction in hyperplastic areas of femoral diaphyses of parathyroidectomized rats fed a magnesium-deficient diet for 26 days. x500

Figure A. Upper left. From a parathyroidectomized rat fed a magnesium-deficient diet. Cartilage is a prominent component of the periosteal hyperplasia. Enzyme is present in pre-cartilage fibroblasts and in the large chondrocytes. Stained by the Burstone procedure.

Figure B. Upper right. From a parathyroidectomized rat fed a magnesium-deficient diet and administered a total of 300 Units parathormone during the last 6 days. The amount of cartilage is greatly reduced. The fibrous mass stains positively for alkaline phosphatase. Stained by the Burstone method.

Figure C. Lower left. Same as Figure A, but stained by the Gomori-Takamatsu method.

Figure D. Lower right. Same as Figure B, but stained by the Gomori-Takamatsu method.

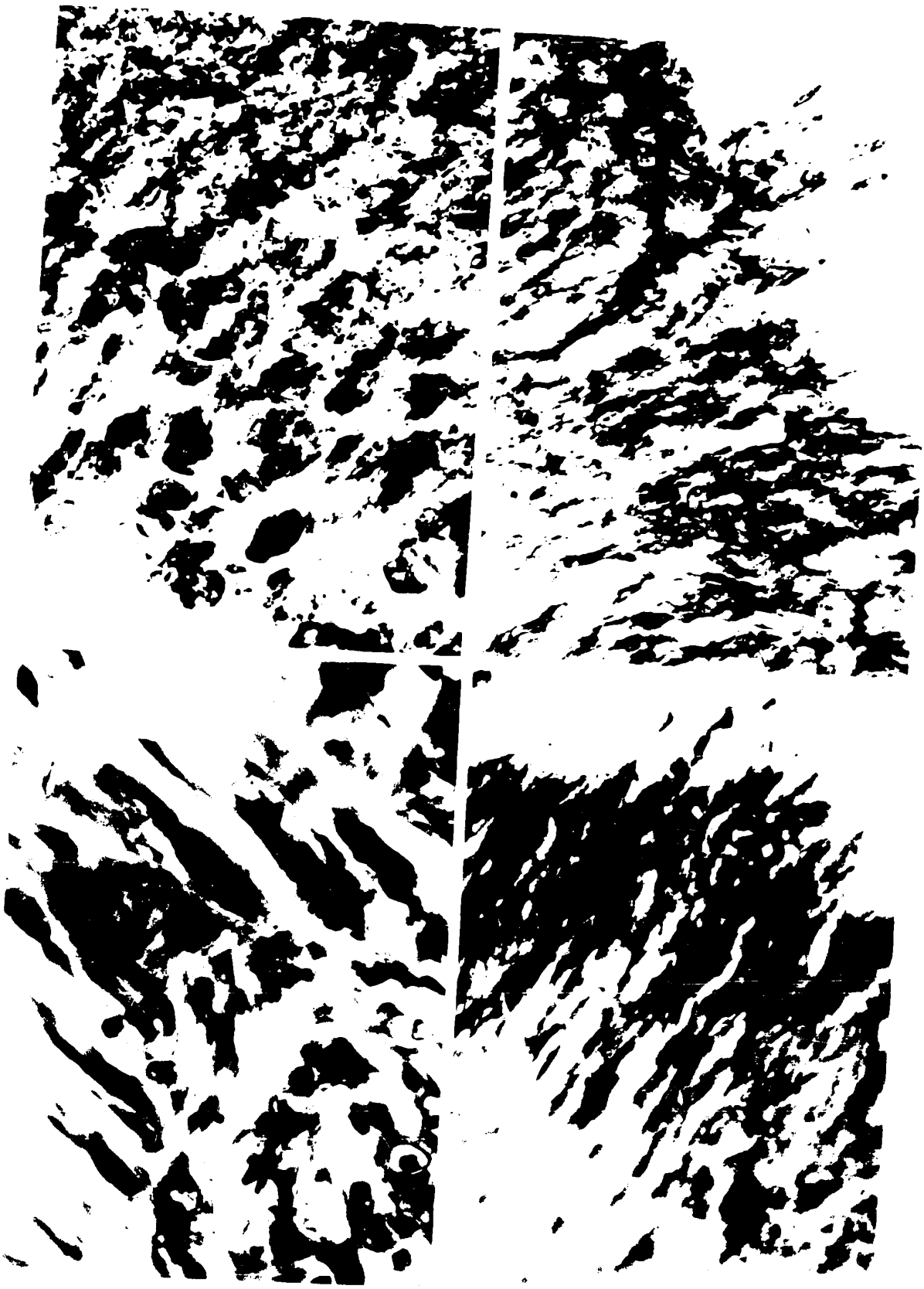


PLATE 18

Alkaline phosphatase reaction (Gomori) in cross sections of femoral diaphyses of parathyroidectomized rats on various diets.

x80

Figure A. Upper. Rat was fed a magnesium-supplemented diet for 20 days.

Figure B. Middle. Rat was fed a magnesium-deficient diet for 20 days.

Figure C. Lower. Rat was fed a magnesium-deficient diet for 20 days, followed by a replenished magnesium diet for 6 days.

The fibrillar portion of the matrix is more evident in the bone of the rat on the magnesium-deficient diet (B), and the number of resorptive cavities appears to be less than in the rat on the adequate magnesium-supplemented diet (A). Following the replacement of magnesium in the diet (C), the bone begins to resemble that of A.

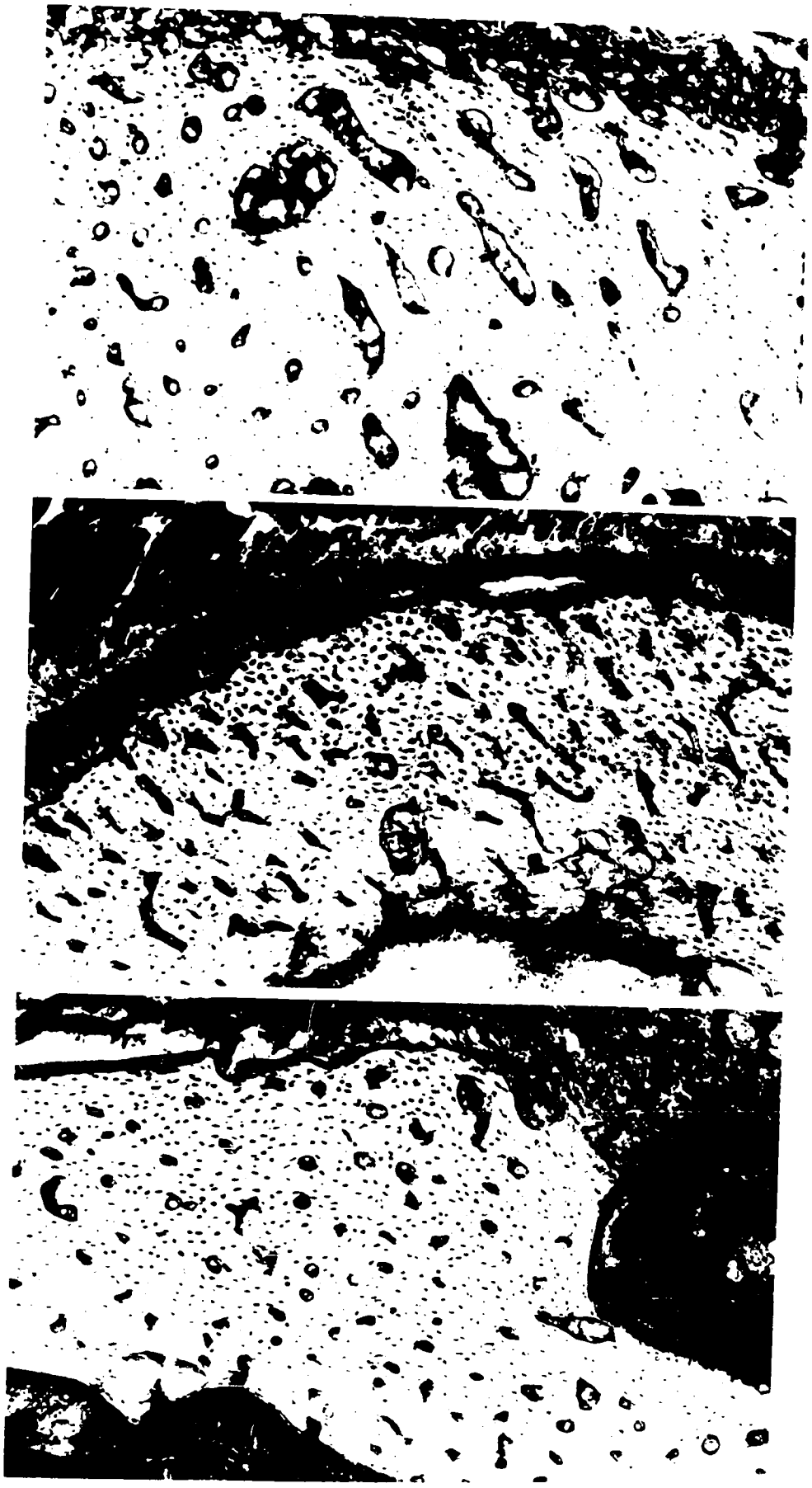


PLATE 19

Alkaline phosphatase reaction in cross sections of femoral diaphyses of parathyroidectomized rats. Gomori method. x200

Figure A. Upper left. Rat was fed a magnesium-deficient diet for 22 days.

Figure B. Upper right. Rat was fed a magnesium-deficient diet for 22 days and administered a total of 100 Units parathormone during the last 2 days.

Figure C. Lower left. Rat was fed a magnesium-deficient diet for 26 days and administered a total of 200 Units parathormone during the last 6 days.

Figure D. Lower right. Rat was fed a magnesium-deficient diet for 20 days followed by a magnesium-supplemented diet for 6 days, and administered a total of 300 Units parathormone during these 6 days.

The fibrillarity of the matrix in the femoral diaphyses of magnesium deficient rats appears to decrease with the administration of parathormone. Compare A, B, and C. The fibrillarity is further decreased when the rat is placed on a magnesium replenished diet (D).

PLATE 20

Alkaline phosphatase activity in the femoral diaphyses of parathyroidectomized rats administered parathormone. x500

Figure A. Upper left. From a rat fed a magnesium-deficient diet for 22 days. As in Plate 16, some osteocytes around Haversian canals stain positively.

Figure B. Upper right. From a rat fed a magnesium-deficient diet for 26 days.

Figure C. Lower left. From a rat fed a magnesium-deficient diet for 20 days followed by a magnesium-repleted diet for 2 days.

Figure D. Lower right. From a rat fed a magnesium-deficient diet for 20 days followed by a magnesium-repleted diet for 6 days.

There appear to be more osteocytes per unit area of bone of magnesium-deficient rats than in the bone of rats given a replenished diet. A loss or lack of formation of new matrix during the period of magnesium deficiency may account for this.

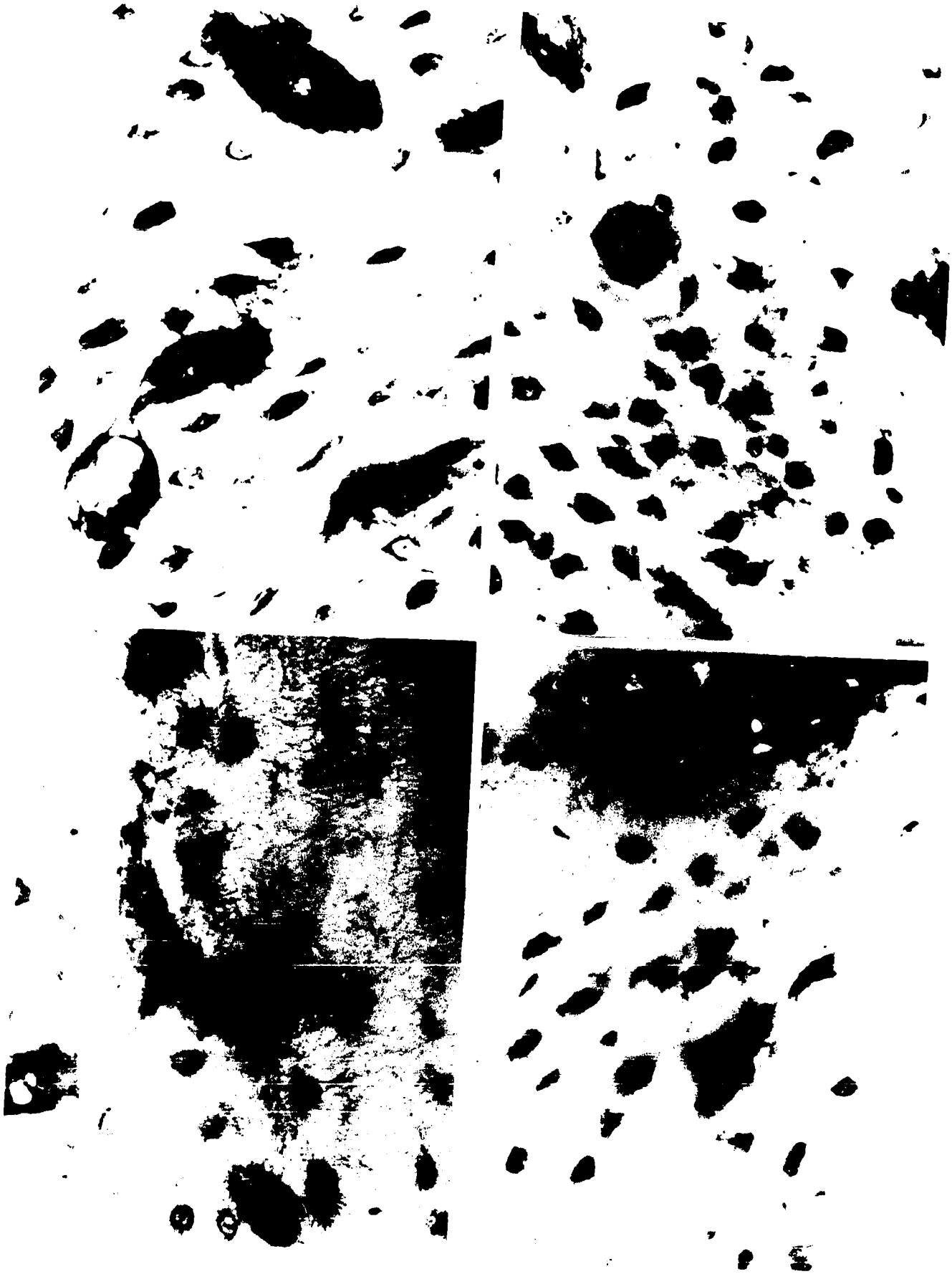


PLATE 21

The cortico-medullary portion of kidneys of female rats.

HPO stain x80

Figure A. Upper left. From an intact rat fed an adequate magnesium-supplemented diet for 20 days.

Figure B. Upper right. From an intact rat fed a magnesium-deficient diet for 20 days. Dark staining mass in the tubules (mainly ascending loops of Henle) are calcareous deposits. Note dilated blood vessels.

Figure C. Lower left. From a thyroidectomized rat fed a magnesium-deficient diet for 20 days. Note that there are some small calcareous deposits (arrows) but far fewer than in Figure B.

Figure D. Lower right. From a thyroidectomized rat fed a magnesium-deficient diet for 20 days and administered daily replacement doses of L-thyroxin. Note that no calcareous deposits are present.

PLATE 22

Longitudinal section through the epiphyseal region of the femur of intact rats. Control sections for Plate 23.

Diluted Wright stain x80

Figure A. Left. From a rat fed an adequate magnesium-supplemented diet for 25 days.

Figure B. Right. From a rat fed a magnesium-deficient diet for 25 days. Note the narrow epiphyseal plate and the short, wide, irregularly-shaped spicules.

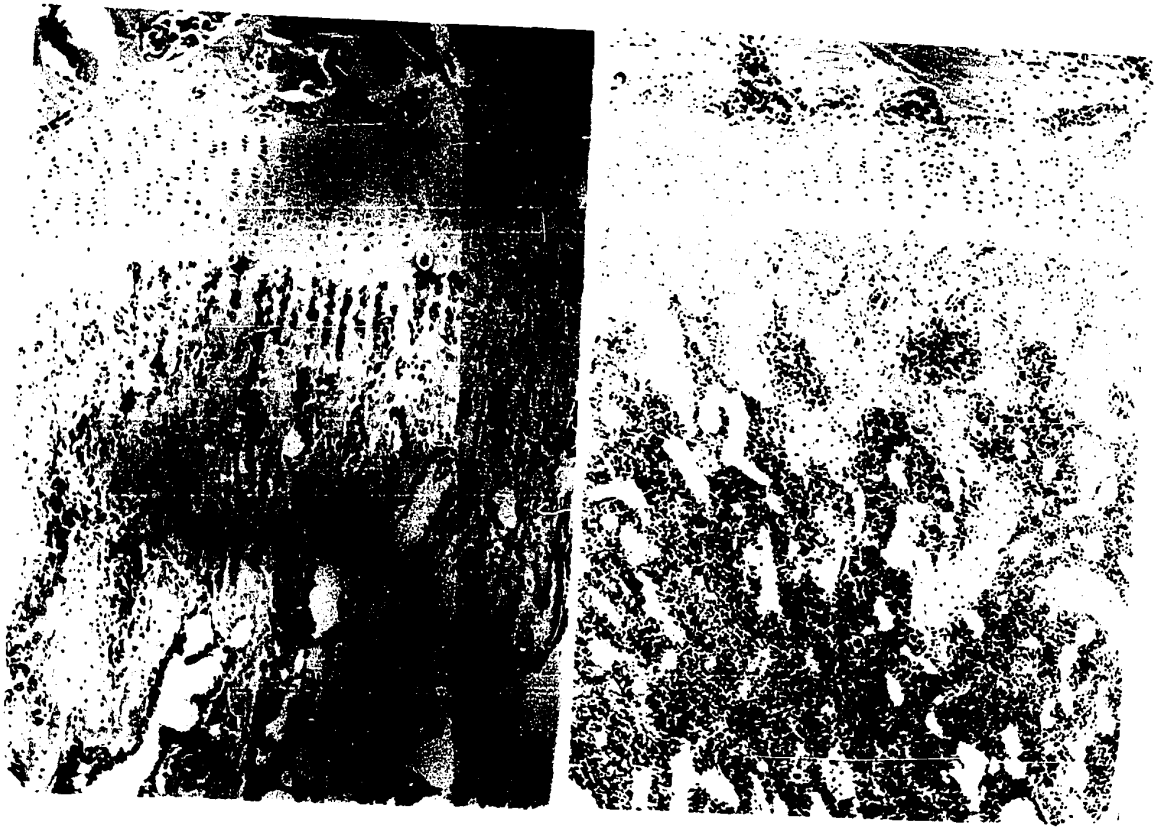


PLATE 23

Longitudinal section through the epiphyseal region of the femur of thyroidectomized rats. Compare with sections in Plate 22.

Diluted Wright stain x80

Figure A. Upper left. From a rat fed an adequate magnesium-supplemented diet. The epiphyseal plate is narrower than normal and subepiphyseal spicule formation is retarded. Compare with Plate 22, Figure A.

Figure B. Upper right. From a rat fed a magnesium-deficient diet for 25 days. The epiphyseal plate is narrow. Spicule formation is extremely sparce. Compare with Plate 22, Figure B.

Figure C. Lower left. From a rat fed an adequate magnesium-supplemented diet and administered daily replacement doses of L-thyroxin. Although not as wide as that of the intact rat, the plate appears normal. Spicule formation has also returned to normal.

Figure D. Lower right. From a rat fed a magnesium-deficient diet and administered daily replacement doses of L-thyroxin for 25 days. The epiphyseal plate has remained small. Compare with Figures B and C. However, the growth of spicules is much increased over that seen in Figure B.

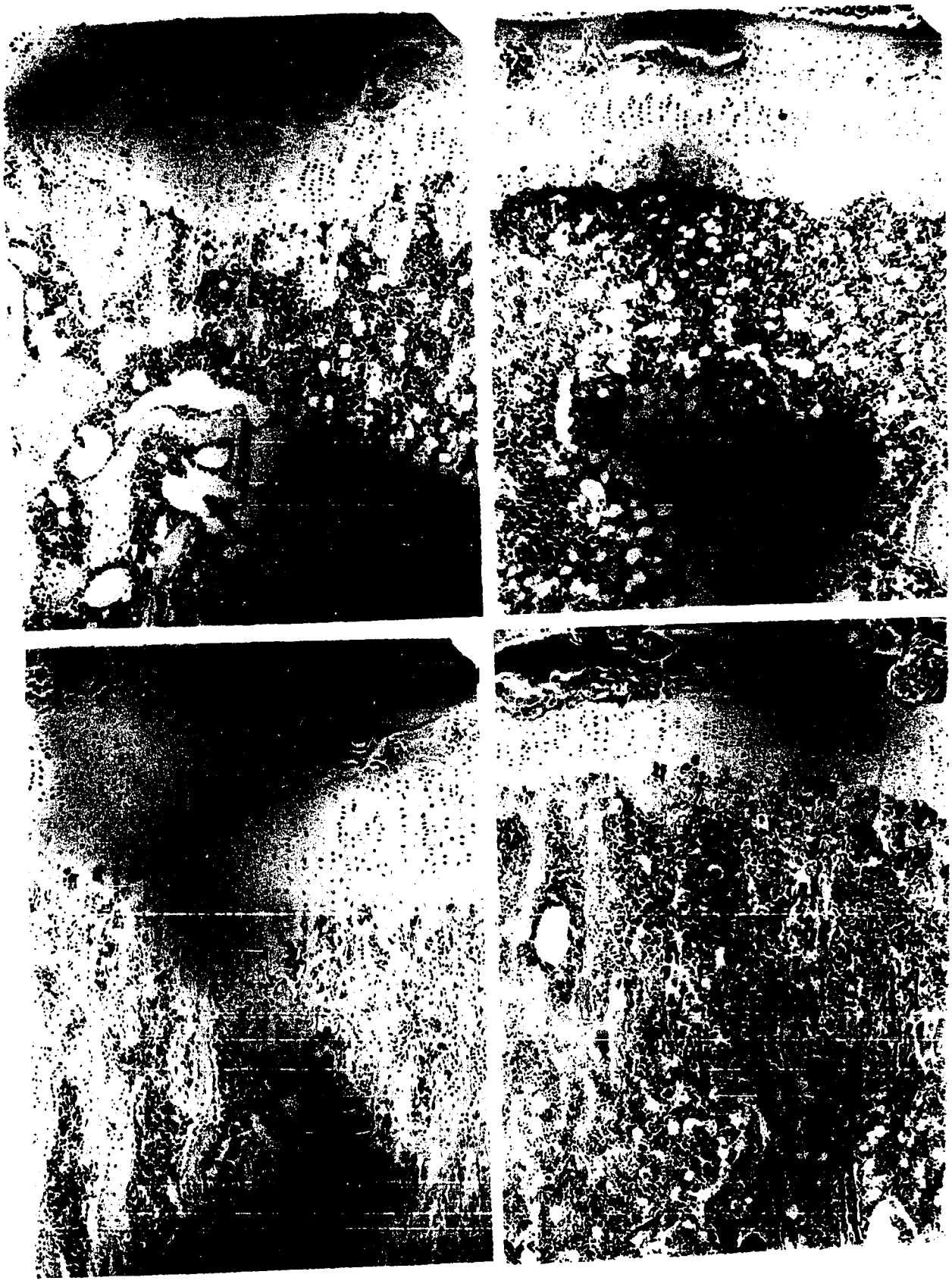


PLATE 24

Alpharadiographs of longitudinal sections through the epiphyseal region of the femur of rats. x80

Figure A. Upper left. From an intact rat fed a magnesium-supplemented diet.

Figure B. Upper right. From an intact rat fed a magnesium-deficient diet for 20 days.

Figure C. Lower left. From a thyroidectomized rat fed an adequate magnesium-supplemented diet and injected daily with replacement doses of L-thyroxin for 20 days.

Figure D. Lower right. From a thyroidectomized rat fed a magnesium-deficient diet and injected daily with replacement doses of L-thyroxin.

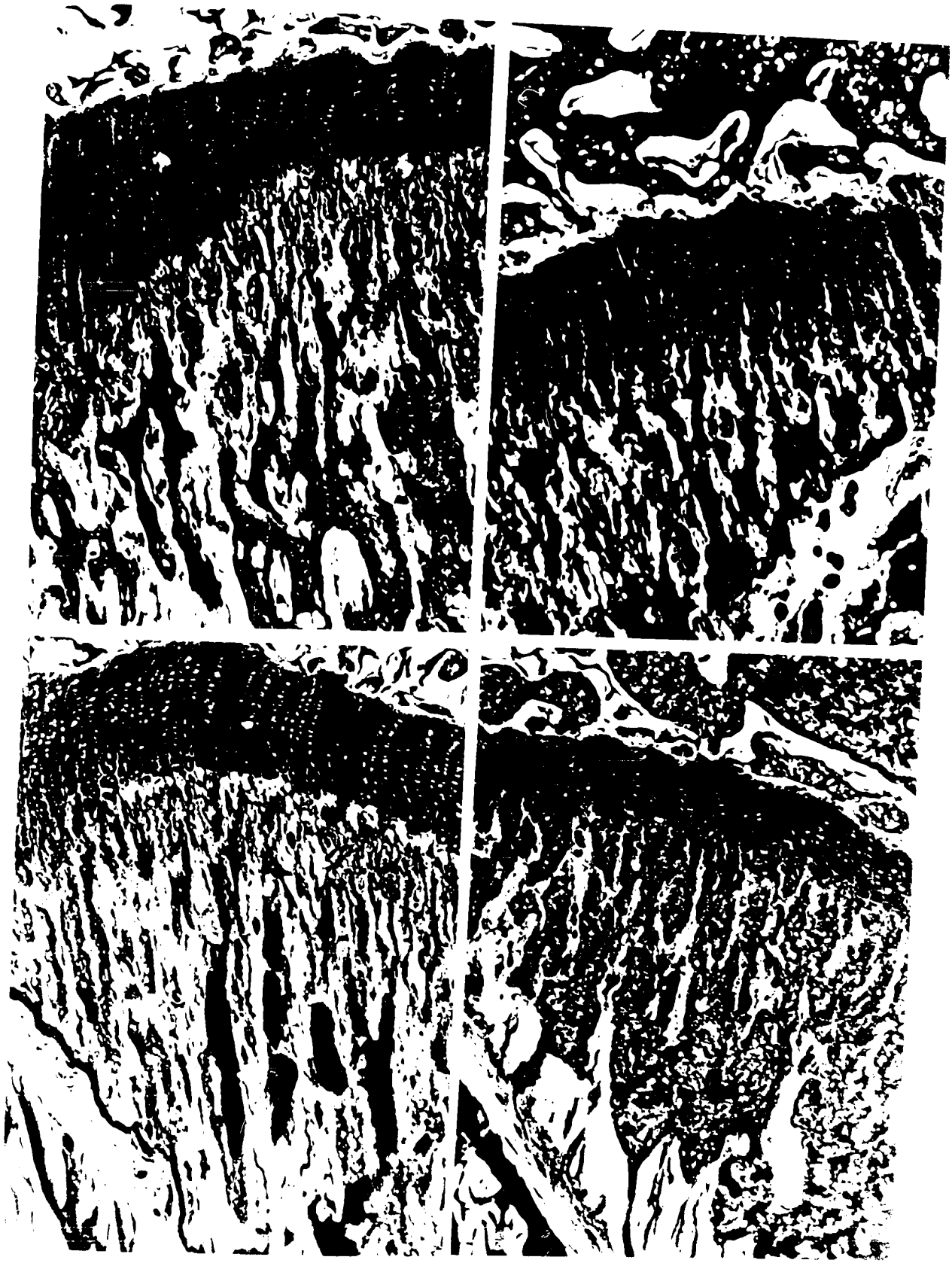


PLATE 25

Alpharadiographs of cross sections through the shaft of the femur of rats. x80

Figure A. Upper. From an intact rat fed a magnesium-supplemented diet for 20 days.

Figure B. Middle. From a thyroidectomized rat fed a magnesium-supplemented diet for 20 days. Compared to Figure A, the width of the shaft is greatly reduced.

Figure C. Lower. From an intact rat fed a magnesium-deficient diet for 20 days. As in Figure B, the width of the shaft is reduced.

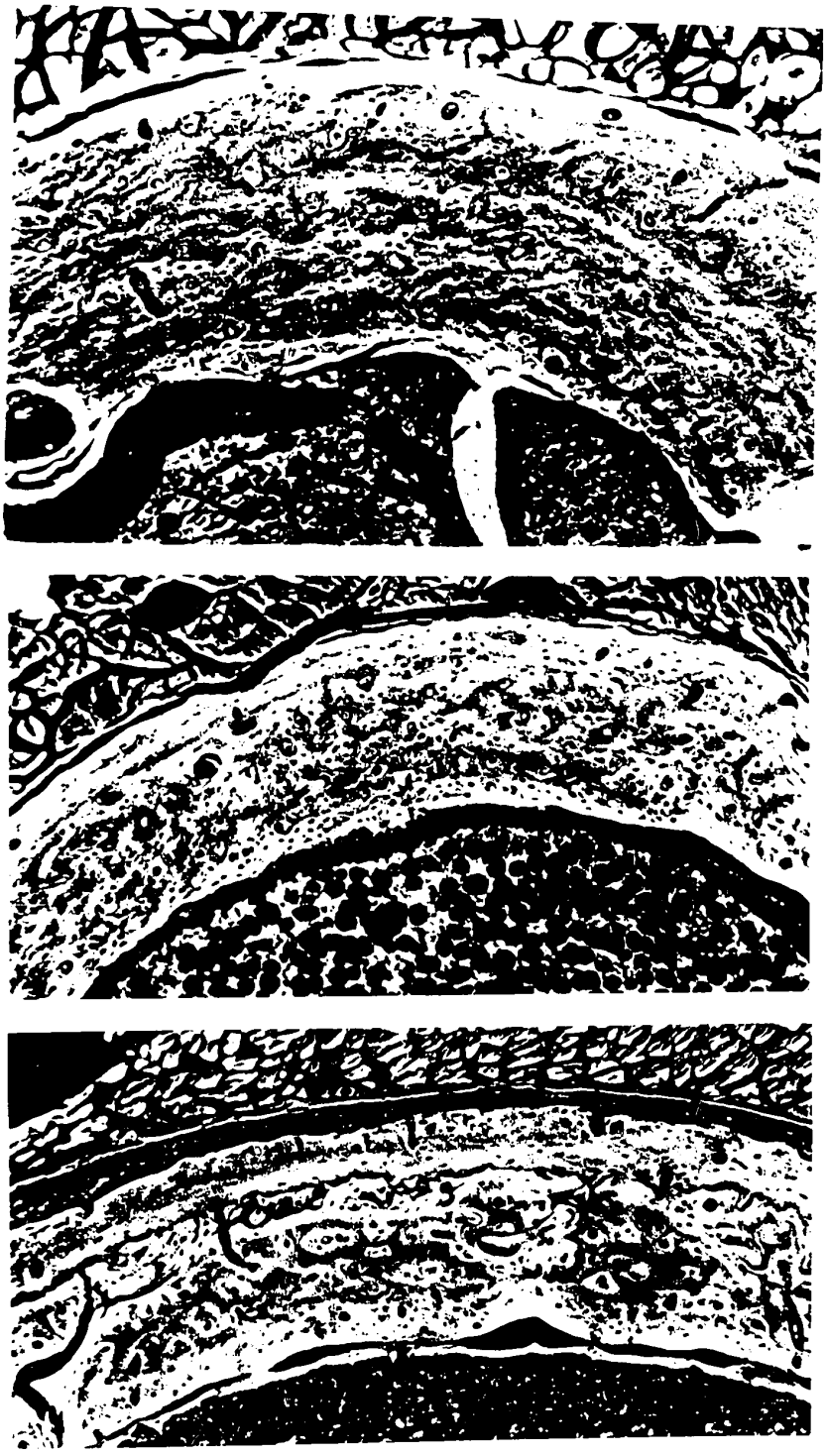


PLATE 26

Figure A. Upper left. The cortical portion of the kidney of an ovariectomized rat fed a magnesium-supplemented diet.

Figure B. Upper right. The cortical portion of the kidney of an ovariectomized rat fed a magnesium-supplemented diet and injected daily with 25 ugms. 17, B-estradiol.

Figure C. Lower left. The cortico-medullary portion of the kidney of a rat treated as in Figure A.

Figure D. Lower right. The cortico-medullary portion of the kidney of a rat treated as in Figure B.

The kidney of the estradiol-treated rat contains engorged blood vessels (Figure B) and focal necrosis of tubules (Figures C and D).

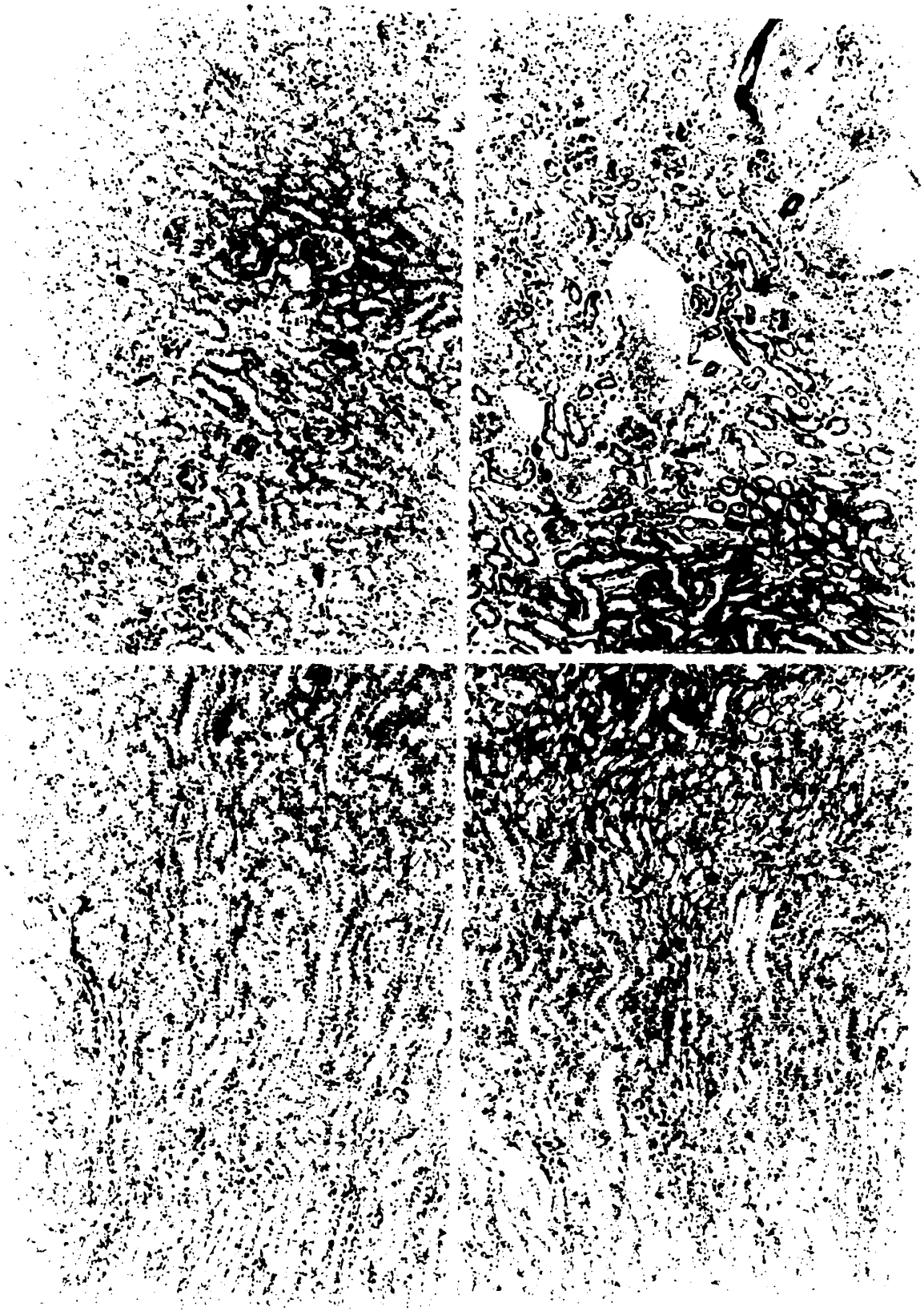


PLATE 27

Figure A. Upper left. The cortical portion of the kidney of an ovariectomized rat fed a magnesium-deficient diet for 20 days.

Figure B. Upper right. The inner cortex in the cortico-medullary area of the kidney of an ovariectomized rat fed a magnesium-deficient diet for 20 days and injected daily with 25 ugms. 17, B-estradiol

Figure C. Lower left. The cortico-medullary portion of the kidney of a rat treated as in Figure A.

Figure D. Lower right. The cortico-medullary portion of the kidney of a rat treated as in Figure B.

Some areas of the kidney cortex appear to show white cell infiltration and/or necrosis especially around dilated blood vessels (Figures A and C). Focal necrosis of tubular cells is more extensive in the kidney of the estradiol-treated rat on the magnesium-deficient diet (Figure C).

Calculi are present in the tubules of the cortico-medullary area of the kidneys of ovariectomized, magnesium-deficient rats whether or not estradiol was administered (Figures B, C, and D). In the kidney of the rat administered the large doses of estradiol (Figure D), the number of tubules affected and the size of the calculi is greater than those in the kidney of the rat without hormone treatment (Figure C).

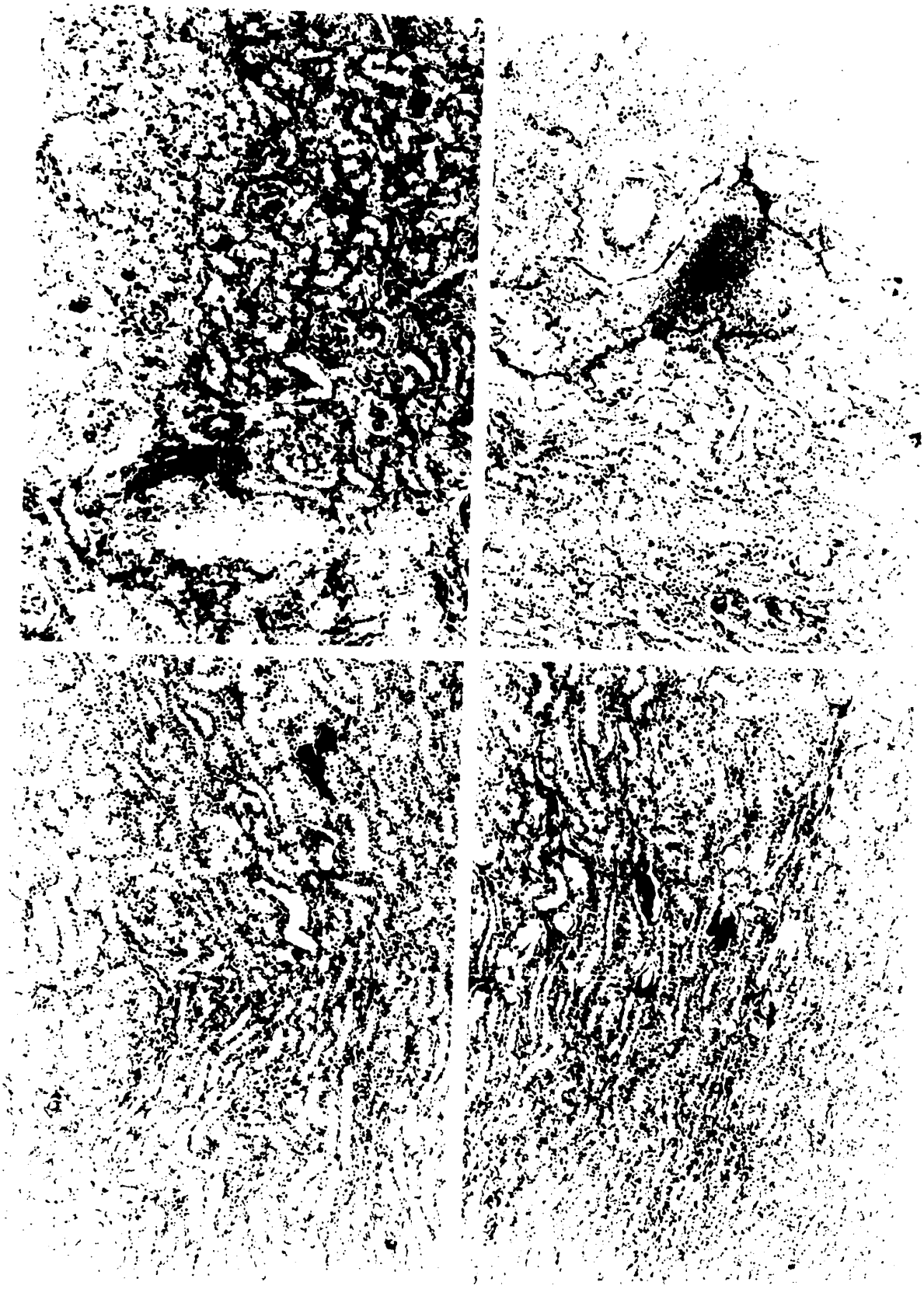


PLATE 28

Longitudinal section through the epiphyseal region of the femur of intact and ovariectomized rats.

Diluted Wright stain x80

Figure A. Upper left. From an intact rat fed an adequate magnesium-supplemented diet.

Figure B. Upper right. From an intact rat fed a magnesium-deficient diet for 20 days. The epiphyseal plate is narrower than in Figure A.

Figure C. Middle left. From an ovariectomized rat fed an adequate magnesium-supplemented diet. The epiphyseal plate is somewhat enlarged in comparison with Figure A.

Figure D. Middle right. From an ovariectomized rat fed a magnesium deficient diet for 20 days. The epiphyseal plate is narrower than in Figure C, but wider than Figure B. Spicule formation is retarded.

Figure E. Lower left. From an ovariectomized rat fed an adequate magnesium-supplemented diet and injected daily for 20 days with 25 ugms. 17, B-estradiol. The epiphyseal plate appears narrower than Figures A or C. Resorption of spicules is retarded.

Figure F. Lower right. From an ovariectomized rat fed a magnesium-deficient diet for 20 days and injected daily with 25 ugms. 17, B-estradiol. The epiphyseal plate is narrower than in Figure D but not than Figures B or E. As in Figure E, spicule resorption appears to be retarded.

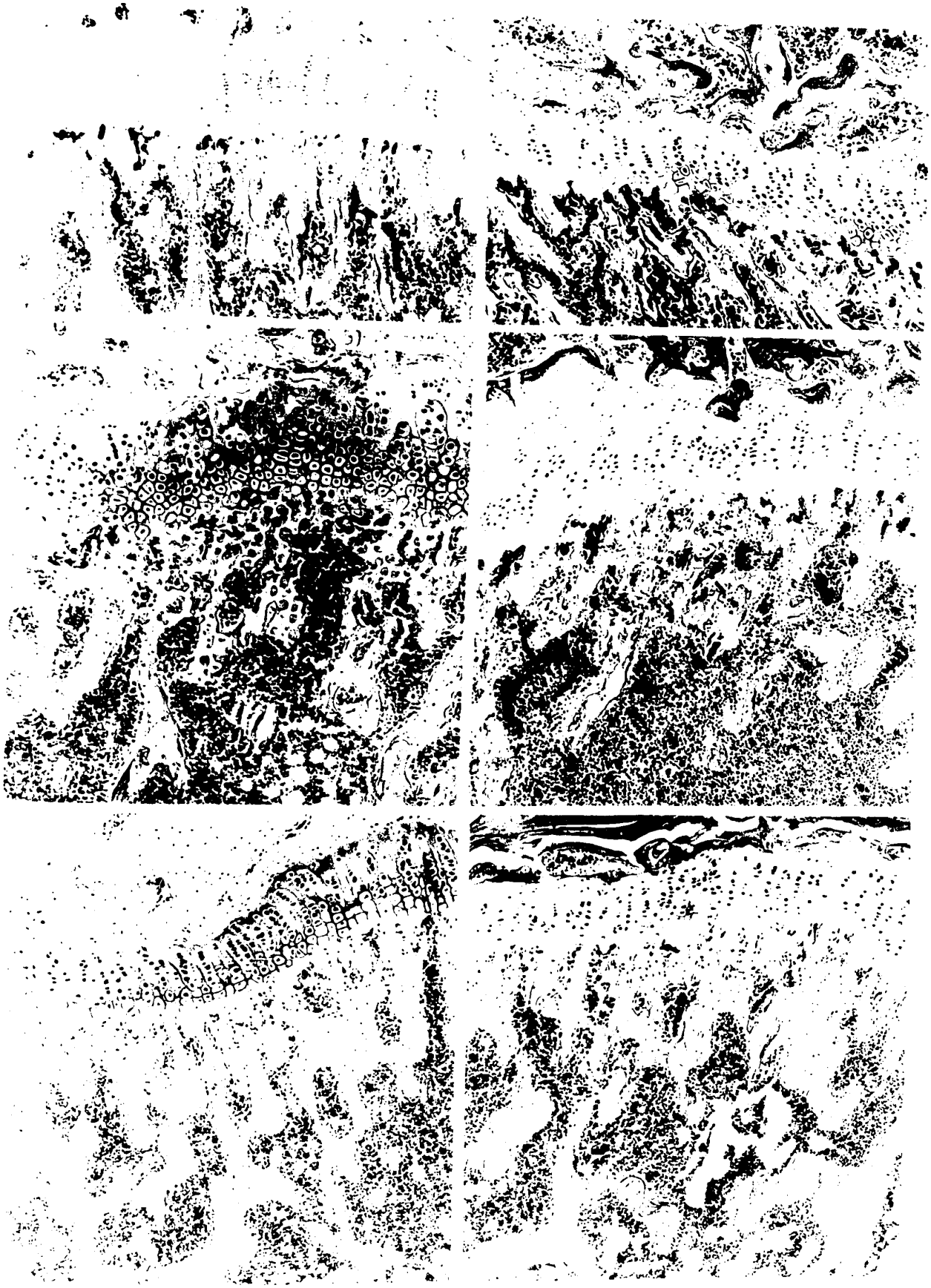


PLATE 29

Alpharadiographs of cross sections through the mid-portion of demineralized tibial diaphyses of rats fed a magnesium-supplemented diet for 20 days. x80

Figure A. Upper. From an intact rat.

Figure B. Middle. From an ovariectomized rat.

Figure C. Lower. From an ovariectomized rat injected daily with 25 ugms. 17, B-estradiol.

Unresorbed cartilage is present in the diaphyses of all animals. The matrix surrounding the cartilage islands (arrows) appears denser than that elsewhere.

PLATE 30

Alpharadiographs of longitudinal sections through demineralized femurs of ovariectomized rats. Compare Figs. A and B.

x80

Figure A. Left. Rat was fed a magnesium-supplemented diet for 20 days.

Figure B. Right. Rat was fed a magnesium-deficient diet for 20 days.

Note the decreased thickness of the epiphyseal plate in Fig. B. In the metaphyseal trabeculae, the osteocytic lacunae are larger in Fig. A, but the matrix is denser in Fig. B.

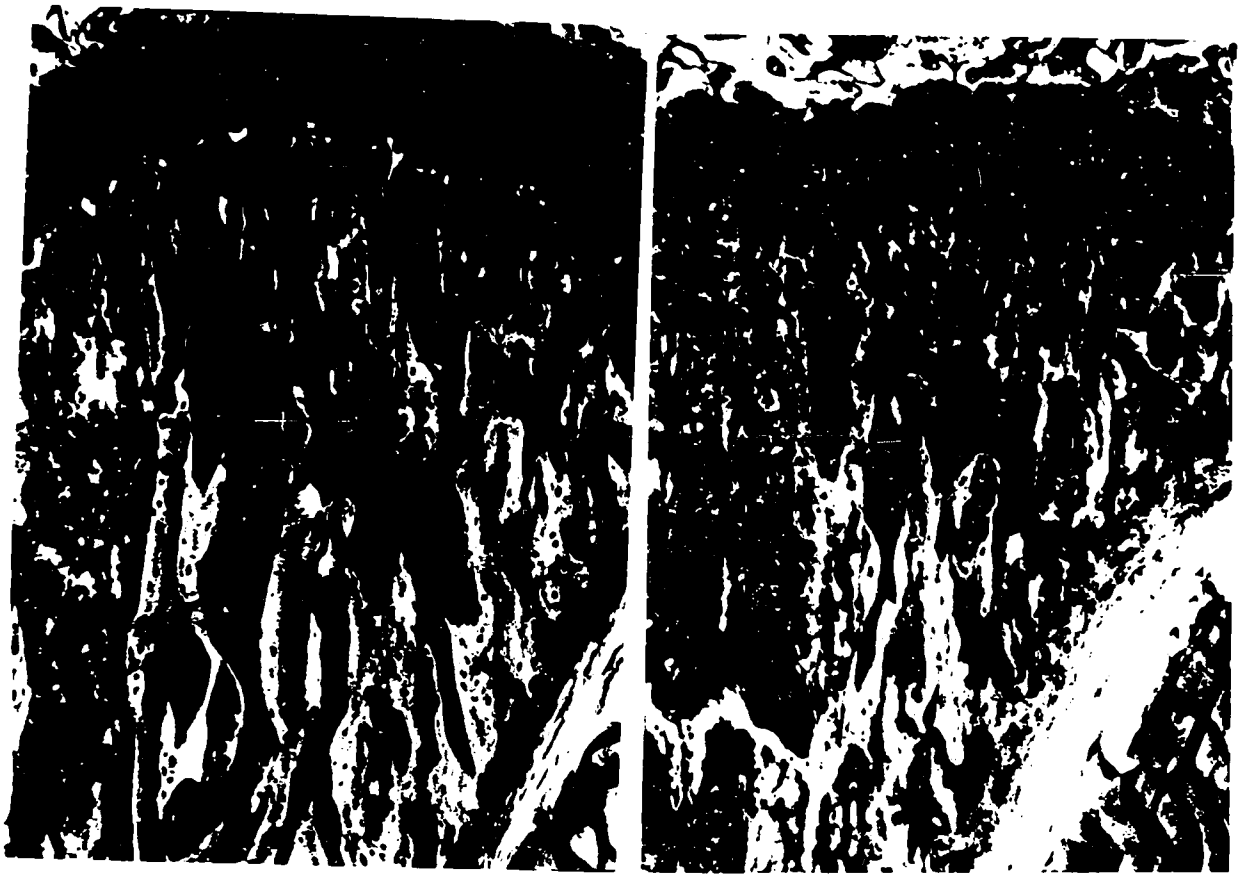


PLATE 31

Longitudinal section through the shaft of the femur of an ovariectomized rat fed a magnesium deficient diet for 20 days.
D = diaphyseal bone; EB = endosteal bone; M = bone marrow.

HPO x80


EB

PLATE 32

Sections through portions of incisors of ovariectomized rats.

HPO x80

Figure A. Upper left. From a rat fed a magnesium-supplemented diet.

Figure B. Upper right. From a rat fed a magnesium-deficient diet.

Figure C. Lower left. From a rat fed a magnesium-supplemented diet and injected daily with 25 ugms. 17, B-estradiol.

Figure D. Lower right. From a rat fed a magnesium-deficient diet and injected daily with 25 ugms. 17, B-estradiol.

Only the ovariectomized untreated rat fed the magnesium-deficient diet shows dentinal striations (alternate bands) and irregularity of the odontoblast layer. The dentinal striations are not seen in the magnesium-deficient rats injected with estradiol.

PLATE 33

Alpharadiograph of a section through the tooth of an ovariectomized rat fed a magnesium-deficient diet. Note bands of alternating densities. Compare with Plate 32, Fig. B. x80

PLATE 34

Autoradiographs of epiphyseal cartilage of femurs of rats injected with 1 mCi. H^3-glycine and sacrificed after 24 hours.
NTB 2 diluted emulsion. Exposure 4 months.

Kingsley stain x500

Figure A. Left. From a rat fed a magnesium-supplemented diet for 20 days. The greatest concentration of label is over the azurophilic matrix of the proliferative cell layer. The chondrocytes are not labeled.

Figure B. Right. From a rat fed a magnesium-deficient diet for 20 days. The cartilage cells and the hyaline cartilage matrix are reduced in size. There is less label in the cartilage matrix of the deficient rat. Compare with Fig. A.

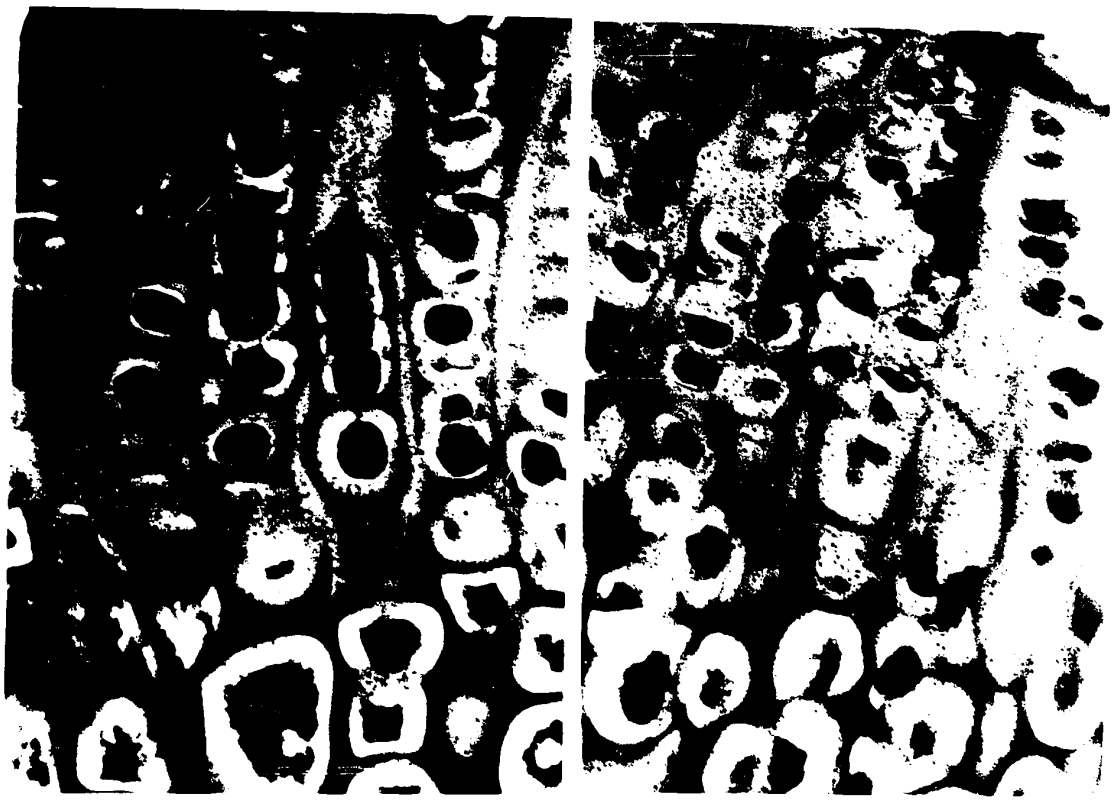


PLATE 35

Autoradiographs of the subepiphyseal region of the femur of rats injected with 1 mCi H^3-glycine and sacrificed 24 hours later. NTB 2 diluted emulsion. Exposure, 4 months.

Kingsly stain x200

Figure A. Upper. From a rat fed a magnesium-supplemented diet for 20 days. Grains are localized along the endosteal surfaces of the spicules.

Figure B. Lower. From a rat fed a magnesium-deficient diet for 20 days. Very few grains can be seen lining the endosteal surfaces of the spicules.

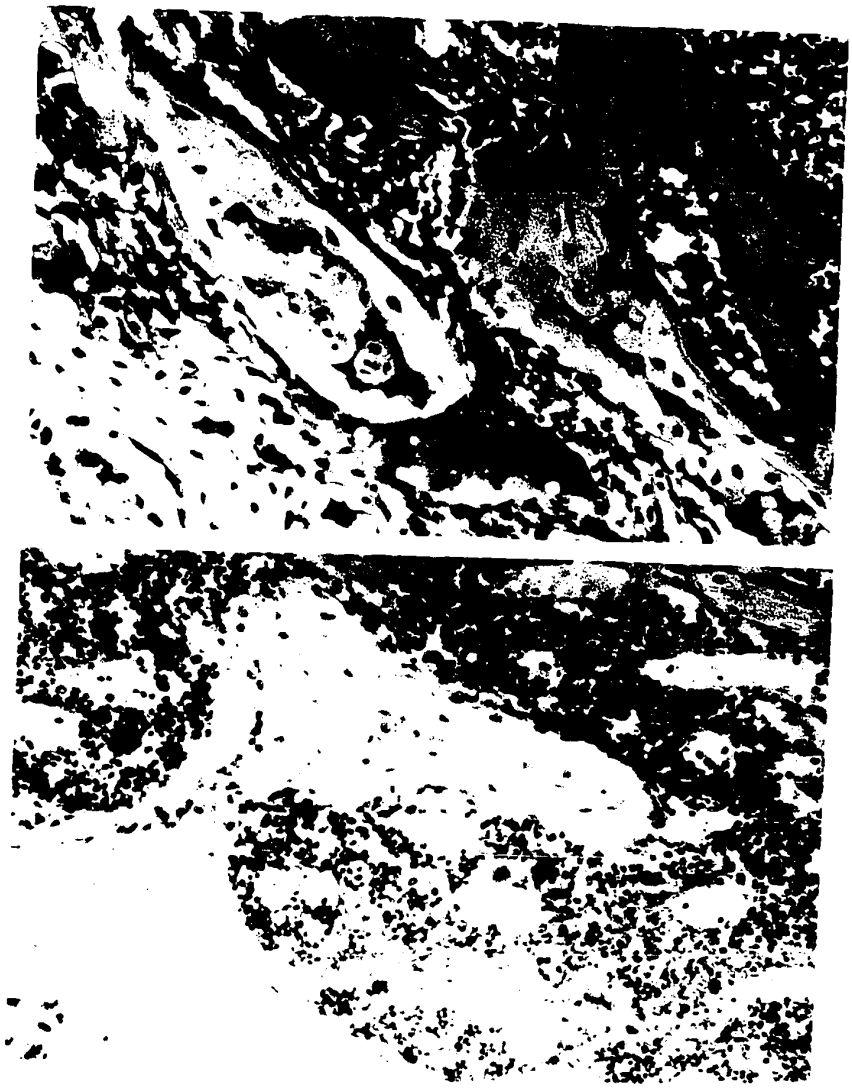


PLATE 36

Autoradiographs of a longitudinal section through the conical portion of the femurs of rats injected with 1 mCi H^3-glycine and sacrificed after 24 hours. NTB 2 diluted emulsion. Exposure, 4 months.

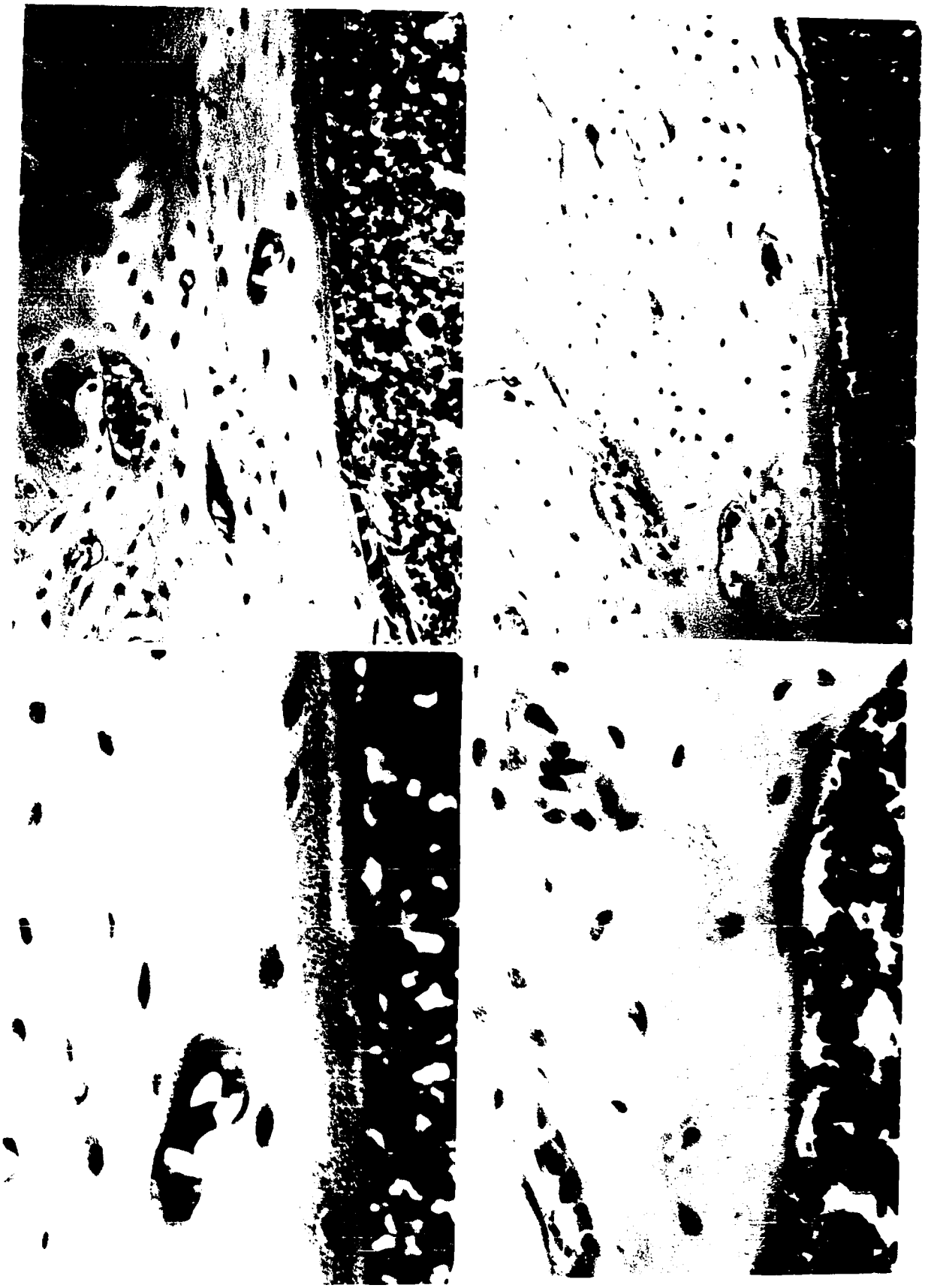
Kingsley stain

Figure A. Upper left. From a rat fed a magnesium-supplemented diet for 20 days. Grains are localized along the length of the endosteal surface of the bone. The osteoblasts lining Haversian canals are also labeled. x200

Figure B. Upper right. From a rat fed a magnesium-deficient diet for 20 days. There are very few grains lining the endosteal surface of the bone. x200

Figure C. Lower left. High power view of Fig. A. x500

Figure D. Lower right. High power view from an area similar to Fig. B. x500



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Appendix 1

Magnesium-deficient Diet[1]

Formula	gms/kg
Casein	300.00
Destrose	499.66
Corn oil	150.00
Salts (see below)	21.48
Sodium bicarbonate	12.60
Potassium bicarbonate	15.10
Vitamin mix (see below)	

Salt mix

Calcium chloride	5.700
Calcium phosphate	14.300
Cupric sulfate	0.013
Ferric citrate	1.240
Manganese sulfate	0.1739
Sodium iodide	0.030
Zinc sulfate	0.024

Vitamin mix

Calcium pantothenate	0.020
Choline chloride	1.000
Niacin	0.040
Pyridozine	0.004
Riboflavin	0.008
Thiamine	0.004
Vitamin A	37,500,00.0 IU
Vitamin D_2	5,400,00.0 IU
1% vitamin fortification mix	

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[1] Ko. K.W.; Fellers, F.X; and Craig, J-M. Lab. Invest. 11:294 (1962)

Appendix 2

Procedures for Staining Histological Sections

1. Diluted Wright Stain

   A. Dilute Wright Stain with equal volume of distilled water

   B. Bring paraffin sections down to water

   C. Stain sections 30 minutes in Diluted Wright stain

   D. Wash sections in distilled water to remove excess stain

   E. Rinse sections quickly in 95% ethanol and 3 changes of absolute ethanol.

   F. Clear sections in 3 changes of xylol and mount them under a cover slip with Permount.

2. Hematoxylin-phloxine-orange G

   A. Bring paraffin sections down to water

   B. Stain sections in filtered Delafied's hematoxylin for 6 minutes

   C. Wash sections in distilled water to remove excess stain

   D. Differentiate with acid alcohol

   E. Rinse sections in distilled water followed by 1 minute in distilled water containing a pinch of lithium carbonate.

   F. Wash sections in tap water for at least 15 minutes

   G. Stain sections in 1% aqueous phloxine for 6 minutes

   H. Wash the sections rapidly with 95% alcohol and 2 changes of absolute alcohol, followed by a 2-minute change in fresh absolute alcohol.

   I. Clear the sections for 2 minutes in each of three changes of xylol; and mount the sections under a cover slip with Permount