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BONE AND CARTILAGE INDUCTION PRODUCED BY GRAFTS OF DEVITALIZED BONE FROM NORMAL AND CATION-DEFICIENT RATS

by

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SCOPE

The induction of new bone by grafting different tissues and materials into muscle or connective tissue of adult mammals, is a well known phenomenon. A very thorough analysis of the mechanisms of bone induction produced by grafts of bone devitalized following various procedures has been conducted for several years by the group of Urist. These studies however have been focussed on the nature of the inductor principle.

Studies on the influence of physiological or pathological variations of the hosts on the induction process are, contrarywise very scarse.

In recent contributions, Bélanger, Robichon and Urist (1975) have studied the effect of magnesium deficiency in the host's response to normal graft.

The present study analyzing further the influence of magnesium - calcium - and zinc deficiency on the evolution of grafts constitutes a continuation of the above mentioned study.

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CHAPTER I

INTRODUCTION

Late in the 19th century, some important surgeons used decalcified bone for filling cavities, which were the result of some bone diseases (Senn, M. 1889). Using the technique developed by Senn, positive results were reported by Makie in 1890.

Nageotte, (1918), Polettini, (1922) and De Bruyn, (1947), reported the use of autogenous bone devitalized by freezing in liquid nitrogen, dry ice, formalin, alcohol or heat, and transplanted to the thigh muscle of the rabbit; in a few instances, there was bone formation. Levander (1938) thought that the stimulating agent might possibly be transferred from the graft to the surrounding area in the form of some substance which is soluble in the tissue lymph. He believed that the different forms of bone regeneration encountered in adult organisms could be explained by the inductive process described by embryologists. In subsequent experimentation, Kimbal (1949), had success in one instance. He produced bone formation using boiled homogenous bone implanted in the anterior chamber of the eye in rabbits and guinea pigs. P. Weiss (1950) found new cartilage surrounding an

implant of freeze dried cartilage in salamander larvae. He explained that this inductive phenomenon is due to the existence of an orientation of molecules on the surface of cells from the graft, while the host cells which are in direct contact with the graft copy that molecular orientation with a high degree of specificity and complexity on the basis of both physical and chemical mechanisms.

Since 1952, Urist's laboratory has produced most of the works related to the use of decalcified and devitalized bone as a substrate for the stimulation of bone formation. Urist and McLean (1952) described the properties of the bone grafts devitalized by freezing and implanted in the anterior chamber of the eyes of rats. The result was formation of new bone which, according to the authors, originated from the cells of the host by contact between the transplanted bone and the tissue of the host. They claimed that the process of "induction" was responsible for the new bone formation. The next year, Urist and McLean (1953) defined the concept of induction and the differences between new bone formation by induction and bone formation from tissues which have osteogenetic potency. They explained

as follows: the term osteogenetic potency describes the capacity of certain tissues to produce new bone. This potency is demonstrated by osteogenetic activity following injury or autogenous transplantation. The term "induction" designates the physical-chemical effect of one tissue upon another in contact with it. The new bone formation is originated from the perivascular connective tissue and is generally associated with absorption of bone or cartilage after injury or transplantation. In subsequent experiments, Selle and Urist (1961) produced damage by injection with various chemical solutions (Alcohol and hydrochloric acid, alcohol in different concentrations). The injury was made in different muscles of the rabbit. Formation of new bone did not occur except with acid alcohol solution. They then concluded that non-specific injury and inflammation did not produce ectopic bone formation. Factors other than mechanical injury and necrosis of tissue are required to produce an inductor system for new bone formation. In later works by Heiple et al, (1963), they demonstrated that the inductive potency of the bone preserved in various ways was altered. They found the highest inductive activity in bone treated by freeze dried and the lowest in deproteinized bone.

In earlier work, Urist and McLean (1952), Chalmer and Sissons, (1959), Zaccalini and Urist, (1964), had found formation of bone produced by implant of bone treated by freeze dried process, in only 30% of the cases. In 1965, Urist gave clear evidence of bone formation (90% positive cases) using fragments of a cylinder of long bone (1 or 2 cm in length, unfixed, decalcified in 0.6 N. HCl and lyophilized), from rabbit and rat. The grafts (diaphysis from long bone) were implanted in a pouch in the muscle, usually rectus abdominus. After three weeks, the grafts showed an envelope of highly vascular inflammatory and fibrous connective tissue. The interstitia of the implanted bone and the old vascular channels were infiltrated with histiocytes, large and small lymphocytes and fibroblasts. The invading cells of the host, chiefly wandering histiocytes, were first related to the process of resorption of the implanted graft. They were identified in excavation chambers and in the vascular channels of the decalcified matrix or between folds of compressed softened matrix, and consisted of nests of proliferating cartilage cells. The new bone gre[illegible]rom the proliferating connective tissue cell associated with vascularization. The bone

and cartilage induction was confined to the spaces created by resorption of the dead matrix. Finally, Urist said that the new bone was produced by autoinduction in which non-specific substances or degradation products of the dead tissue stimulated or attracted the wandering histiocytes to migrate into the interior of the implant and the induction process produced an entirely new ossicle with a marrow cavity.

Many authors have done similar experiments. Unfortunately, the experiments were done in slightly different ways by each investigator; each one employed a different solution for decalcification.

Van de Putte and Urist, (1966) implanted cylinders of bone previously treated with various decalcified solutions in the abdominal muscle of the rabbit. They found that bone decalcified with HCl 5% and EDTA (ethylenediaminetetracetic acid) produced new bone in approximately 90% of the cases. This report confirmed the suggestion of Ray and Holloway (1975), who said that decalcified bone is absorbed more rapidly than undecalcified bone, and thereby may be replaced sooner by new bone. This was also confirmed by Sharrard and Collins, (1961) who reported favourable results after

the use of EDTA decalcified homogenous bone in spinal fusion in children with scoliosis. In subsequent publications, Urist et al, (1967) described a bone induction principle (BIP), which is defined as protein macromolecules of unknown structure present in dentin and bone matrix, which produce differentiation of the mesenchymal cells in the osteoblasts.

Also using grafts, Burwell and Oswestry (1966), confirmed earlier experiments by showing that the induction capacity depends upon the way in which the bone had been treated before implantation. Buring (1968, 1969) found that there was a loss of inductive properties of bone after irradiation, the magnitude of the loss depending on the dose applied. This finding however, contradicts an earlier paper by Chalmers and Sisson (1959), who did not note a destructive influence of radiation on the inductive properties of bone grafts. In 1971, Urist observed the evolution of the implanted bone grafts prepared according to his standardized techniques, using electron micrographic and chemical techniques. In this report he suggested that both the three dimensional complex structure of the substratum and chemical components of bone tissue act

jointly to impose a bone morphogenetic pattern on proliferating mesenchymal cells. He also said that the collagen must be very well preserved in order to retain its morphogenetic properties.

Most of the work at this time was directed to researching the nature of the inductor. Urist (1967) reported that the inductor is an acid insoluble, macromolecular protein(s), which is devoid of antigenic activity, and can be transmitted for short distances, but is not free in solution in the extracellular space. It is not inactivated by freezing and thawing, radiation or by protein denaturating agents. Later, Urist et al, (1973) prepared bone grafts which were treated with different combinations of six extractive solutions. (Two examples of these solutions were: chloroform-methanol, to extract lipid and inhibit or extract endogenous enzymes, and EDTA to remove residual calcium and extract phosphoproteins). The residue of bone insoluble matrix (Insoluble bone gelatin, Urist, 1968), produced new bone when it was implanted in allogenic rats. The yield of bone was lower than the one produced in previous studies. Urist explained that the agents responsible for the degradation of the "bone morphogenetic protein"

(Urist, 1971), were extracted by chemical solutions. Nogami and Urist (1974), claimed that in tissue culture the insoluble bone gelatin induces the development of cartilage only while in intact animals, it evokes differentiation of bone as well as cartilage. Electron microscopical observations (Urist 1971, Nogami and Urist, 1974) showed that filopodial extensions of the plasma membrane of neighbouring mesenchymal cells project into the bone matrix substratum in the period between the second and third day after implantation. They suggested that the filopods may be a means of transferring biochemical or biophysical information from the matrix to the mesenchymal cell membrane. Deposits of filament beaded with ruthenium stained fine granules were also observed in the extracellular space. Similar experiments made by Terashima and Urist, (1975) found that the bone matrix gelatin in tissue culture produced cartilage. They think that the bone morphogenetic protein stimulated the mesenchymal cell differentiation into prechondroblasts during their migratory phase, while the effect of the culture medium is to provide the bionutritional requirements for synthesis of cartilage. In recent works,

Nogami and Terashima (1976) put the bone gelatine (Urist, 1968) after digestion in collagenase, into a special double-chamber millipore device. When implanted, the inductor-factor must diffuse through the interstitial fluid for a distance of at least 2000μm which is the thickness of the upper compartment of the duplex chamber before it can act on host tissues.

New bone was deposited in the external surface proving that the inductor is diffusible. Since bone is not deposited when the insoluble bone gelatin is not treated with collagenase. The authors concluded that collagenase digestion of the bone gelatin and subsequent action of other enzymes in vivo after implantation may facilitate release of the bone morphogenetic protein and enhance the bone morphogenetic response. Studies to characterize and isolate bone morphogenetic protein are being pursued by Urist's group.

INFLUENCE OF MAGNESIUM IN BONE

Magnesium is the fourth most abundant cation in the skeleton. The skeleton contains about half of the total amount of magnesium in the body. An adult man weighing about 70 kg has a total body content of approximately 2,400 mEq of magnesium. The remainder being equally distributed between muscle and non-muscle soft tissues (Aikawa, 1963). The magnesium requirement of adult males has been estimated by balance techniques to lie between 200 mg and 300 mg a day. The magnesium plasma level in normal human beings is in the range of 1.5 - 1.8 mg per 100 ml (Foster, 1968). England, Denton and Randle (1967) have estimated by measuring the aconitase equilibrium that the free magnesium ion concentration in heart mitochondria is in the range of 250-500 μm or approximately twice that in the cytosol. Magnesium has an important role as an activator for a large number of enzymes, some of which are especially important in the skeleton such as alkaline phosphatase and the pyrophosphatases (Tenenhouse and Rasmussen, 1968; Rasmussen, 1972). Large amounts of cellular magnesium are in a

complex formation, such as ATP Mg^{--} in either cytosol or mitochondria. Magnesium is involved in all reactions in which ATP participates and may serve as a dual role as a part of the substrate $ATP\sim Mg^{2-}$ and as an activator of the enzyme (see review Rasmussen and Bordier, 1973). Deprivation of magnesium in the diet produces numerous biochemical and histological alterations which are variable, presumably because the factors involved in magnesium homeostasis are complex.

An examination of the cause of magnesium deficiency should not lead one to the obvious conclusion that variation in magnesium intake is the only factor responsible for the induction of magnesium deficiency. Morris and O'Dell (1963) found that the magnesium requirement in guinea pigs is directly related to the percentage of calcium and phosphorus in the diet. Magnesium deficiency in young growing animals produces a characteristic syndrome. This syndrome has been described by Orent, Kruse and McCollum, 1932 and confirmed later by Watchorn and McCance, 1937; Tufts and Greenberg, 1938; Bélanger, 1957; MacIntyre and Davidson, 1958; Martindale and Heaton, 1964;

Whang and Welt, 1963; Heggtveit, Herman and Mishra, 1964; Clark and Bélanger, 1967; Hunt, 1971 and Hunt and Bélanger, 1972). These authors described initial hyperemia and edema of the extremities of the ears, followed by hemorrhagic and necrotic skin lesions. One of the characteristic aspects of the magnesium deficiency syndrome is the formation of mineralized plaques in various soft tissues of the body. One tissue consistently affected in all species is the kidney. Calcification of other soft tissues, such as the liver or skeletal muscle, occurs less frequently. Increases in tissue calcium content have been found in chronic magnesium deficiency (for example, forty fold increase in calcium in the heart of the dog, Chiemchaisri and Phillips, 1965). The direct cause or causes for mineralization of soft tissues during low intake of magnesium is not known. serious modifications of the lymphatic system, such as formation of thymic tumors and changes in the morphology of lymphatic cells have been reported (Bois, 1964; 1966; 1968) in magnesium deficient rats. During the period of magnesium deprivation, neuromuscular irritability and general growth

retardation are present.

Some authors have found hypercalcemia in magnesium deficient rats (Gitelman, Kukolj and Welt, 1968; Smith and Nisbit, 1968; MacIntyre, 1963; Whang and Welt, 1963; Foster, 1968) and other authors found hypocalcemia (MacManus and Heaton, 1970; MacManus, Heaton and Lucas, 1971). In 1970, MacManus and Heaton stated that hypocalcemia has been reported in cattle, sheep, dogs and pigs, and they think the discrepancy in rats may be due to differences in the calcium content of the diets used. Histological changes in bones from rats under magnesium deficiency have been described by Bélanger (1958), Bernick and Hungerford (1965), Clark and Bélanger (1967), Smith and Nisbit (1968) and Hunt and Bélanger (1972).

These changes are characterized by a decrease in the width of epiphyseal plates and in the thickness of cortical bone. A reduced mucopolysaccharide and protein synthesis was suggested by the results of histochemical and radioautographic techniques (Bélanger, 1972). Hunt and Bélanger in 1972 reported the formation of localized

bone tumours at the level of linea aspera of the femora, as well as the development of an increase in the number and size of medullary trabeculae in growing rats under magnesium deficiency. Similar results were obtained by Bogoroch and Bélanger (1975). The mechanisms by which these histological changes are produced have not been clarified. It has been suggested that magnesium deficiency could alter bone function by affecting its response to parathyroid hormone. Thus, MacManus and Heaton (1970) and MacManus, Heaton and Lucas (1971), working with rat bone in tissue culture, found that the reduction of magnesium concentration to 0.3 - 0.4 mM decreases resorption in parathyroid-treated bone. They suggested that this shows that changes in ion concentration can alter bone resorption and its regulation by hormones.

Changes in bone growth produced by magnesium deficiency do not appear to be related only to interference with the action of parathyroid hormone. It was previously discussed that magnesium acts as a co-factor in numerous enzymatic reactions, including the activation of ATP, and that its

deficiency produces a deficit in protein and mucopolysaccharide synthesis. It is evident that the alterations in bone growth observed in magnesium deficiency could be the result of a defect in one or several of the biochemical processes for which mangesium is a necessary co-factor. At this time it is not possible to identify those co-factors which are principally involved.

INFLUENCE OF CALCIUM IN BONE

The adult human body contains about 1,100 gr of calcium, approximately 1.5 of the body weight. Most of the calcium is in the skeleton. The plasma calcium normally is about 10 mg/100 ml (5 mEq/liter) according to Smith, Davis and Fourman (1960), Peacock and Nordin (1973). Calcium is present in the plasma in three forms: ionized, protein-bound and combined with citrates and other organic acids. Calcium is the most important cation in the process of bone mineralization. Changes in the concentration of calcium in the blood will produce alterations in different systems in which it is involved such as nerve conduction, muscle contraction, membrane transport and calcification in bone. These are some of the reasons for which it is essential that the concentration of this cation should remain constant in plasma and in extracellular and intracellular fluids.

Experimental animal deficiency of calcium without deficiency of vitamin D produces osteoporosis and not rickets, since there is a loss of bone but no increase in width of osteoid or in the amount

of the osteoblastic activity (Sherman, Pappenheimer, 1921). In the rat, a diet poor in calcium and in vitamin D produces a combination of osteoporosis and rickets (McClendon and Blaustein, 1965; Mathieu et al., 1966). Osteoporosis from simple calcium deficiency has been produced only in growing animals (Shah et al., 1967). It has been recognized by several authors that calcium homeostasis has a main role in the mineralization of the bone matrix (Engstrom, 1972; Pyke, in Review by Isadore Zipkin, 1973). The level of calcium in the plasma is maintained by complex, interacting factors whose relation to one another is still not clearly understood. At present, it is recognized that vitamin D through its metabolites, plays an essential role in the strict regulation of plasma calcium, reacting in a complex way mainly with the parathyroid gland, kidney, intestine and skeleton. Important studies about this have been undertaken by Kodicek (1956); DeLuca (1966, 1972); Boyle, Gray and DeLuca (1971).

In experiments in which parathyroid gland was surgically removed, there followed a remarkable fall in blood calcium associated with tetany and convulsions, which could be relieved by calcium

administration (MacCallum and Voegtlin, 1909). After a series of experiments made by different authors such as Collip (1925), Hasting and Huggins (1933), Copp (1960), McLean, in 1957, postulated a feedback mechanism to control secretion of the hormone. Hypocalcemia stimulates its production while hypercalcemia suppresses it. In relation with the histological evidence, parathyroid hormone has at least two effects on calcium mobilization. It rapidly stimulates the translocation of calcium from bone to extracellular fluid. It stimulates osteocytic osteolysis (Bélanger, 1969) and it also promotes the development or arrival of new multinucleated cells which is a later event. The first is seen within 2 hours or at least 24 hours must elapse before any histological evidence of increased resorption or increased numbers of osteoclasts can be seen microscopically (Raisz and Neimann, 1967; Bingham et al., 1969, Reynolds and Dingle, 1970). Following parathyroid hormone administration to a variety of species, there is evidence of a rapid depression of the metabolic activity of osteoblast (Gaillard, 1961, 1967). Flanagan and Nichols (1965) working with bone chips, incubated in vitro, noted

a depression of C^{14} glycine incorporation.

It is already demonstrated (Copp, 1961) that there is a plasma calcium lowering hormone, calcitonin, produced by the C cells of the thyroid gland. They were first recognized as the source of calcitonin in 1964 by Foster, MacIntyre and Pearse. It is also clear, the opposing action of calcitonin and parathyroid hormone in calcium homeostasis (Copp, 1969). Calcitonin is far more effective in young than in old animals and in children rather than adults, since sensitivity to calcitonin is related to rates of bone turnover. There is numerous experimental evidence that calcitonin exerts a direct action on bone resorption. This was first suggested by Milhaud and co-worker (Milhaud et al., 1965). In young animals, a fall in the level of both plasma calcium and phosphate is the most obvious effect of injecting calcitonin.

Radiographic and histological examination of the vertebrae of parathyroidectomized rats given calcitonin show a decreased number of osteoclast (Foster et al., 1966). Whalen and his colleagues have described changes in the bones of young growing rats in which an excess of calcitonin was induced either

by chronic administration of calcitonin or parathyroidectomy or both (Whalen et al., 1973).

Anast and Conaway (1972) have reviewed the evidence. They conclude that in vitro, the resorption of both matrix and mineral is inhibited by calcitonin. Reynolds and Dingle (1970) claimed that calcitonin prevents the differentiation of new osteoclast and decreases the effectiveness of existing osteoclast.

According to Foster et al., 1972, the model of action of calcitonin is unknown. Rasmussen (1972) has proposed a model in which involved cyclic AMP for the events in bone cells when activated by either parathyroid hormone or calcitonin. He admits that the least well established part of the model concerns a postulated intercellular messenger which increases after calcitonin addition and is postulated to alter the uptake of calcium into subcellular compartments.

One of the major relationships in bone mineral homeostasis is in between vitamin D and parathyroid hormone. It is recognized today that vitamin D through its metabolites plays an essential role in the strict regulation of plasma calcium, and to some extent, plasma phosphate, reacting in a complex way

with the parathyroid gland, the intestine, kidney and the skeleton. In 1937, Nicolaysen conclusively demonstrated by a variety of physiological methods, that vitamin D acted by increasing intestinal calcium absorption in rachitic rats. Vitamin D is present in the body in the form of several metabolites with some differences in the physiological activity (De Luca, 1971). When the vitamin D is absorbed from the intestine into the blood stream, it is hydroxylated in the liver to 25-hydroxycholicalciferol 25-OHD_3, then further hydroxylated in the kidney to 1,25-OHD_3 (Fraser and Kodicek, 1970) or to 24,25-$(OH)_2D_3$ depending on the physiological state of the animal. Under hypocalcemic or hypophosphatemic condition, the kidney produced 1,25-$(OH)_2D_3$ (Boyle, Gray and De Luca, 1971). It has long been disputed as to whether vitamin D has a direct action on bone or whether the skeletal lesions associated with vitamin D deficiency are secondary to the faulty absorption of calcium and phosphate. According to Vaughan (1975) present evidences make it clear that the active metabolite 1,25 $(OH)_2D_3$ has a most potent effect on osteoclastic resorption while there is less well defined information suggesting that the metabolite may well

exert an effect on matrix metabolism.

In rickets, endochondral calcification is defective. Calcification along the column of hypertrophic cells does not take place, the invasion by blood vessels is disorderly, and the zone of provisional calcification is not resorbed. In the skeleton, osteoid is laid down on bone surface (see review of Vaughan, 1975).

There is also evidence that vitamin D affects bone matrix and osteoblastic activity. Baylink et al., 1972, using quantitative histological studies, in rats deficient in vitamin D describes a reduced rate of matrix formation, and a slowed rate of mineralization.

The effect of vitamin D deficiency on the skeleton, however mediated, is to cause a deficiency of calcification affecting both bone and cartilage matrix. In children, these results in rickets and in adults in osteomalacia (Mellanby, 1919, Lindquist, 1952). In both, the distinguishing feature is an excess of uncalcified bone.

The administration of excessive amounts of vitamin D promotes serious disorders of calcium metabolism. These can occur in an individual of any

age who receives abnormal amounts of the vitamin. The toxic effects of vitamin D mimic the action of an injection of parathyroid hormone and are manifest as hypercalcemia with numerous clinical consequences, such as, demineralization of bone (Aurbach and Potts, 1967), renal calculi and metastatic calcifications in soft tissues (Barnicot, 1948). The serious toxic manifestation may be seen at calcium blood levels of 15 mg/100. According to Goth (1972), marked hypercalcemia is more likely due to bone resorption of calcium from the intestine. According to Brickman and collaborators (1972), the metabolites responsible for the toxicity of vitamin D seem to be 25-hydroxycholecalciferol which has a greater osteolytic effect than cholecalciferol which has almost none. The most potent bone-removing agent is the 1,25-dihydroxy-derivative.

INFLUENCE OF ZINC IN BONE

About one century ago, it was demonstrated that zinc is essential in the nutrition of some fungus (Aspergillus niger) Raulin (1869). In later research, zinc had been shown to be a constituent of hemosycotypin, the respiratory pigment of the snail Sycotypus (Mendel and Bradley, 1906). In 1934, Todd and co-workers showed the first indisputable evidence that zinc is a dietary essential for the rat. Raper and Curtin (1953) reported that a combined diet of cobalt and zinc prevented dermatitis in pigs. In subsequent experiments in poultry, O'Dell and associates (1958) showed that zinc is required for growth, feathering and skeletal development. Zinc deficiency has been linked with the occurrence of dwarfism and hypogonadism in boys in Egypt and Iran (Prasad et al., 1963, 1966, 1970; Holsted et al., 1972). Concurrently with these nutritional investigations, intensive studies were undertaken of zinc metabolism and the influence in living cell processes. On the biochemical level, zinc has been reported to exert an effect upon enzymes and enzymatic function,

carbohydrate metabolism (Mikoc-Devic, 1970), protein synthesis (Fujoka and Liekerman, 1964 and Williams et al., 1965), and bone formation where this element appears to be instrumental in the mechanism of mineralization (Haumont and Vincent, 1961; Haumont, 1961; Haumont and McLean, 1966).

The total content of zinc in the body is estimated to be 1.4 to 2.3 gr in a man weighing 70 kg (Widdowson et al., 1951). The studies by Forbes and Yohe (1960) have indicated a minimum zinc requirement for growing rats of 12 to 18 ppm, depending on the protein source. It is very well known that the concentration of zinc in the bone of man and experimental animals is higher than in most tissues (Bergman, 1970; Day and McCollum, 1970; Hove et al., 1938; Prasad et al., 1967; Swenerton, 1968; Williams and Mills, 1970).

Reports on the effect of zinc deficiency on growth vary considerably in the literature. It seems that minor variations in zinc content can be associated with considerable growth differences. With 2-4 ppm, Hurley (1963) has observed "very severely impaired growth". At 5 ppm, Millar et al.

(1958) have reported decreased growth. At 15 ppm, "slow" growth has been observed by O'Dell et al. (1958). The normal requirement for rats is unknown, according to Hurley (1963), but the minimum is considered to be 18 ppm. For the human child, the minimum is considered to be 30 ppm (Hurley, 1963).

Congenital deformity is one of the effects which has been described by numerous authors such as Hurley and co-workers (1968, 1971). They reported that pregnant rats maintained on a zinc deficient diet through gestation produced fetuses with congenital malformation. These malformations encompassed a wide variety of organs and systems, including the skeletal system. The skeletal defects were characterized as missing or short mandible, vertebrae, digits, deformities of the skull, abnormal curvature of the spinal column, and missing centre of ossification (Hurley and Swenerton, 1971). Histological changes in bones of zinc deficient rats were reported in 1941 by Follis et al. They observed that the epiphyseal cartilage was narrower and hypertrophic cells were few in zinc deficient animals. Subsequently, Hoekstra, 1969, reported alterations in the epiphyseal plate in zinc

deficient chicks. The cells near blood vessels were larger and rounder and separated by abundant extracellular matrix. Bergman et al., in 1972 found that animals on zinc deficient rations, revealed a reduction of cell proliferation in areas of the mandibular condyle and the proximal epiphysis of the humerus. Thus, they suggested that the cellular proliferation of undifferentiated mesenchyme is affected by a zinc deficient diet.

Zinc has an important role in the reaction of a number of enzymes as an essential part of the enzyme molecule or loosely bound ion (Mikoc-Devic, 1970). In 1939, Keilin and Man showed that zinc is a constituent of the enzyme carbonic anhydrase, but Hove, Elvehjem and Hart (1940) and Day and McCollum (1940) found no changes in carbonic anhydrase activity in cases of zinc deficiency. Later Tupper, Watts and Wormall (1952) showed in studies with Zn^{65} that zinc in carbonic anhydrase was firmly bound to the protein. These results are in agreement with the observations of Keilin and Man (1940) who said that zinc is undoubtedly firmly bound to the protein and is not removed by dialysis, even when the enzyme has become

inactivated by long standing. Later investigations described alterations of alkaline phosphatase activity in magnesium deficiency. Westmoreland and Hoekstra (1969) studying zinc deficient chicks, reported that alkaline phosphatase activity decreased only in cells remote from blood vessels. Prasad et al. (1967) reported that lactic dehydrogenase, alcohol and malic dehydrogenase and alkaline phosphatase are decreased in the bone of zinc deficient rats.

In addition to the previous works, it is also important to consider the effect of zinc excess diet in bone formations. In 1951, Sadasivan noted that dietary zinc excess brought about a reduction in the weight and ash content in the rat femur. These observations have some support by the experiment of Ferguson and Leaver (1972) in which zinc excess appears to have a competitive effect on calcium and consequently to exert a porotic effect upon bones and teeth.

INDUCTION

In interpreting the mechanisms of induction produced by deviltalized bone graft, Urist and co-workers have compared the phenomenon to the embryonic induction of various structures and tissues.

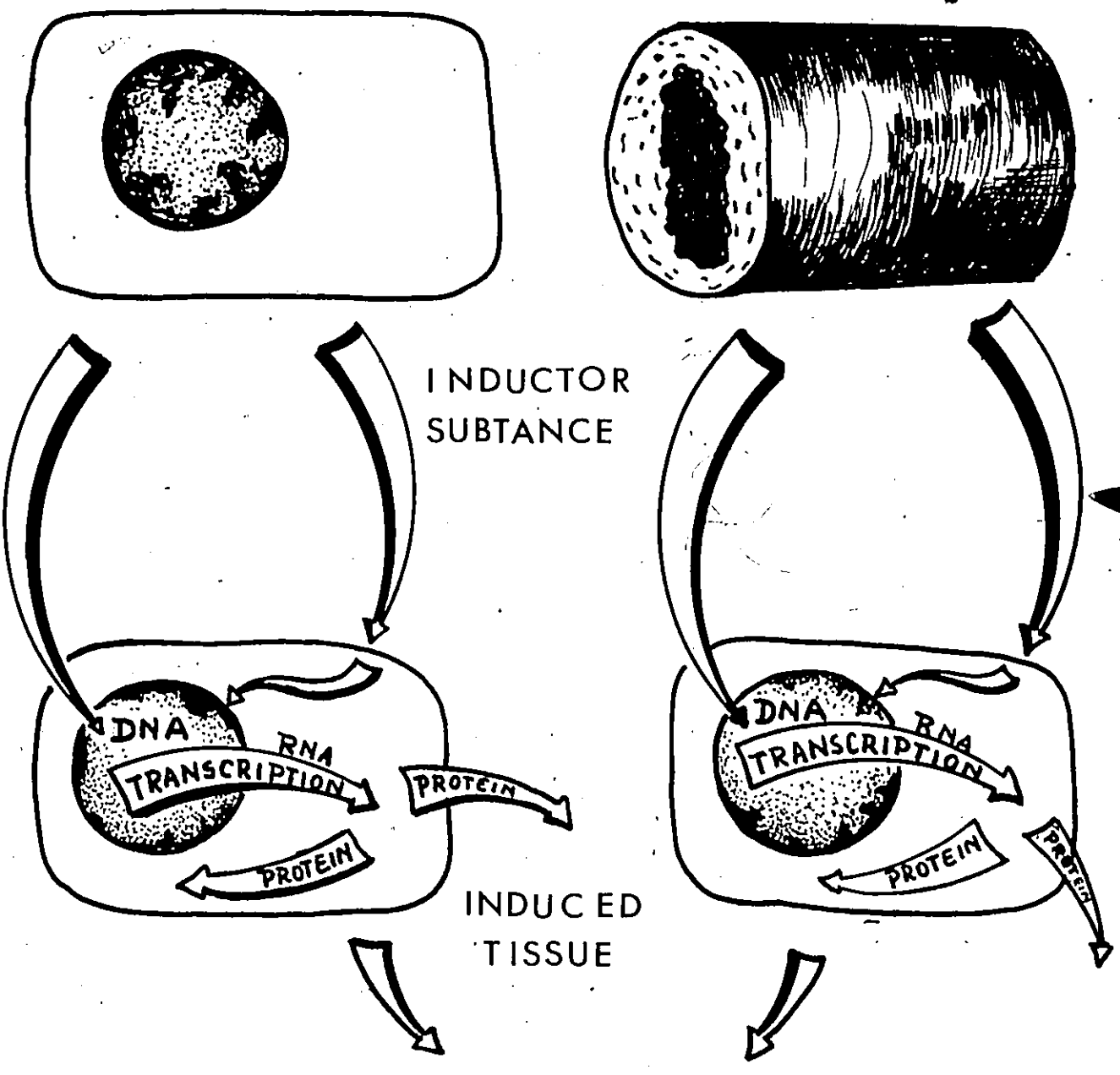
In the typical embryonic induction (see Scheme page 30), a cell or group of cells (Inductor Tissue) liberates a diffusible agent (Inductor Substance), believed to be a nucleo-protein or a protein, and this agent acts on a second cell or group of cells (Induced Tissue) which as a result of the stimulus reacts by differentiating into a new cell type. It has not been established yet if the inductor substance penetrates into the second cell in order to reach its nucleus, or if it only acts on the surface of the cell where it stimulates the production of a second messenger of the type of the cyclic AmP-calcium system. One way or the other, it finally acts on the genome of the cell and by altering the process of transcription (RNA Messenger) alters the differentiation of the cell.

This phenomenon can be compared with the bone induction process as described by Urist. In the case of embryonic induction, of course the inducing agent is a living cell, while in Urist's experiments, the inducing agent is a dead tissue. However, similar to the case of the embryonic induction, in Urist's

INDUCTOR TISSUE

LIVING TISSUE

DEVITALIZED BONE



INDUCED TISSUE

DIFFERENTIATION

experiments, the inductions is conveyed by a diffusible substance. This was demonstrated by Urist and co-workers by showing that bone induction occurs even when extracted bone is enclosed in a millipore chamber. He later showed that collagen, isolated from devitalized bone, is capable of producing similar induction and this shows that the same is in embryonic induction, the agent is a protein. Moreover, he later successfully repeated his experiments using collagen digested with collagenase, thus indicating that the inductor agent is a protein fraction of smaller molecular weight. Urist's studies have successfully investigated the nature of the inductor agent. However, in studying embryonic induction, embryologists have focused their attention on the inductor principle and on the nature of the reacting cell or tissue, and have demonstrated a general principle which can be expressed as follows: the inductor principle acts as a trigger and the type of induction resulting depends more on the nature of the target cell (See Saxén and Toivonen in Primary Embryonic Induction, 1962).

In order to find out if this principle applies to the phenomenon of bone induction in the adult animal, we have tried in the following experiments, to demonstrate that the response to our inducing agent, that is, devitalized bone, can be altered by experimentally changing the physiological status of the responding tissue. We tried to accomplish this by submitting the hosts to cation-deficient diets.

CHAPTER II

MATERIALS AND METHODS

A. Animals

A total of one hundred and twenty-two white Sprague Dawley rats supplied by Canadian Breeding Farm were used. All of the animals were males whose weight was between eighty and one hundred and fifty grams. The experimental rats were individually housed and kept at a temperature of 74°F. The animals were grouped in (six experimental) series described in Table I (Page 60). The state of the animals was observed daily and any change in symptoms was recorded. The amount of food which the rats with zinc deficient diet ate in each feeding was also recorded. The body weight was recorded once a week.

B. Diets

1. Standard

The standard diet consisted of the regular feed (Rockland chow pellet) used in our animal house and water ad-libitum.

2. Experimental Diets

Three kinds of special diet (magnesium, calcium and zinc deficient) were used.

All the diets were obtained from General Biochemicals, Chagrin Falls, Ohio. These diets were started one week before the operation and continued until four weeks after.

The magnesium deficient diet was supplied as a pellet.

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	gms/kg
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

The magnesium content in this diet was analyzed by absorption spectrophotometry by Dr. E.C. Goodhue (National Research Council of Canada). It varied from 0.925 to 1.2 mg./100 gm dry weight (0.880 gm./100 gm wet weight) for the magnesium-deficient diets and from 260 to 265 mg./100 gm dry weight for the standard diet. The calcium content was 0.698 g./100 gm dry weight (0.660 g./100 gm wet weight) in the magnesium-deficient diet.

Calcium Test Diet [1]

Formula	gm/kg
Casein, GBI Vitamin Free Test	240.00
Sucrose	684.00
Oil, Corn	50.00
Salt Mix (see below)	26.00

Salt Mix

Cupric Sulfate	0.012785
Ferric Citrate	1.171939
Magnesium Sulfate	2.130798
Manganese Sulfate	0.1917718
Potassium Iodide	0.0340928
Potassium Phosphate Dibasic	13.743648
Sodium Chloride	6.008851
Sodium Phosphate Monobasic	2.684806
Zinc Chloride	0.02130798

Vitamin Mix

p-Aminobenzoic Acid	0.3024
Calcium Pantothenate	0.01992
Choline Chloride	1.0008
i-Inositol	0.01128
Menadione	0.006

[1] Kenny A.D., and Munson, P.L.' (1959) Endocrinology, 64, 513.

	gm/kg
Nicotinic Acid	0.02496
Pyridoxine HCl	0.003504
Riboflavin	0.003504
Thiamine HCl	0.003504
α-Tocopherol	0.050
β-Carotene, Synthetic	0.011
Vitamin D_2	3,000 IU

Zinc Deficient Diet [1]

This special diet was supplied as powder. The powder was mixed with distilled water and poured in a small porcelain pot. This mixture was replaced every two days. Glass distilled water was also supplied during the experimental period.

Formula.

	g/kg
Egg White Solids, Spray Dried	200.0
Dextrose, Hydrate, Technical	631.053
Fiber, Non-Nutritive	30.00
Oil, Corn	100.00
Salt Mix* (see next page)	
Vitamin Mix (see next page)	

[1] Luecke, R.W., Olman, M.E., Baltzer, B.V. 1968. J. Nutrition 94, 344.

Salt Mix*

	g/kg
Calcium Carbonate	9.94405
Calcium Phosphate Dibasic	3.1489
Cobalt Chloride	0.00185
Cupric Sulfate	0.009945
Ferric Citrate	0.911542
Magnesium Sulfate	3.38106
Manganese Sulfate	0.008791
Potassium Iodide	0.026518
Potassium Phosphate Dibasic	14.0044
Sodium Chloride	5.55198

Vitamin Mix

Biotin	0.004
B_{12} (0.1% in Mannitol), Vitamin	0.020
Calcium Pantothenate	0.016
Choline Chloride	1.500
Chlortetracycline **	0.250
Folic Acid	0.0005
Menadione	0.00033
Niacin	0.025
Pyridoxine HCl	0.004
Riboflavin	0.006
Thiamine HCl	0.010

** An antibiotic added to vitamin mix.

	units/kg
Vitamin A Palmitate	10,000.000 IU
Vitamin D_2	1.250.000 IU
Vitamin E Acetate	110.000 IU

This deficient diet was analyzed by atomic absorption for zinc and magnesium by Dr. E.C. Goodhue (National Research Council). It gave the following results: 0.9 ppm of zinc and 440 ppm of magnesium.

A group of six rats was fed with the same diet but supplemented with zinc acetate. The analysis by atomic absorption for zinc and magnesium in this supplemented diet was 48 ppm of zinc and 440 ppm of magnesium. The supplementary diet was prepared in the laboratory by mixing 150 mg of Zinc Acetate with one kilo of zinc deficient diet. To mix the food, a laboratory jar mills was used (Stoneware Okrou Ohio, Coleman-Palmer, 275 R.P.M.) for six hours.

C. Technique for Graft Preparation

1. General

The femur was used for preparing the grafts of devitalized bone. In Series I, II, III, IV, the donors were normal rats. In Series V and VI, the bones came from rats which were fed for four

weeks with magnesium and calcium-deficient diets.

2. Decalcification

All of the bones were obtained after autopsy and immediately processed as follows:

The bones were cleaned of the surrounding tissue and put in decalcifying solution (0.6 N. HCl according to Urist et al., 1965) without fixation. They remained in HCl for 20 hours at -4°C on a magnetic stirrer. After cleaning the bones in distilled water, a cylinder segment of the diaphyses one centimeter in length was cut with a razor blade. The bone marrow was taken out and the marrow cavity was cleansed with distilled water. After the excess water was removed with filter paper, the fragments of bone were frozen on dry ice and left overnight until the

lyophilization on the next day.

3. Lyophilization

A. Equipment

The equipment used was a Virtis Histological Freeze drier (model no. 10-165), a vacuum pump, Precision Scientific model 25, and a McLeod Gauge, Virtis no. 10-22A. (see Fig. 1)

The Virtis histological freeze dryer consists of several parts:

a) Stainless steel cold bath (Fig 1 Part A) which has three ports for vacuum tubing connection. One of the base ports is connected to a vacuum pump using suitable rubber vacuum tubing. Between the stainless steel cold bath and the vacuum pump was a container with drierite. The McLeod Gauge was connected with a rubber tube to the second base port in the stainless steel cold bath. Finally, the pyrex-brand glass freeze dryer (Part B) was connected with the top port of the stainless steel cold bath using the 19/38 pyrex adapter and rubber vacuum tubing.

b) The pyrex glass freeze dryer (Fig 1) is formed by two parts: an upper one which is a cylindrical double-walled container for the refrigerating

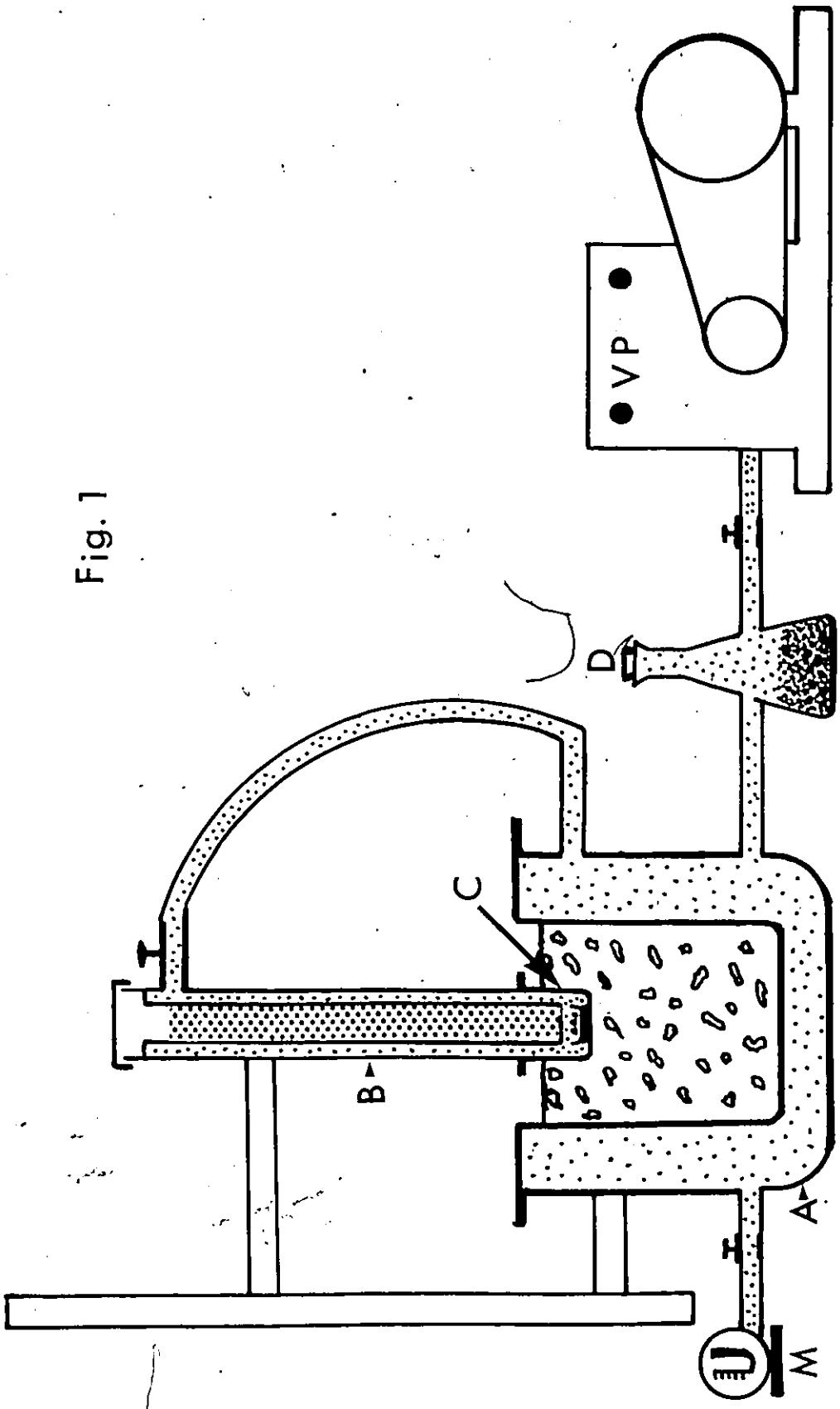


Fig. 1

bath and the lower one which forms part of the vacuum chamber which when connected with the upper portion, forms a closed vacuum chamber. The connection of both portions consists of ground glass flanges which are greased with a high vacuum silicone grease in order to obtain a better grip.

B. Preparation

The stainless steel bath was filled with methyl cellosolve as a heat transfer solvent; in order to obtain temperatures between -20°C to -40°C, varying amounts of dry ice were added to it at intervals. The upper part of the dryer was filled with liquid nitrogen. This container remained full at all times; nitrogen was added constantly during the process.

C. Method

The mid-diaphysis fragment of bones that were already frozen, were put in groups of six in small Petri dishes. These dishes were put inside the lower glass (Fig. Part C) of the dryer apparatus. Immediately when this chamber was closed, the system was ready for operation. The vacuum pump was started and the glass dryer was then moved down

until half of the lower chamber of the dryer was submerged in the bath of methyl cellosolve. When the level of the vacuum was 5 mm of mercury or 5 micron, the freeze-dryer system was left working for six hours under permanent control. For the last hour, the glass dryer was moved up from the methyl cellosolve bath and left at room temperature. At the end of this process, the lower chamber was removed and kept with the samples in an air-evacuated glass dessicator for no more than three days. From each group of prepared bones, two samples were taken: one was incubated in aerobic culture media and the other was incubated in anaerobic culture media. This process was used in order to ensure that the devitalized allografts were completely sterile. In this manner, the grafts were ready for implantation.

D. Surgical Procedure

Implantation

The operation was performed in a Clean Bench Horizontal flow with positive pressure (Edgegord Hood. The Baker Co. Inc., Stanford, Maine). The characteristics of this special chamber are important in creating sterile surgical conditions necessary to obtain objective results

of the host response. The horizontal laminal flow clean bench has a motor blower (1/3 H.P. - Phase 1 - cycle 60 - R.P.M. 1075) located under the bench. This motor blower blows air in through a series of two kinds of filters; a HEPA filter (rater capacity, 1125 c.f.m.) and a prefilter made of Scott foam. The blower has been selected to provide air at a minimum of 90 F.P.M., maximum 120 F.P.M. over a range of pressure drop which insures a sufficient air supply when the filter is loaded with dirt. In addition, slots are located at the front edge of the work surface and sidewalls in which influx air from 800 to 900 F.P.M. to insure effective results. When the clean bench starts to work, the motor blower blows air in through the prefilter and through the (HEPA) filter horizontally across work area. The characteristics of the laboratory confirm that 99.7% of particles larger than 0.3 μm are removed according to special testing to insure a steril area of work: The Clean Bench was started half an hour before the operation. The surgical instruments were sterilized at 180°C for three hours in dry oven.

Before the surgical procedure, the animals received an intraperitoneal injection of anesthetic

(Innovar-Vet) 0.3 ml per 100 gm of body weight. Each rat was shaved in the lumbar area and the operation zone was cleaned with antiseptic solution of Zephiran (Brand of Zenzalkonium chloride Solution U.S.P. Winthrop). A dorsal incision was made in the skin about two centimeters in length, parallel to the vertebral column. The superficial tissues and aponeurosis of the dorsal muscles were sectioned, and the fibers were separated with forceps. A cylindrical fragment of mid-diaphysis of devitalized bone was implanted in the muscular pocket. The muscle was sutured using sterile chromic surgical gut (medium 00). The skin was closed with sterile silk (4-0) suture, silicone treated. Before the animal was put back into the cage, the wound was cleaned with the antiseptic solution again. No more than 12 rats were operated on at one time. After four weeks they were sacrificed.

E. Technique for Morphological Studies

1. Fixation and Decalcification

During the autopsy of the experimental rats, the grafts were removed together with abundant surrounding tissue. These tissues were prepared with different procedures according to the histological or histochemical reactions to be

performed. Small portions of some of the grafts were separated, fixed in neutral formalin and processed for microradiography (according to Bélanger (unpublished)). The rest of the tissues were fixed in AFA (15 parts 95% ethanol; 4 parts formaldehyde and 1 part acetic acid). After remaining in fixation for 24 hours, the tissues were decalcified in 10% (saturated) EDTA (ethylenediaminotetra-acetic acid) (sequestrine Geigy) in distilled water (pH approx. 5.5) for ten days according to the constant replacement method of Bélanger et al., (1965). This technique consists of placing the bones in a flask to which one drop per second of fresh EDTA solution from a reservoir is constantly added to replace a drop that is lost from the flask by overflow. After this process, the bones were washed in running water for thirty minutes and then the specimens were embedded in paraffin for sectioning.

2. Embedding and Staining

After decalcification, the tissues were embedded according to the following method:

Dehydration in graded ethanols

- Two changes of 70% ethanol of 8 hrs each
- Two changes of 95% ethanol of 8 hrs each
- Three changes of absolute ethanol of 8 hrs each
- Three changes of Toluol of 8 hrs each
- Five changes of paraffin of 5 hrs each

The final step of this process was the formation of blocks with the tissue already embedded.

Sections, five microns thick, were cut on a Spencer A.O. microtome and stained with routine and special stain. As a routine stain, haematoxylin-phloxine-organ G (H.P.O.) (Bélanger unpublished) was used.

Method of H.P.O. Stain - (Van Campenhaut; Bélanger, unpublished)

A) After deparaffinization with xylene, the sections were taken through the usual series of ethanol solutions down to water.

B) Stained in filtered Delafield's hematoxylin for six minutes.

C) Washed in distilled water to remove excess of stain.

D) Differentiated with acid alcohol (1% HCl in 50% alcohol).

E) Rinsed with distilled water and lithium carbonate 1 minute.

F) Washed in running water for at least 15 minutes.

G) Stained in 1% aqueous phloxine 6 minutes.

H) Changed rapidly once in 95% alcohol.

I) Stained in 1% Orange G in 95% alcohol 1½ minutes.

J) Washed quickly in 95% alcohol followed by two changes in absolute alcohol 2 minutes each and one change in absolute alcohol for 2 minutes.

K) Changed three times in xylol 2 minutes each and mounted in permount.

3. Microradiography

The undecalcified portions which had been fixed in neutral formalin were transferred to 80% ethanol. Using a dental drill, cross sections of the same were cut. Dehydration of those sections was completed in 95% ethanol, three six hour changes of absolute ethanol and two 8 hour changes of acetone. The bone samples were then infiltrated with increasing concentrations of Epon resin according to the following schedule. (Luft, 1961).

Two main solutions were used:

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)

Subsequently, these solutions were mixed in different proportions:

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

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

Mixture 3: Solution A: Solution B: Acetone 3:3:2 to which is added 3% DMP-30 accelerator (2,4,6, trimethyl amino ethyl phenol)

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

The procedure for embeddement was the following:

a) During the first hour, the bones in test tubes containing Epon mixture 1 were centrifuged at 1800 R.P.M. and then stored in a desiccator overnight.

b) In the morning, the bones were again centrifuged the first hour with mixture 2, the second hour with mixture 3.

c) Finally, the bones were infiltrated under vacuum (18 mm. Hg) for four hours with mixture 4.

d) 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 and the capsules were stored for thirty minutes under vacuum at room temperature.

e) 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^{o}, 45^{o} and 60^{o}.

f) When the Epon was completely polymerized, the capsule and its contents were trimmed with a hand saw and cemented onto a thin plate of similar material fitting the cutting table of the Gillings and Buonocore (1959) cutting machine. Sections of 100 microns were obtained. The uniformity of the section 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.

g) 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).

The emulsions were purchased from the Canadian Kodak Sales Co., Toronto 15, Canada on 1X3 inch glass plates coated with 10 micron thick layer of NTA nuclear emulsion of 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.

Process

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.

The bone section adhering to the tape was lifted from the storage glass slide and transferred to the emulsion covered plate so that the bone section now was put in between the tape and the photographic emulsion. Intimate contact between bone and emulsion was essential to insure high resolution. All these processes were made in the dark room. The bone sample was now ready to be microradiographed.

Exposure to X-Ray

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^{o} 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 graft bone studied was uniform. Only the extent of calcification of the bone varied.

Development and Fixation

In the dark room, the exposed emulsion plate and adhering section and tape were removed from the camera, and the adhesive tape bearing the bone section was transferred 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.

4. Histochemistry

Three main techniques were used; two of them correspond to mucopolysaccharide identification. For Acid mucopolysaccharides, the Alcian Blue-Basic Fuchin was used (Bélanger, unpublished); P.A.S. Reaction was used for identification of neutral mucopolysaccharides and glycoprotein. Also, the third technique corresponded to the Von Kossa Reaction, which was used in order to identify the deposits of bone mineral in the tissue.

Description of the method:

A. Acid Mucopolysaccharides

Alcian Blue-Basic Fuchsin

Method -

1. Sections were deparaffinized and hydrated in alcohol of decreasing concentrations down to distilled water.

2. They were then stained in alcian blue 0.5% for 45 minutes. The alcian blue solution was prepared with the following buffer:

Sodium acetate	50 cc
Hydrochloric acid 1M	100 cc
Distilled water	100 cc

The solution was around pH 1.

3. Rinsed briefly in buffer for 10 seconds.

4. Washed in running water, 15 minutes.

5. Stained with basic Fuchsin 0.055% for 10 minutes.

6. Fast dehydration in absolute alcohol (three changes of alcohol, 2 minutes each).

7. Three changes of xylol, 2 minutes each.

8. Mounted in permount.

Acid mucopolysaccharides were stained pale blue.

B. Neutral Mucopolysaccharides and Glycoproteins

P.A.S. Reaction (Periodic Acid Schiff) (Barka and Anderson)

Solution I

0.5% periodic acid diluted in distilled water.

Solution II

Schiff's Reagent: It was prepared by dissolving 1 gm basic Fuchsin in 200 cc distilled water.

After cooling it to about 50°C, 1 ml hydrochloric acid and 2 gm of sodium bisulphite were added.

The flask tightly stopped, it was shaken and then let stand at room temperature for 24 hours.

0.5 gm of absorbent charcoal was added and the mixture was then shaken again and filtered.

The mixture was kept refrigerated.

Solution III

2% solution of sodium bisulphite.

Method -

1. Stained with Solution I for 10 minutes.
2. Washed in running water for 10 minutes.
3. Sections were placed in Solution II for 12 minutes (10-15 minutes).
4. Rinsed in distilled water.
5. Placed in Solution III for 1 minute (1-2 minutes).
6. Washed for 5 minutes (5-10 minutes).
7. Counterstaining was made with Hematoxylin followed by dehydration, drying and mounted as usual.

C. Von Kossa Technique (Barka and Anderson)

Bone sections, undecalcified and fixed in AFA were used.

Method -

1. The sections were deparaffinized and hydrated until water.
2. Left for one hour in 1% acqueous solution of $AgNO_3$ (Silver Nitrate) in the dark.
3. The slides were exposed to light for an hour (Reduction process).
4. Rinsed in distilled water.
5. Immersed in 5% acqueous solution of sodium thiosulphate for 30 seconds.

6. Washed in tap water for 5 minutes.
7. Nuclei were counterstained with 0.025% basic fuchsin for 30 secs. to one minute.
8. Dehydration in alcohol - cleaned in xylene and mounted in permount.

CHAPTER III

RESULTS

TABLE I

SERIES #	RATS	HOST	DONOR
Series I	31	Normal	Normal
Series II	10	Mg deficient	Normal
Series III	12	Ca deficient	Normal
Series IV	12	Zn deficient	Normal
	6	Zn Suppl.	Normal
Series V	18	Normal	Mg deficient
Series VI	10	Normal	Ca deficient
	6	Normal	G Mg deficient
Series VII	5	Normal	G Mg deficient & testosterone
	5	Normal	G
	7	Normal	G & testosterone

(1) Zn Suppl: Supplementary diet: 48 pp.m.

G: Gonadectomized

Testosterone Supplementary: 25 u.g. β-estradiol daily.

GENERAL

Table I groups all the series of explants to be analyzed. Several common characteristics were found in the evolution of grafts in all experimental series. Connective tissue of the host surrounded the grafts and filled their cavity. Although in some cases, giant cells were found in connection with grafts, implying the existence of some kind of foreign body reaction, significant rejection of grafts was not observed in any case. In all the experimental series, new bone was formed. It was localized inside and/or outside the graft. Wherever the new bone grew in groups of trabeculae, bone marrow-like tissue was always present. In addition to the above mentioned common characteristics, the grafts in the different series, had distinctive features which will be analyzed in the following description.

SERIES I

GRAFTS OF NORMAL BONE IN NORMAL RATS

Microscopic Observations

The microphotography in Figure 2 shows with low magnification a cross section of a typical implant of a normal bone into a normal host. The boundary in between the graft and the skeletal muscle is clearly visualized as a narrow layer of connective tissue surrounding the implanted bone. Some of the vascular channels in the thickness of the wall still appear empty. These spaces have different shapes and sizes. The central cavity of the grafted bone is also filled with connective tissue with numerous blood vessels (Fig. 2). In some grafts resorption spaces communicated the central cavity with the outside with connective tissue.

The host response to the grafts implanted in the muscle was in all cases characterized by infiltration of the grafts by connective tissue cells followed in nineteen out of thirty-one cases by differentiation of bone and/or cartilage. In addition, bone marrow tissue usually appeared in connection with the newly differenciated bone. The process can be described as

follows: connective tissue cells which probably have migrated from the adjacent muscle, invade the old vascular channels of the bone matrix, and the central cavity of the graft (Fig. 3); they also form a layer of connective tissue around the implant (Fig. 3). Wherever this new connective tissue is present, it is possible to identify fibroblast-like cells, macrophages multinucleated giant cells (Fig. 4) and many cells which look like histiocytes and contain a brownish material probably of hematic origin. These macrophages are usually more abundant in the central cavity. Together with the infiltration of connective tissue, small capillaries reach the bone marrow cavity forming a network of new blood vessels which also occupies the empty channels which are present in the thickness of the wall of the implanted bone. Often clumps of fibrin stained in red with the H.P.O. stain were found in the central cavity; they were easily distinguished as compared from collagen fibers which stained orange with the same technique. The new bone appears as trabeculae containing lacunae with typical osteocytes and surrounded by a layer of osteoblasts in a linear formation (Figs. 5-6). In most cases, the bone

trabeculae are inside the cavity of the grafts; but not infrequently bone trabeculae are also found at the periphery of the graft (Figs. 5-6). This deposit of bone was generally observed in areas of the graft in which the wall of the implanted bone was thin. Bone marrow tissue type usually accompanies osteogenesis. This type of tissue differentiated among the bone trabeculae (Fig. 6). However, it is not evident when the bone grows as a large single mass of tissue (Fig. 8). The new bone marrow tissue contains large blood vessels, groups of fat cells and small rounded cells which look like lymphocytes. It is also possible to see few rounded multinucleated giant cells; they appear to be megakaryocytes (Fig. 11). In ten out of the thirty-one implants, small islands of cartilage were found. They were usually formed by one or two cartilage cells and were located most frequently in spaces within the dead bone matrix of the graft (Fig. 12). The new cartilage cells were very clearly recognized with the alcian blue stain, a blue ring appears surrounding each cell (Figs. 12-13). In few instances, cartilage cells were also found

in the connective tissue filling the central cavity (Fig. 13). These cells usually were localized in areas in which the irrigation was not evident, none of these allografts showed characteristics of any infection.

Microradiograph techniques applied in this experiment showed that the new bone trabeculae formed inside and in the periphery of the graft were well mineralized (Fig. 14).

SERIES II

GRAFTS OF NORMAL BONE IN MAGNESIUM DEFICIENT RATS

Symptomology

The ten rats in this series showed the clinical symptoms which are typical of growing rats fed a magnesium deficient diet. At the end of the first two weeks, there was erythema mainly in the extremities and the ears; hyperemia and scaling of the skin in the tail were also observed as well as an increase in their irritability by all kinds of noises. Differences between their body weight and that of

controls was noticed (Chart 1).

Microscopic Observations

Figure 15 represents a low magnification microphotography of the cross section of the intramuscular implant in a magnesium deficient rat. It shows that similar to implants in normal rats, the bone graft is surrounded by newly formed connective tissue (Fig. 19) which has also penetrated the central cavity and some of the old vascular channels of the graft. The connective tissue surrounding the implant is characterized by the presence of fibroblast and multinucleated giant cells, some of which contain amorphous or fibrillar material which appears to originate from fragments of demineralized bone matrix. The central cavity is invaded by mesenchyme-like tissue with fibroblasts and multinucleated giant cells. These giant cells are organized in circular structures (Fig. 16) around some material, probably a piece of devitalized bone matrix. In addition to the giant cells, there are zones infiltrated by small rounded cells and macrophages with some material in the cytoplasm. In a few cases, skeletal muscle fibers were found

inside the central cavity. This tissue was represented by myoblast-like cells with double or single nuclei and red staining myofibrils (Fig. 16). They probably entered the cavity from the muscle tissue surrounding the graft. Some clumps of fibrin are found in the marrow cavity. Five out of the ten grafts in this group presented bone trabeculae located both in the marrow cavity and the periphery of the graft. The bone trabeculae were small and thin compared with the control, and did not occupy a large space in the central cavity (Fig. 22). Typical osteoblasts in a linear formation surrounding these trabeculae were also found in the internal surface of the graft, facing the cavity (Fig. 22). None of the grafts showed any formation of marrow tissue. The most important observation in the grafts of this group was the presence of large amounts of newly formed cartilage. All of the implants showed chondrogenesis usually localized in spaces within the matrix of the implanted bone. In some zones, two or three cartilage cells occupied small spaces probably derived from lacunae or old blood vessels channels of the implanted bone (Fig. 17). In other zones, groups of ten or more cells formed large islands located in

large spaces, probably produced by resorption of the implanted bone (Figs. 18-20). These large islands of growing cartilage were represented by small cells possibly chondroblasts and large cells or chondrocytes surrounded by deeply alcianophilic material. These areas could be considered poorly differentiated. In only one case cartilage was found in the central cavity. It was represented by a mass of chondrocytes of different sizes and stages of differentiation (Fig. 17). The zone corresponding to the linea aspera of the implanted femur was the elective site for cartilage induction (Fig. 23).

SERIES III

GRAFTS OF NORMAL BONE IN CALCIUM DEFICIENT RATS

Symptomology

During this experiment, the physical condition of the host rats deteriorated rapidly. The symptomology was characterized mainly by hemorrhagic skin lesions in areas of the extremities and snout. In the second and third week, the animals showed loss of equilibrium: the rats fell easily when

walking, skin areas shaved before the operation did not grow hair again. Three out of the twelve rats in this group died during the course of the experiment. In observation of this material after using the microradiography technique, calcification over the new bone was not found: mineralization that was evident in implant of normal bone into normal host.

Microscopic Observations

The same as in previous groups, the grafts were surrounded by connective tissue which also penetrated into the central cavity. Vascularization of this central cavity appeared to be better developed than in the other groups. The connective tissue contained few fibroblasts; multinucleated cells were rarely found. Multinucleated giant cells were present in few areas in which the reabsorption was evident. Small amounts of new bone were found in six out of twelve grafts. This bone was not found in the form of free trabeculae, but as a continuous layer mainly in the inner surface of the grafts. It is very highly stained with alcian blue which indicates that it is immature type. The organization of the osteoblast did not show the typical linear formation

identified in implants in which characteristic trabeculae appears (Fig. 7). The bone was so soft that sections could be cut without previous decalcification. Von Kossa's technique showed positive reaction in only one implant indicating the absence of calcium phosphate in most of the new bone. This new bone is similar to the immature type or osteoid described by early histologists (Figs. 24 and 25). The strong characteristics of this tissue in sections treated according to P.A.S. and alcian blue technique were also similar to those described for osteoid. Only in one case was found some cartilage in the form of isolated cells, localized in the deep part of the implanted bone matrix. Bone marrow-like tissue was never found in this group. This was to be expected since bone did not grow in the form of trabeculae. Deserving special mention, is the fact that resorptive spaces were found with less frequency in this group of implants. The implanted bone looks more compact; not many vascular channels in the thickness of the wall can be observed.

SERIES IV

GRAFTS OF NORMAL BONE IN ZINC DEFICIENT RATS

Symptomology

Contrary to what happened with rats kept on magnesium-deficient or calcium-deficient diets, those maintained on a zinc-deficient diet, did not present detectable symptomology. Although their body weight progression was different from those of normal rats, (Chart 2) this difference was not as pronounced as the one found with the other diets.

Microscopic Observations

All the grafts in this series showed formation of large amounts of osteoid. This was localized mainly in the central cavity, where it grew in the form of large masses in which lacunae containing osteocyte can be identified; osteoblasts were found at the periphery of the masses. These bone masses had an irregular shape and staining characteristics showed that they consisted of uncalcified material, similar to the osteoid formed previously in the grafts in Series III (calcium-deficient host). These masses of immature bone or osteoid were

surrounded by a layer of connective tissue cells in active proliferation; these proliferative cells appeared to be differentiating into new osteoblasts (Fig. 25), thus providing for the growth of the osteoid. In about half of the cases, bone in the form of trabeculae was found inside of the cavity (Fig. 28 and 29) or in the external surface. This trabeculae did not have the typical characteristics presented by the control. They were usually wide and the osteoblast that normally formed a linear structure around it, seemed to be reduced in comparison with the quantity of new bone. Another interesting observation was the fact that the proportion of the implanted matrix which was resorbed was much larger than the one found in the previous series. The resorbed matrix was in this case always replaced by osteoid. In some regions of the graft and especially in the external surface of it, many multinucleated osteoclast-like cells (Fig. 27) were still found engaged in active resorption. In addition to this multinucleated giant cells, a layer of connective tissue was present in between the muscle tissue and the graft. In all the cases in which bone trabeculae were found, bone marrow tissue was present in the neighborhood.

The bone marrow contained large blood vessels, megakaryocyte-like cells and rounded mononuclear cells.

Zinc Supplementary Diet

In all the cases in which the same type of diet was supplemented with zinc, new formation of bone in and around the graft was in the form of typical trabeculae. Typical lacunae with osteocytes were very easily recognized. Osteoblasts were limited around the new bone. All the implants among the trabeculae showed a bone marrow tissue with the same characteristic that was presented in a previous experiment (Series 1). Wherever the new bone appeared, it showed all the characteristics of normal growth, no osteoid was identified in this group. The infiltration of connective tissue around these grafts does not show any difference with the implant in rats fed with zinc-deficient diet.

SERIES V-VI

GRAFTS OF MODIFIED BONE IN NORMAL RAT

General

In all the preceding series, I have explained the evolution of implants of normal bone treated according to Urist's procedure into normal host rats. In order to establish if the quality of the two implanted bones can affect the evolutions of this graft, several experiments were made in which the implants were obtained from experimentally treated rats and were grafted into normal hosts. These include:

Series V: Bone from mangesium-deficient rats.

Series VI: Bone from calcium-deficient animals.

SERIES V

GRAFT OF BONE FROM MAGNESIUM DEFICIENT RATS INTO NORMAL HOSTS

Microscopic Observations

Fifteen out of eighteen animals showed heterotopic osteogenesis in connection with the implant. The new bone grew both outside and inside the grafts usually in the form of a few small groups of trabeculae (Fig. 30 and 31) surrounded by osteoblasts. It showed the same characteristic arrangement usually observed in new bone produced by implantation of devitalized normal bone (see description page 40). One of the implants

showed an interesting formation: the new bone formed inside the cavity of the graft had the shape of a hollow cylinder containing marrow-like tissue (see cross-section of the same in Fig. 35). In the connective tissue that always invaded and surrounded the implant, numerous multinucleated giant cells were observed in addition to the fibroblasts and some small rounded cells. This cellular picture appears to represent a rejection reaction to the foreign body (Figs. 32 and 33). In most of the cases, this characteristic reaction was observed in those areas facing the linea aspera of the central cavity of the graft, there were zones containing intercellular substance with large amounts of fibrillar material (Figs. 34 and 35). In ten out of the eighteen implants, bone marrow tissue was present. This was characterized by the presence of adipose cells, large blood vessels, many small rounded cells as well as megakaryocyte-like cells (Fig. 36). Although no precise measurements were made, it appeared evident that in this group of implants, the proportion of marrow relative to the amount of new bone found was much greater than in previous experimental groups. In one instance, isolated cartilage cells were observed in spaces within the implanted matrix.

SERIES VI

GRAFT OF BONE FROM CALCIUM-DEFICIENT RATS IMPLANTED IN NORMAL HOSTS

Five out of ten implants showed bone growth. It was represented by small masses of bone tissue surrounded by osteoblasts (Fig. 37). Usually these masses projected from the inner surface of the graft. In other cases, the new bone formed a thin layer which covered old vascular channels and empty spaces of the implanted matrix (Fig. 38). Three of the implants with a few small new bone trabeculae (Fig. 38) were accompanied by bone marrow-like tissue having the same characteristics as anterior series, in which bone marrow was present (Series I-III-V-VI).

The central cavity as usual contained mesenchymal tissue, with fibroblasts and macrophages with some pigment material in the cytoplasm.

Isolated cartilage cells were found in one case. They were localized in the spaces of the implanted matrix.

TABLES

TABLE 2

SUMMARY OF OBSERVATIONS

SERIES	GRAFT	HOST	#	BONE	CARTILAGE	BONE MARROW
I	Normal	Normal	31	Large amount. Bone trabeculae located in the cavity and within the wall of the graft. 61% positive cases.	Few isolaged cells located mainly in spaces of the implanted matrix. 32%	Megakaryocytes, small rounded cells and fat cells. 29%
II	Normal	$Mg^{(-)}$	10	Very small amount. Few small and thin bone trabeculae attached to the inner wall of the graft. 50% positive cases.	Large number isolated cells or small groups of cells localized in spaces of implanted matrix and specially in areas facing linea aspera of the graft. 100% positive cases.	Not found
III	Normal	$Ca^{(-)}$	19	Osteoid, small amount. 66%	Isolated cells in the spaces of the implanted matrix. 11%	Not found
IV	Normal	$Zn^{(-)}$	12	Large amount of osteoid and some mineralized bone trabeculae. Both localized in the cavity and within the wall of the graft. 83%	Not found	Megakaryocytes, small rounded cells and fat cells. 41%

TABLE 2 (Continuation)

SERIES	GRAFT	HOST	#	BONE	CARTILAGE	BONE MARROW
V	$Mg^{(-)}$	Normal	18	Small amount typical bone trabeculae located outside and inside the cavity. 83%	Isolated cells located in the spaces of the graft. 27%	Large amount. 55%
VI	$Ca^{(-)}$	Normal	10	Small amount of few trabeculae, mainly in the inner surface of the graft wall. 50%	Isolated cells in the implanted bone matrix. 10%	Marrow-like tissue, no megakaryocytes. 30%
VII	∅ $Mg^{(-)}$ ∅ $Mg^{(-)}$ + Test.	Normal	11	Very small amount. 45%	Isolated cells located in the spaces of the graft. 0.9%	Megakaryocyte 0.9
	∅ ∅ + test.	Normal	13	Small amount. No trabeculae. 53%	Small group of cells. 23%	Megakaryocyte 0.7

CHARTS

CHART 1

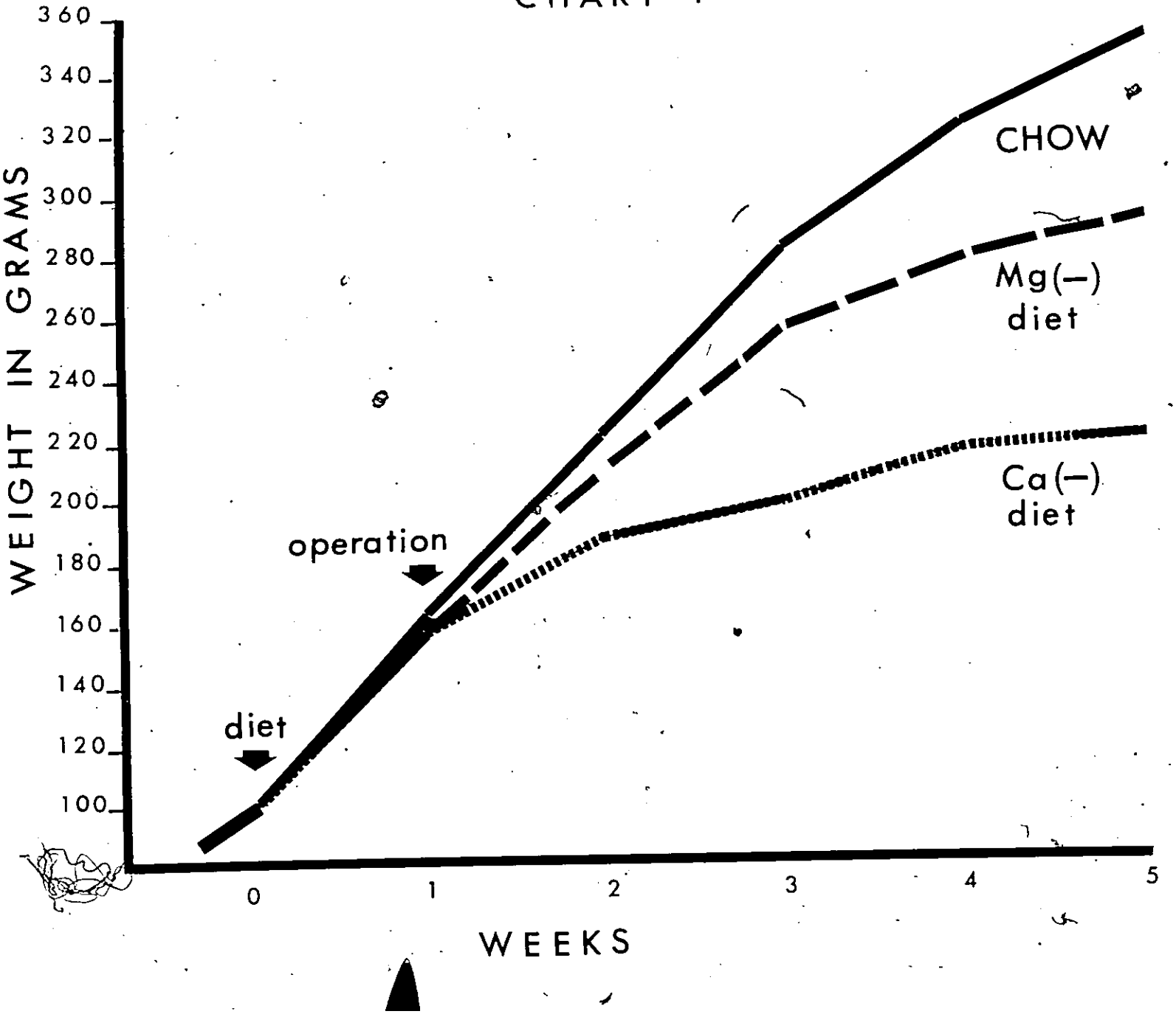
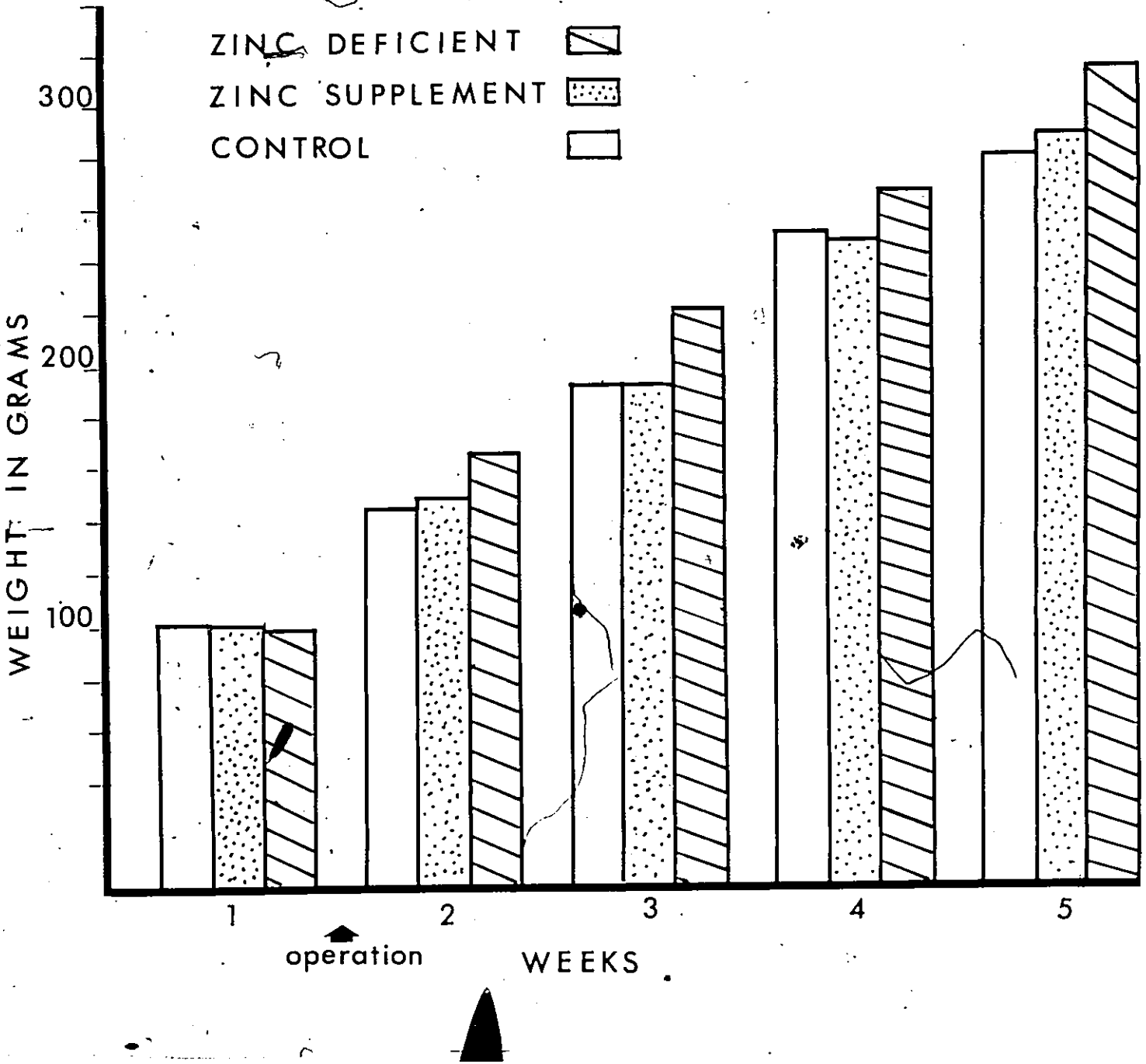

WEIGHT IN GRAMS
360
340
320
300
280
260
240
220
200
180
160
140
120
100
CHOW
Mg(–) diet
Ca(–) diet
operation
diet
0
1
2
3
4
5
WEEKS

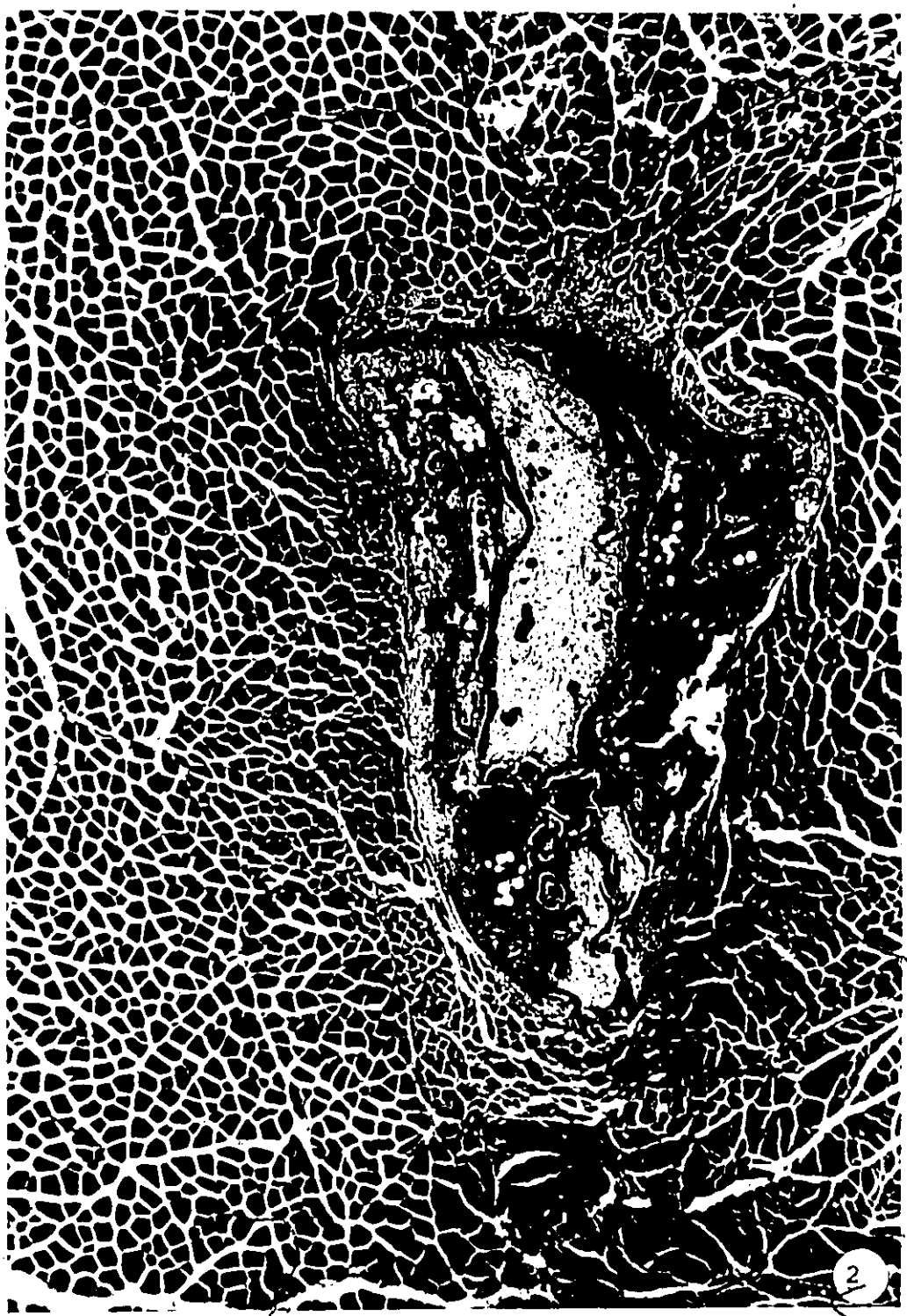
CHART 2


ZINC DEFICIENT
ZINC SUPPLEMENT
CONTROL
300
200
100
WEIGHT IN GRAMS
1
2
3
4
5
operation
WEEKS

LIST OF ABBREVIATIONS USED IN FIGURES

NBT:	New bone trabeculae
IB :	Implanted bone
OS :	Osteoid
OB :	Osteoblast
BM :	Bone marrow
BV :	Blood vessels
M :	Muscle
GC :	Multinucleated giant cells
CT :	Connective tissue
C :	Cartilage
HPO:	Hemotoxylin Phloxine Orange G
OT :	Osteocyte
CC :	Central Cavity

Figure 2 Microphotography of a cross section of a typical implant of a normal bone into normal host. Trabeculae of new bone in the inner and outer surface of the graft, solid green areas on the transparency.
New bone marrow tissue type (areas with dotted green).
Connective tissue in spaces of the implanted matrix and in the central cavity (CC) can be identified as skeletal muscle around the graft.
H.P.O. Stain 34X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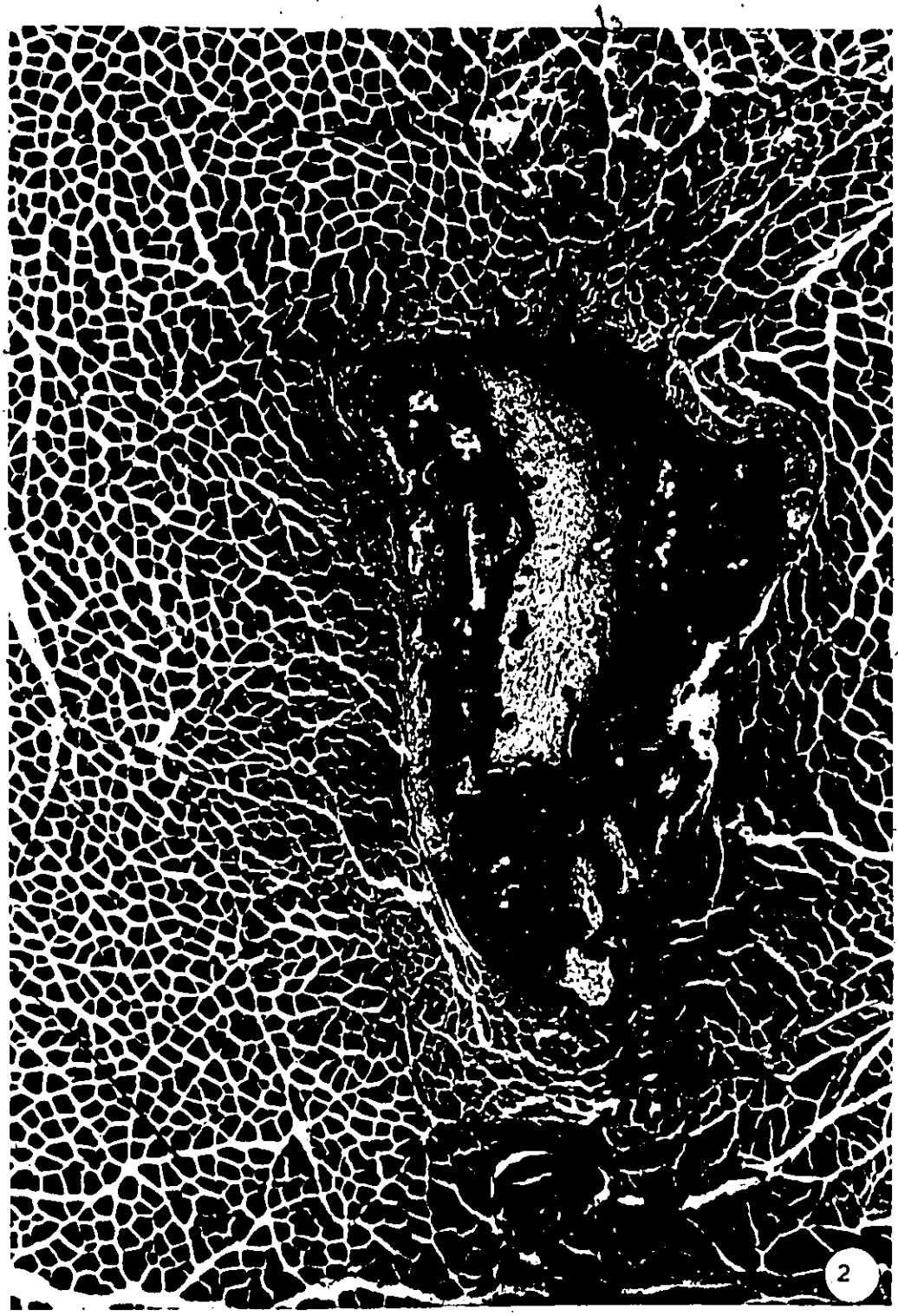

2

Figure 3 . A portion of the outer border of an implant of normal bone into normal rat. The implant (IB) is extensively invaded by connective tissue as well as the central cavity (CC), new bone formation (NBT) is also present.
Alcian blue and basic fuchsin stain 92X

Figure 4 Shows some area of invasion observed in Figure 3. Multinucleated giant cells (arrow) can be identified attached to the implanted bone matrix (IB). Some new cartilage cells are seen (C) in the interface of the wall graft.
Alcian blue and basic fuchsin 240X


IB
3

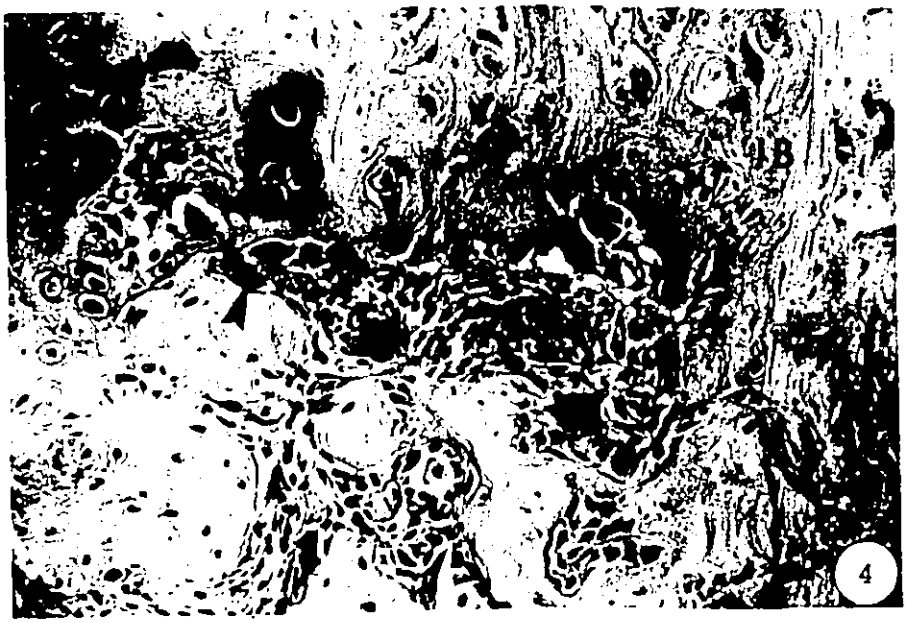

IB
4

Figure 5 From implant of normal bone in normal host. This section shows in all the thickness of the wall graft new bone trabeculae (NBT). Connective tissue in the central cavity is present (CT)
Alcian blue Stain 100 X

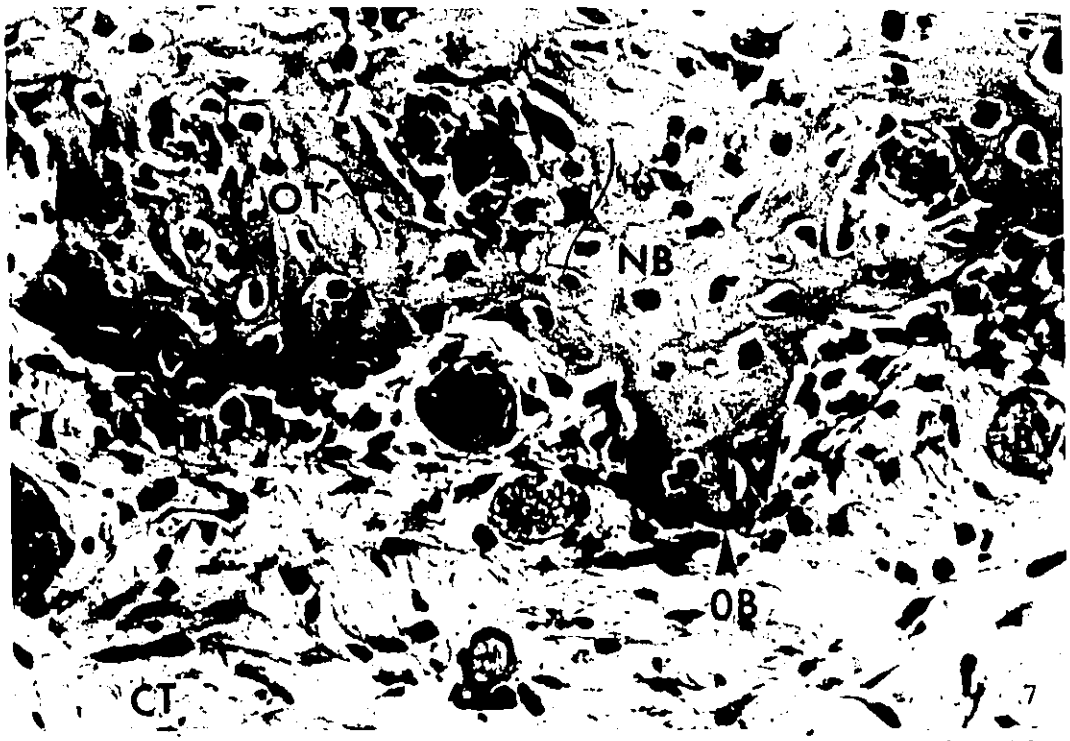
Figure 6 From implant of normal bone in normal rat.
Typical new bone trabeculae can be seen (NBT). Bone marrow tissue is present in between the new trabeculae (BM). The central cavity is occupied by connective tissue (CT).
H.P.O. Stain 100X


BT
CT
5


NBT
BM
6-

Figure 7 From implant of normal bone into normal host. Part of trabeculae of new bone (NB) with the typical osteocytes (OT) can be identified. Osteoblasts in a linear structure (OB) and cross section of blood vessels are also present (BV). H.P.O. Stain 400X

Figure 8 From implant of normal bone into normal rat. There is a mass of new cartilage (C) and one large trabeculae of new bone (NB). The new tissue is occupying most of the central cavity.
H.P.O. Stain 250X



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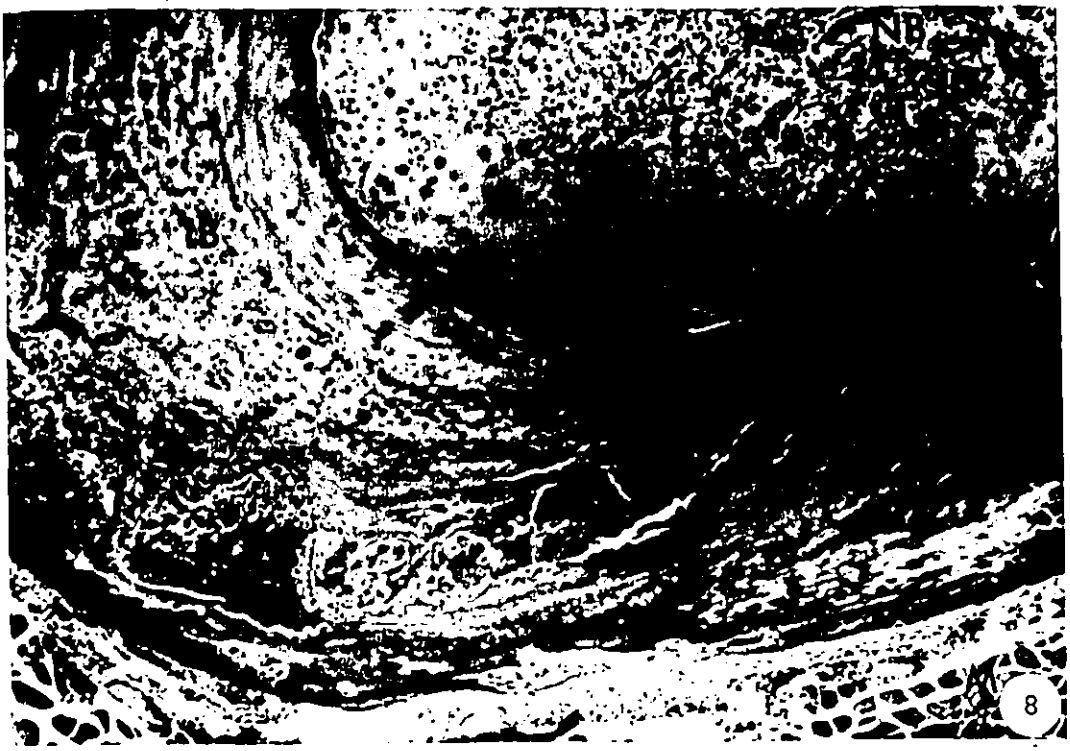


8

Figure 9 Corresponds to implant of normal bone into normal host.
It is a case in which few trabeculae of new bone (NBT) are present.
H.P.O. Stain 34X

Figure 10 Bone marrow from implant of bone into normal host.
New bone marrow tissue, full of rounded cells and three megakaryocytes (arrows) can be seen.
H.P.O. Stain 400X

Figure 11 Section shows a typical multinucleated giant cell (arrows) adjacent to the implante bone matrix (IB).
H.P.O. Stain 400X

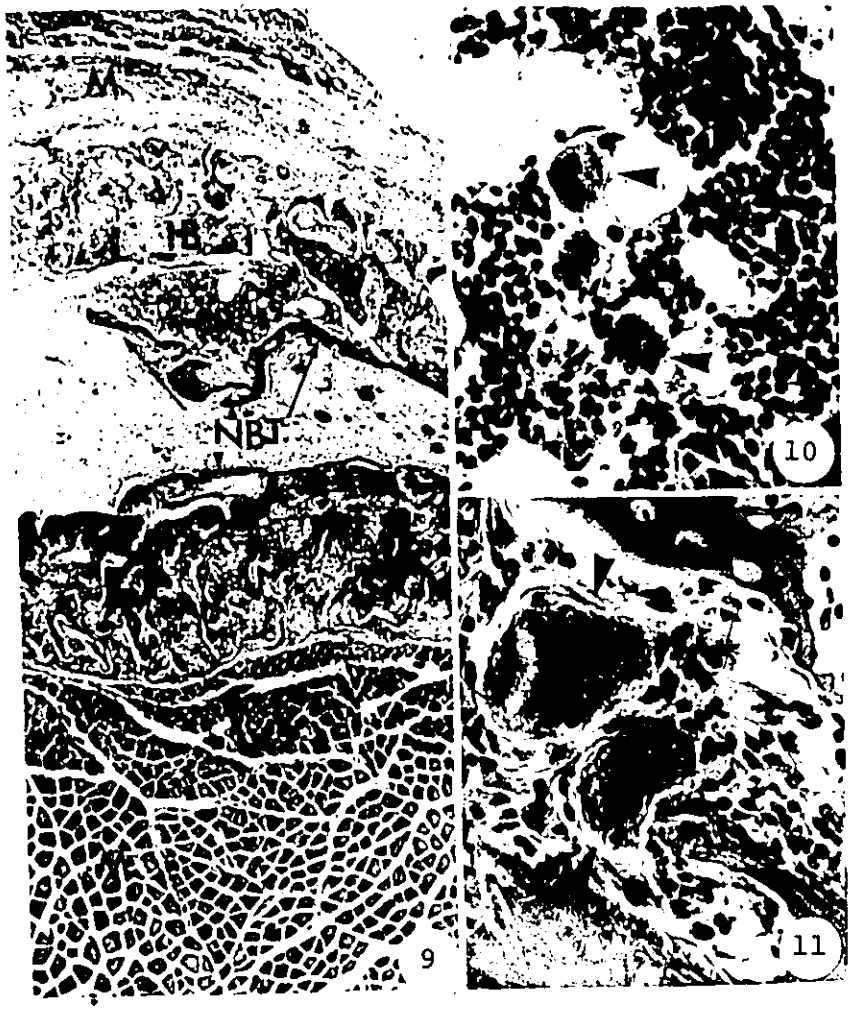
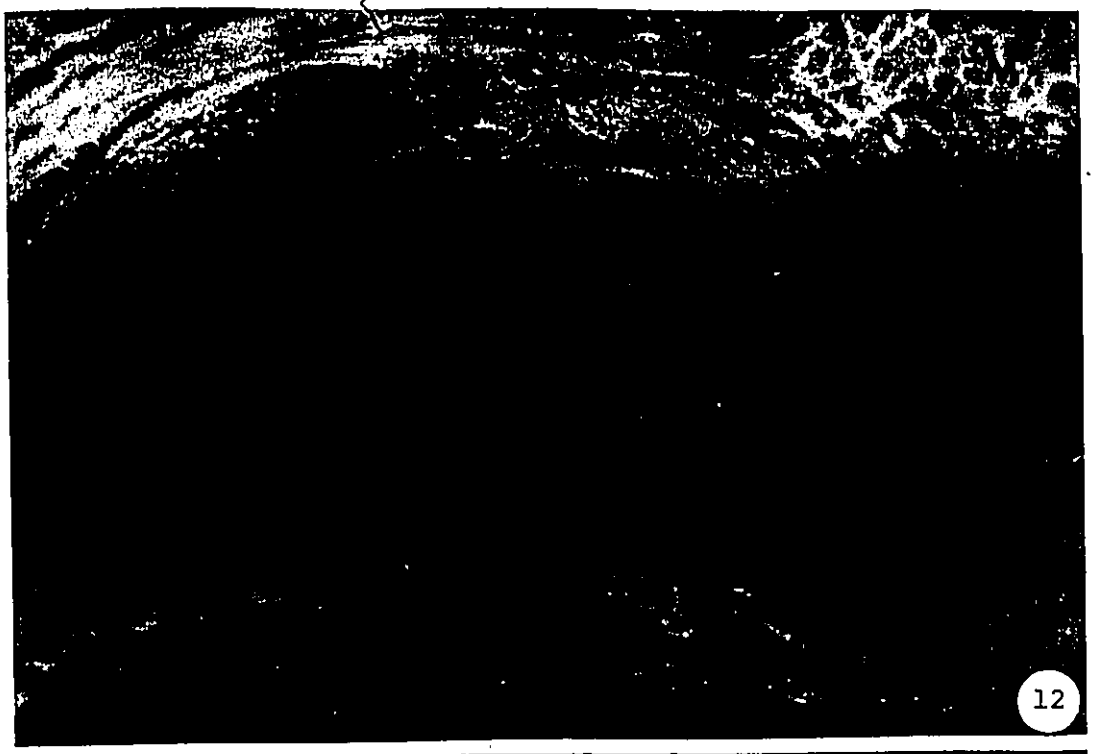

NBT
9
10
11

Figure 12 Section of implant of normal bone into normal host.
Isolated cartilage cells (C) are present in the thickness of the implanted matrix (IB).
Alcian blue Stain 100X

Figure 13 From implant of normal bone into normal host. The specimen shows a cross section of a marrow cavity in which new bone trabeculae (NB) are present. Also present are cartilage cells (C) adjacent to the inner surface of the implanted matrix.
Alcian blue Stain 100X



12

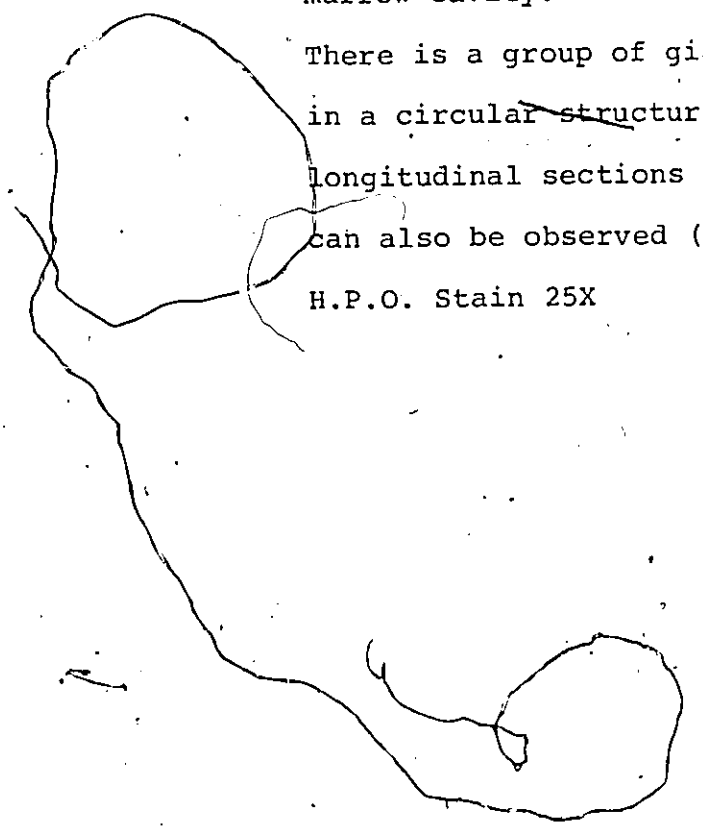
13

Figure 14 Microradiograph of cross section of implant of normal bone into normal host. The new bone trabeculae (white) in the central cavity and the periphery of the implant are well mineralized. 37 X


14

Figure 15 Cross section of whole implant from normal bone implanted in magnesium deficient host.
Multinucleated giant cells (GC) are present outside and inside the graft.
H.P.O. Stain 34X

Figure 16 High magnification of a zone of the bone marrow cavity.
There is a group of giant cells organized in a circular structure (GC). Cross and longitudinal sections of muscle fibers can also be observed (FM).
H.P.O. Stain 25X



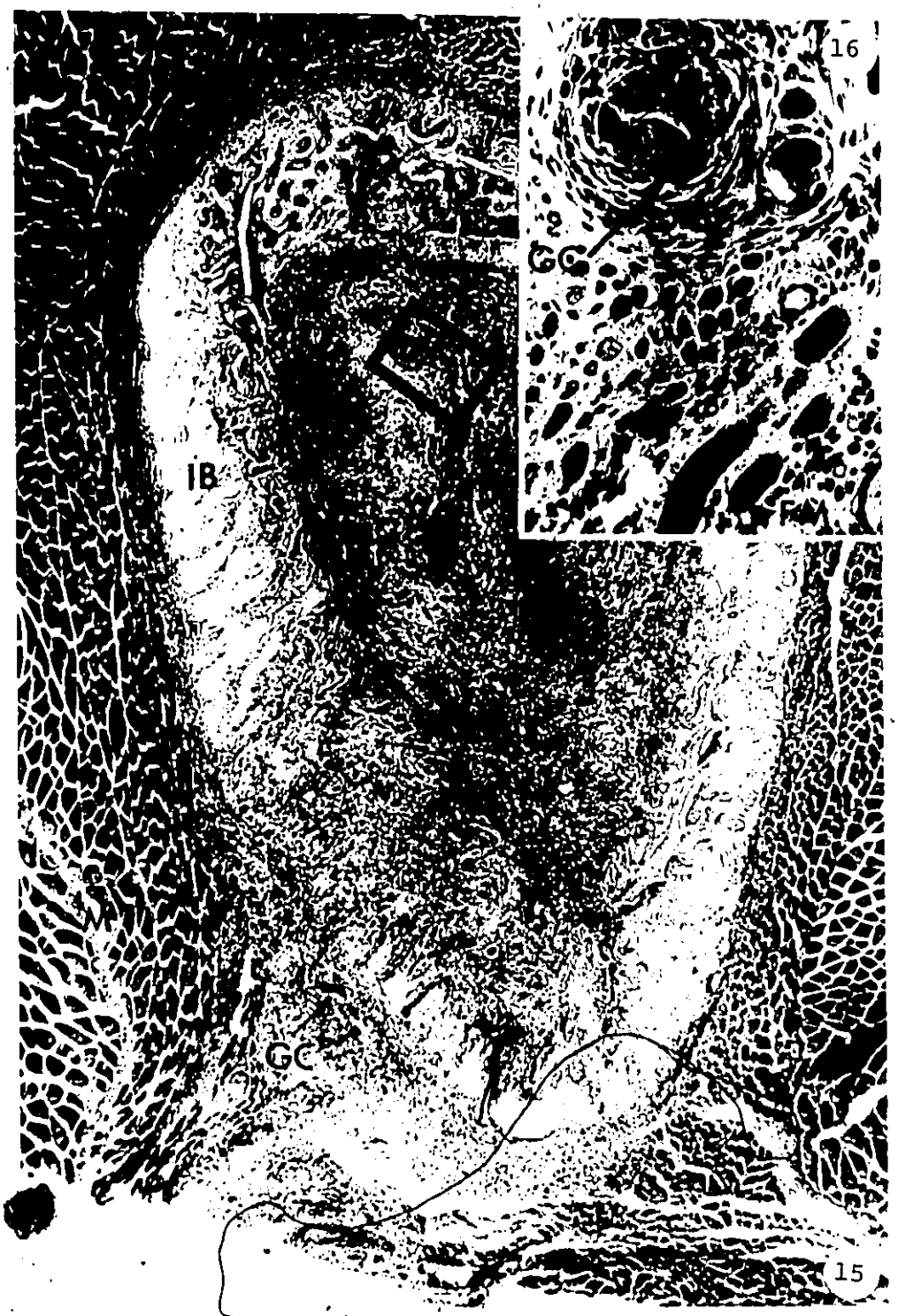
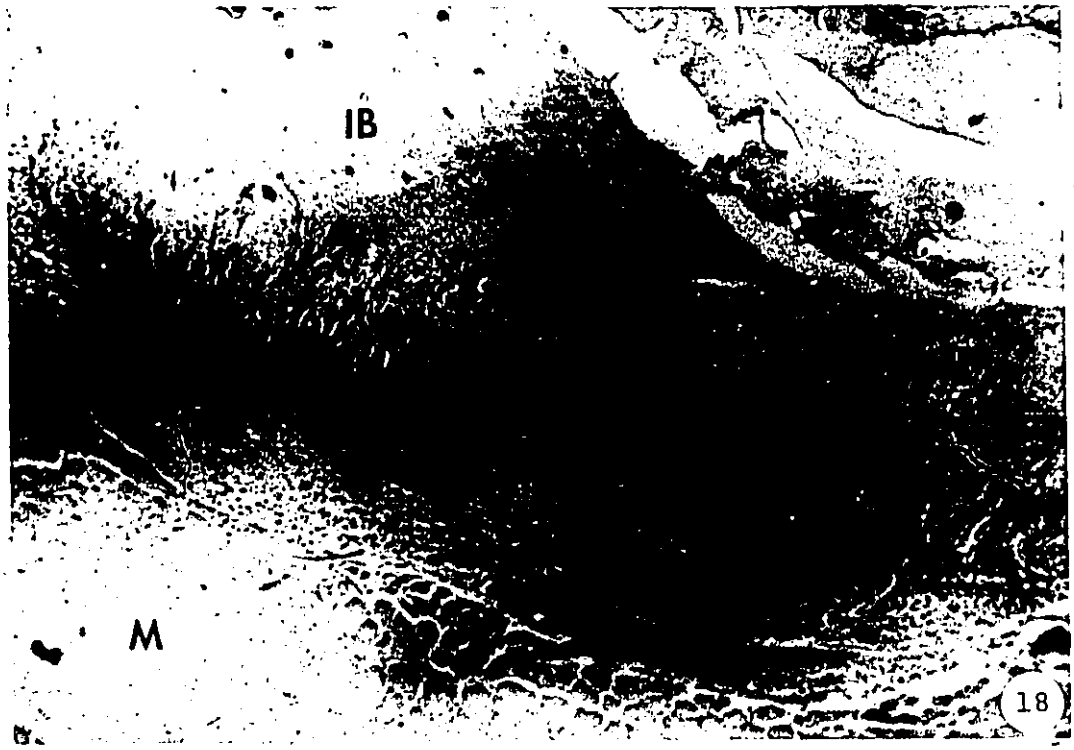

16
IB
GC
15

Figure 17 From implant of normal bone into magnesium deficient host.
Cartilage formation represented by one or more cells (C) localized in the thickness of the graft can be identified. There is also a mass of new cartilage occupying one area of the central cavity.
Alcian blue Stain 100X

Figure 18 Shows a large area of new cartilage localized in the zone of the implanted bone facing the linea aspera.
This cartilage was produced by implant of normal bone in magnesium deficient host.
Alcian blue Stain 100X

17



18

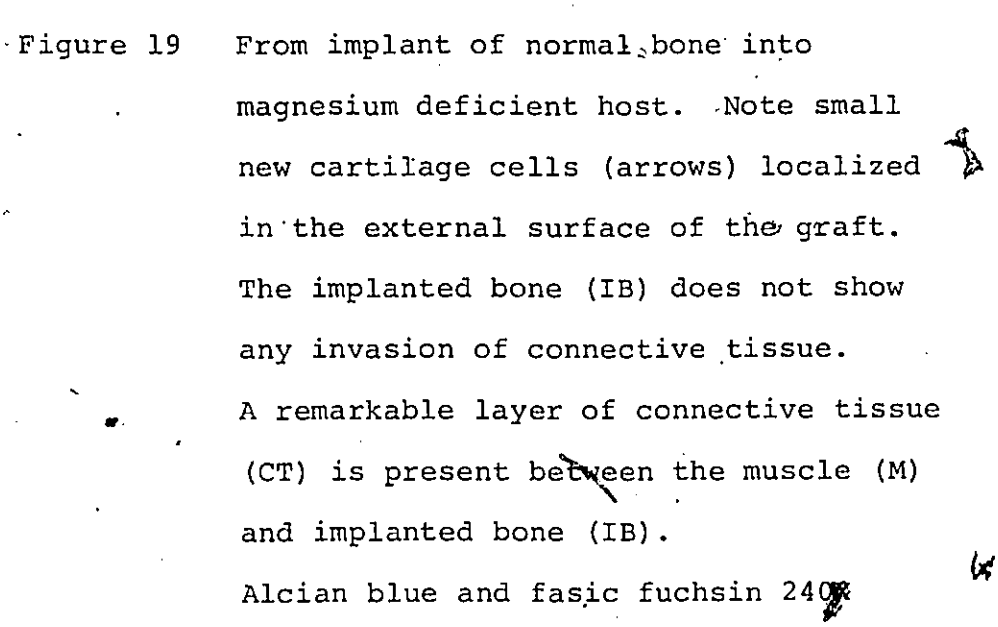


Figure 19 From implant of normal bone into magnesium deficient host. Note small new cartilage cells (arrows) localized in the external surface of the graft. The implanted bone (IB) does not show any invasion of connective tissue. A remarkable layer of connective tissue (CT) is present between the muscle (M) and implanted bone (IB).
Alcian blue and fasic fuchsin 240X

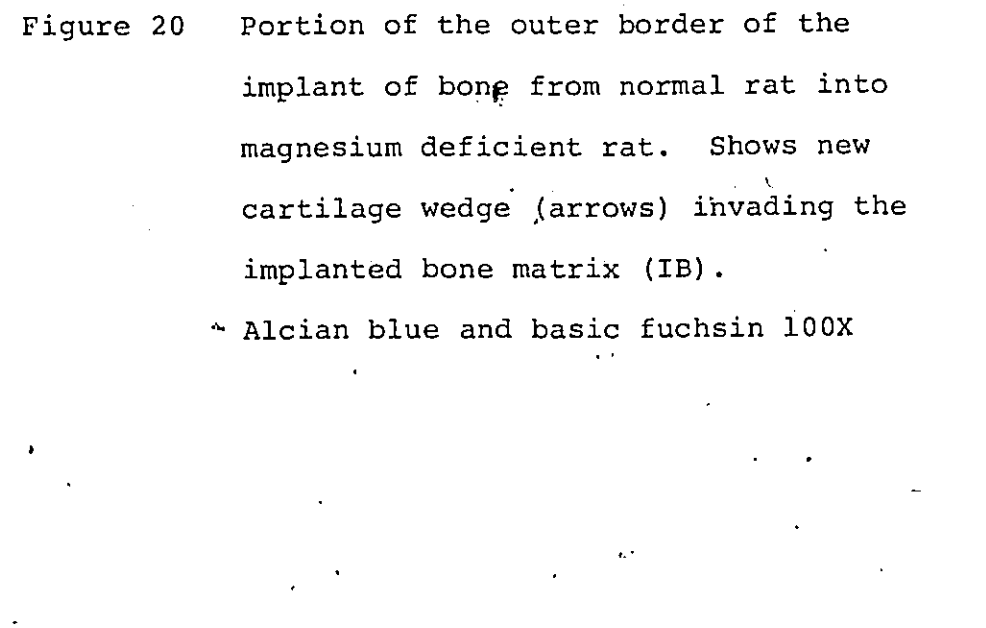


Figure 20 Portion of the outer border of the implant of bone from normal rat into magnesium deficient rat. Shows new cartilage wedge (arrows) invading the implanted bone matrix (IB).
Alcian blue and basic fuchsin 100X

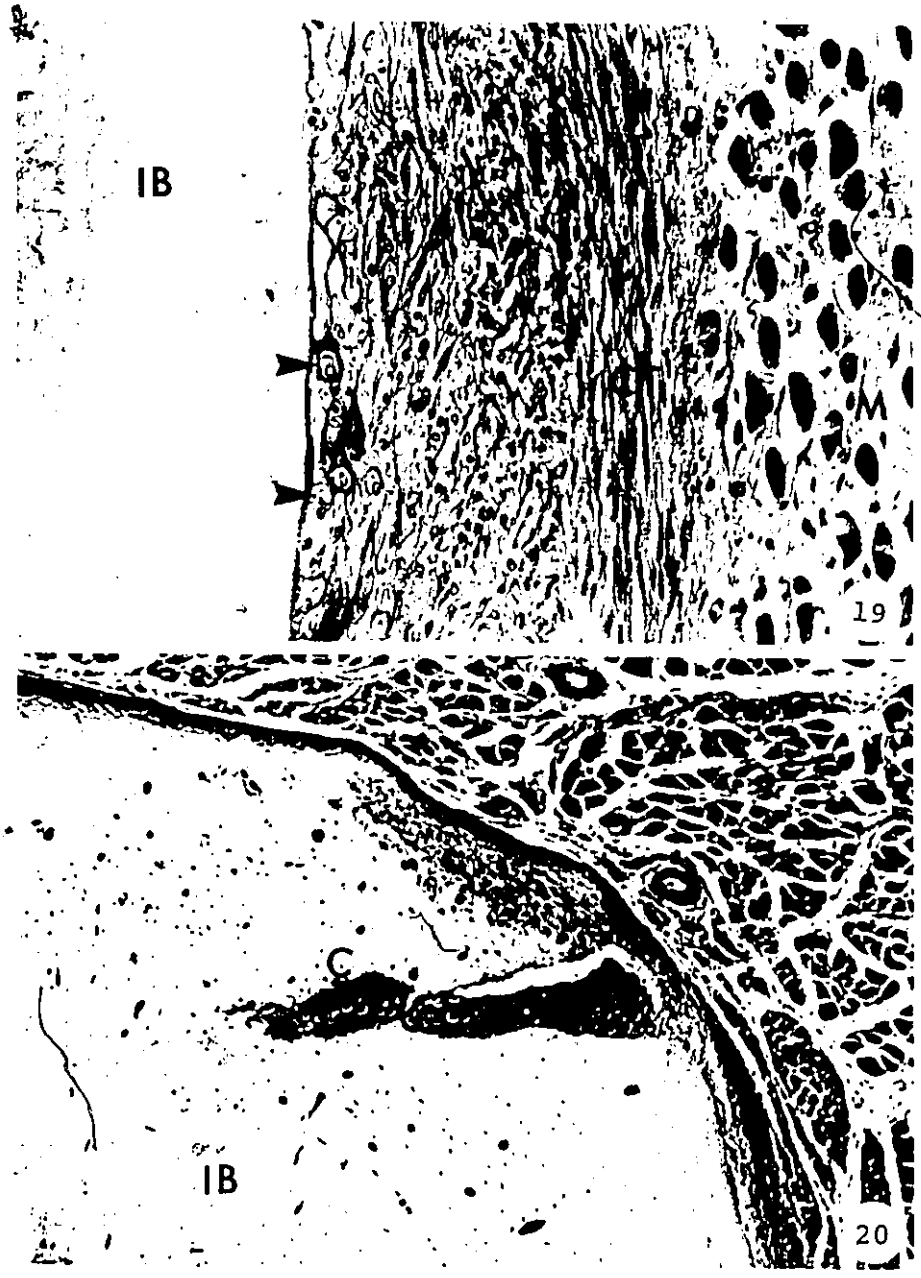
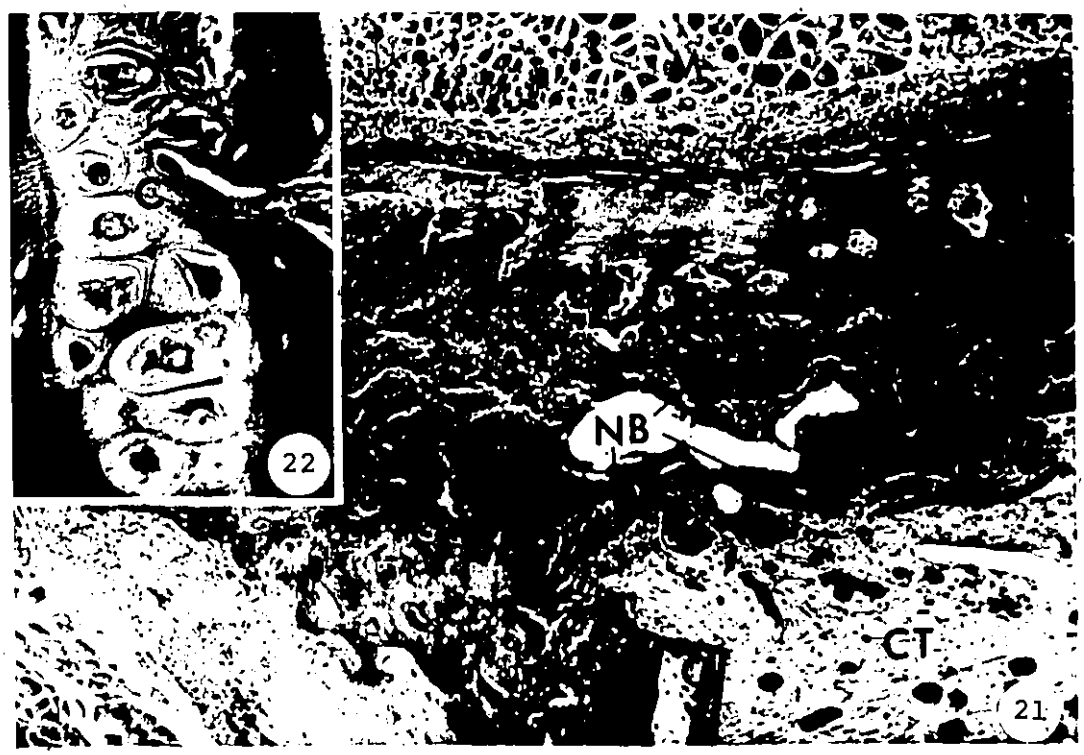
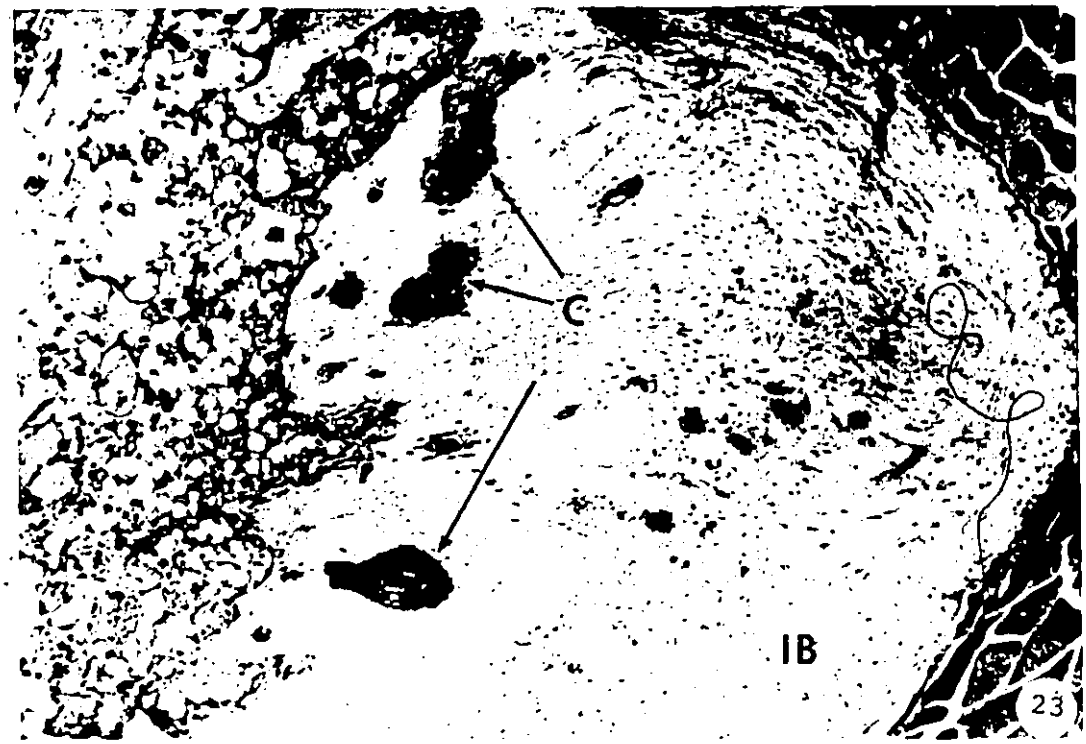

IB
M
19
C
IB
20

Figure 21 From implant of normal bone into magnesium deficient host.
Shows a small new bone trabeculae and one island of new cartilage cells (C). Large blood vessels in between the trabeculae can be observed (BV).
H.P.O. Stain 100X

Figure 22 High magnification from the island of cartilage present in the anterior Fig. 21. Big cartilage cells (C).
H.P.O. Stain 400X.

Figure 23 Belongs to the same series as Fig. 21. It shows islands of new cartilage (C) clearly stained with alcian blue. This area corresponds to the linea aspera of the implanted bone matrix.
Alcian blue Stain 100X


22
NB
CT
21


C
IB
23

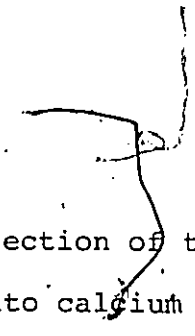


Figure 24 Cross section of the implant of normal bone into calcium deficient rat. The central cavity of the graft shows large amounts of blood vessels (BV). In the inner wall of the graft, there are areas less stained than the implanted bone matrix (IB) which corresponds to the osteoid (OS).
H.P.O. Stain 34X

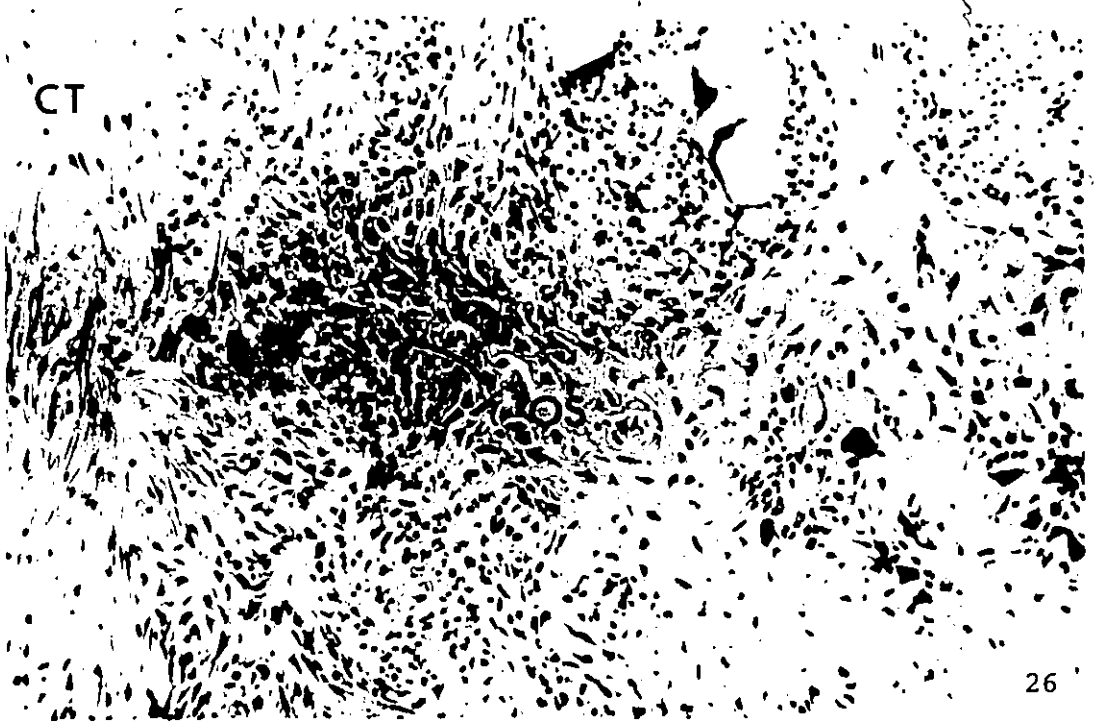
Figure 25 High magnification of one area of osteoid from Fig. 18.
Shows deposits of osteoid mainly around large blood vessels.
H.P.O. Stain 100X

24

25

Figure 26 From implant of normal bone into zinc deficient host. A large area of osteoid (OS) in the central cavity. Notice the disorganization of the new growth.
H.P.O. Stain 100X

Figure 27 From implant of normal bone into zinc deficient host. Areas of osteoid formation (OS) are present inside the cavity adjacent to the inner wall of the graft. Also present in the outer surface of the graft, are a group of multinucleated giant cells (GC).
H.P.O. Stain 250X



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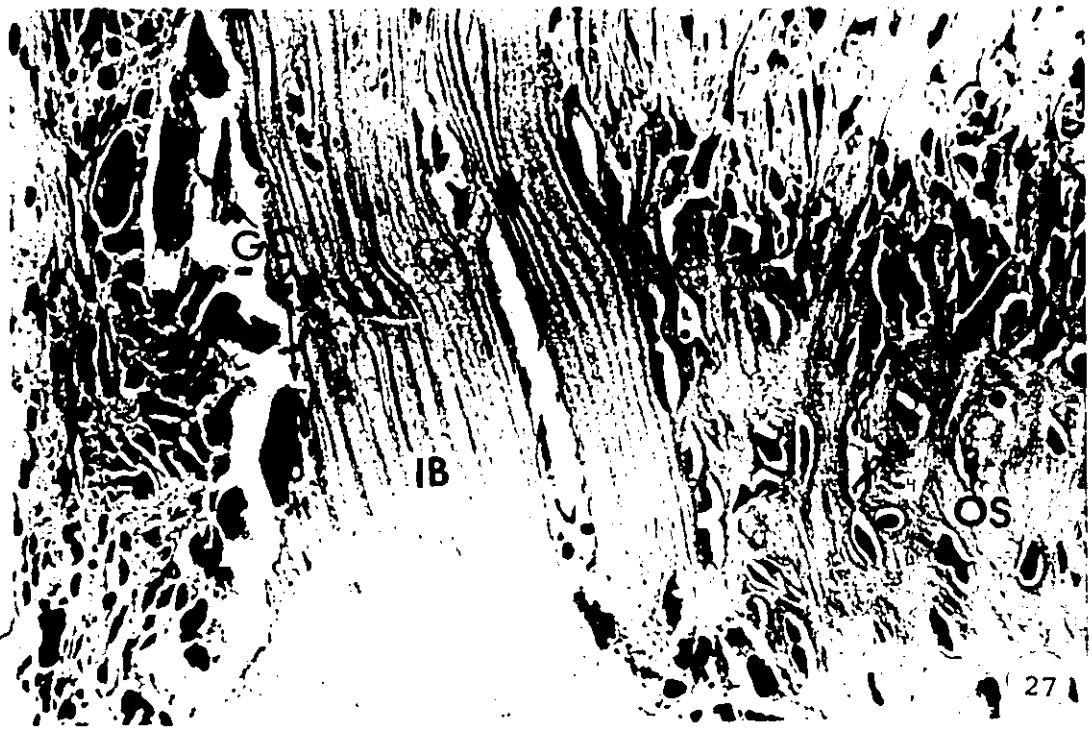
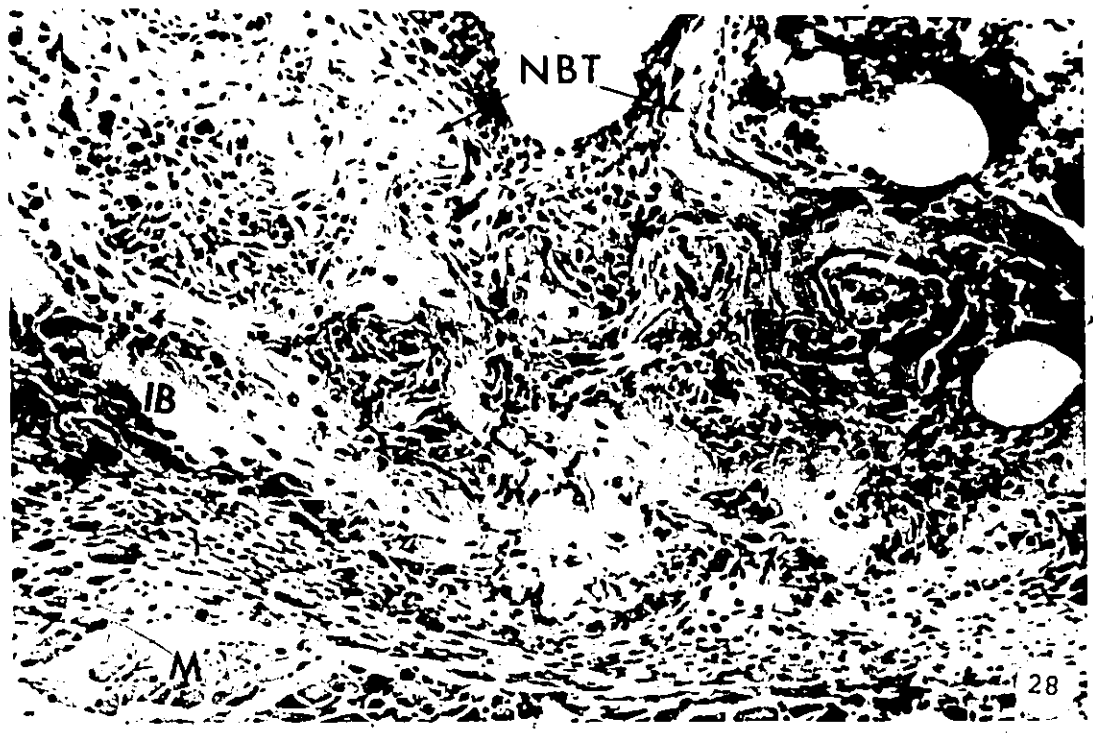


27

Figure 28 From implant of normal bone into zinc deficient host. A large group of new bone trabeculae (NBT) localized in the area that was occupied by implanted bone before reabsorption. Some giant cells (GC) can be identified near a fragment of implanted bone matrix (IB).
H.P.O. Stain 250X

Figure 29 High magnification of a new bone trabeculae from Fig. 28. Lacunae with osteocytes (OT) and osteoblast (OB) can be identified.
H.P.O. Stain 400X



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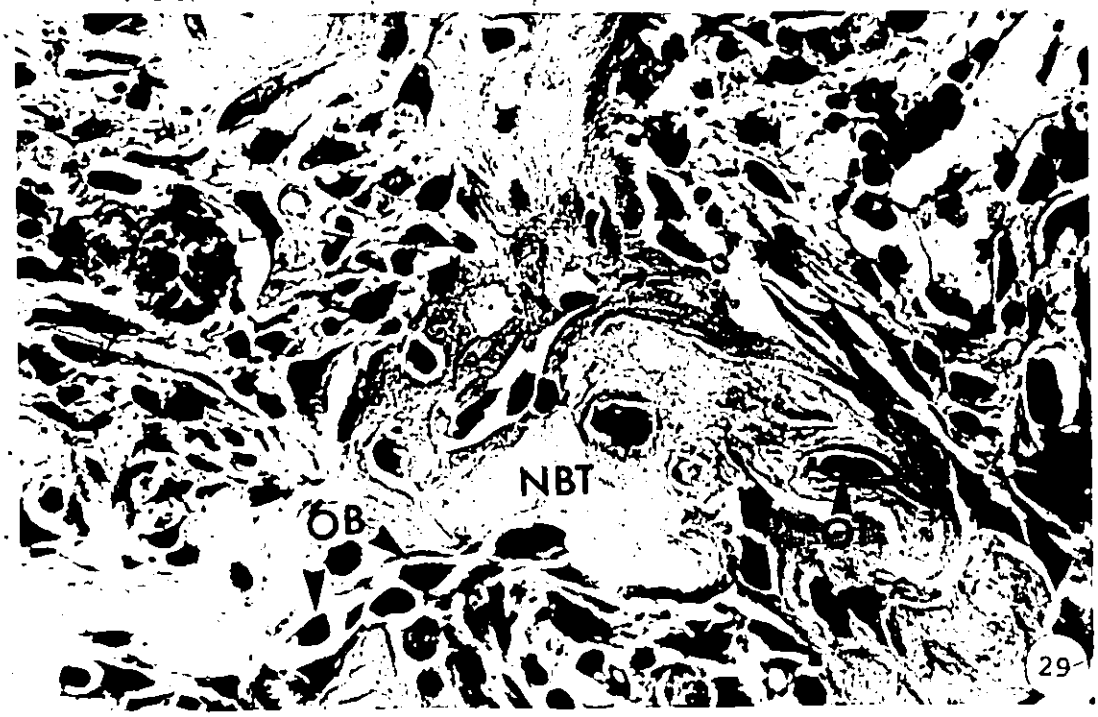
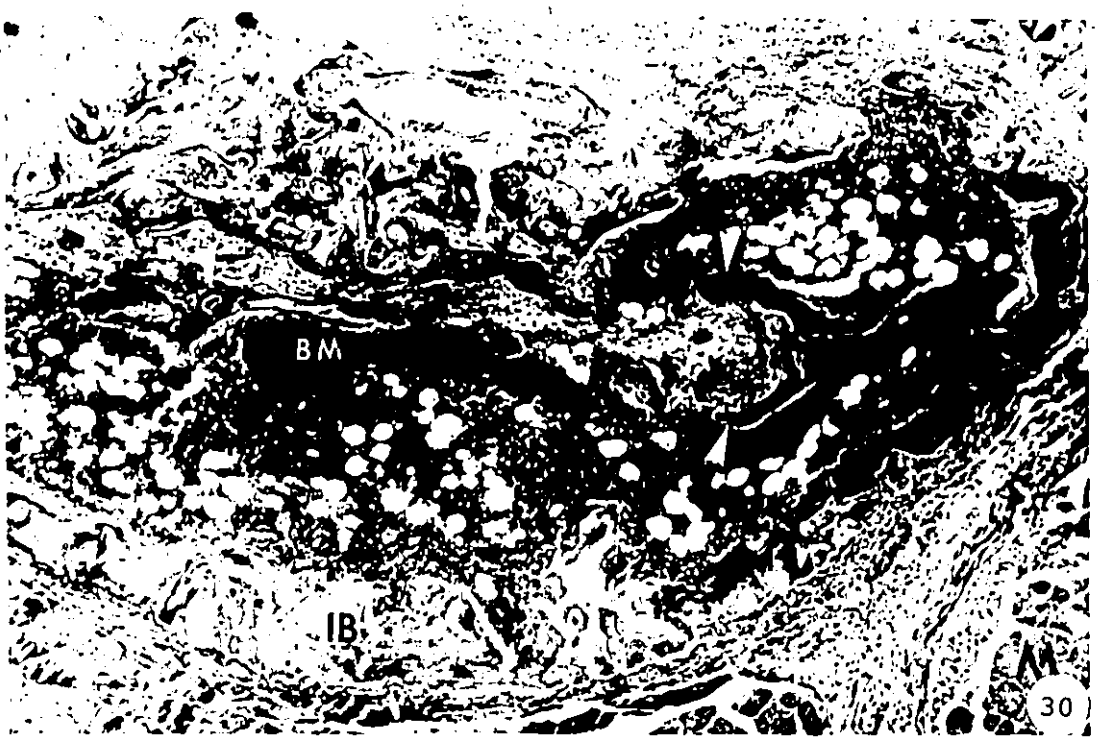


29

Figure 30 From implant of magnesium deficient bone into normal host. Few trabeculae (arrow) of new bone are present. Bone marrow tissue type is occupying the space among the trabeculae and implanted bone matrix (IB).
H.P.O. Stain 100X

Figure 31 From implant of magnesium deficient bone into normal host.
The long trabeculae of new bone (arrow) are present in and outside the central cavity. Connective tissue and marrow type separates the new bone trabeculae from the implanted bone matrix (IB).
H.P.O. Stain 100X

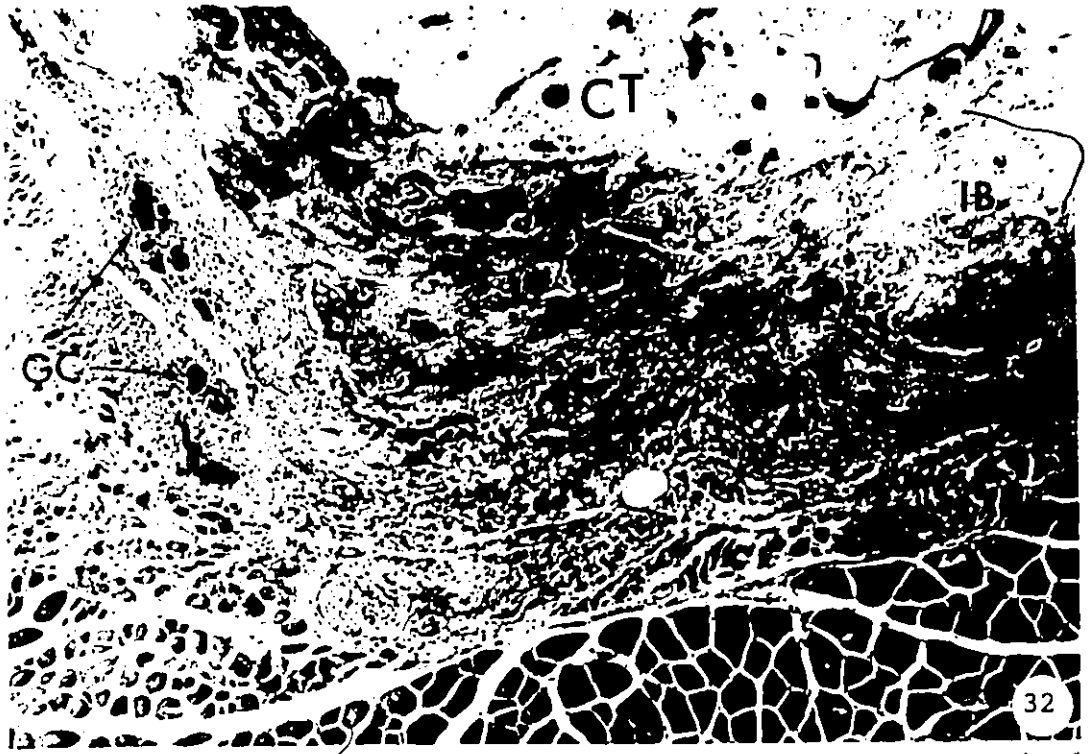


30

31

Figure 32 Shows a segment of implanted bone matrix (IB) from implant of magnesium deficient bone into normal host. There is a group of multinucleated giant cells (GC) in the connective tissue surrounding the graft. The central cavity as usual is occupied by connective tissue (CT). H.P.O. Stain 100X

Figure 33 From implant of magnesium deficient bone into normal host. Shows numerous multinucleated giant cells (GC) facing the linea aspera. Neither growth of bone nor cartilage is present in the implanted bone matrix. H.P.O. Stain 100X


CT
IB
GC
32

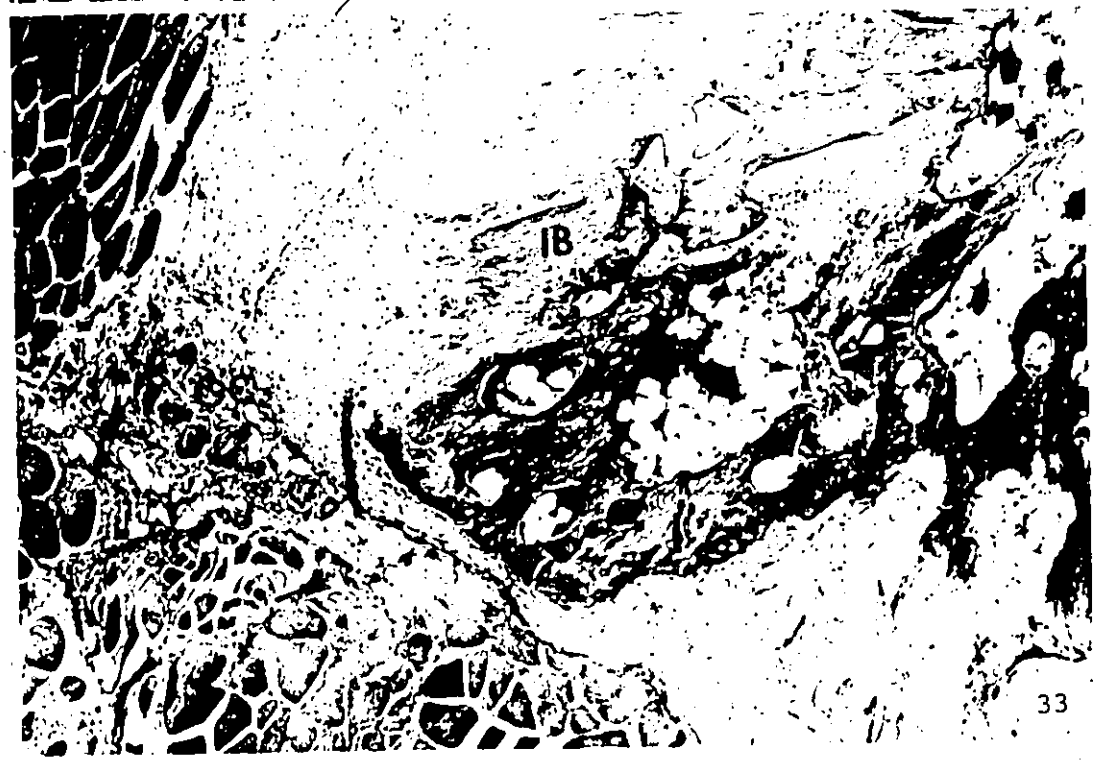

IB
33

Figure 34 From implant of magnesium deficient bone into normal host. Few new trabeculae (NBT, arrows) are present in the inner and outer surface of the graft. New bone marrow tissue (BM) is also present among the new bone trabeculae. The central cavity is filled with connective tissue.
H.P.O. Stain 100X

Figure 35 Belongs to an implant of magnesium deficient bone into normal rat. The new bone formed in the central cavity (NBT) shows an interesting structure in localization. Inside this structure, new bone marrow is present (BM). Connective tissue (CT) surrounds the outside.
H.P.O. Stain 100X


IB
BM
CT
34

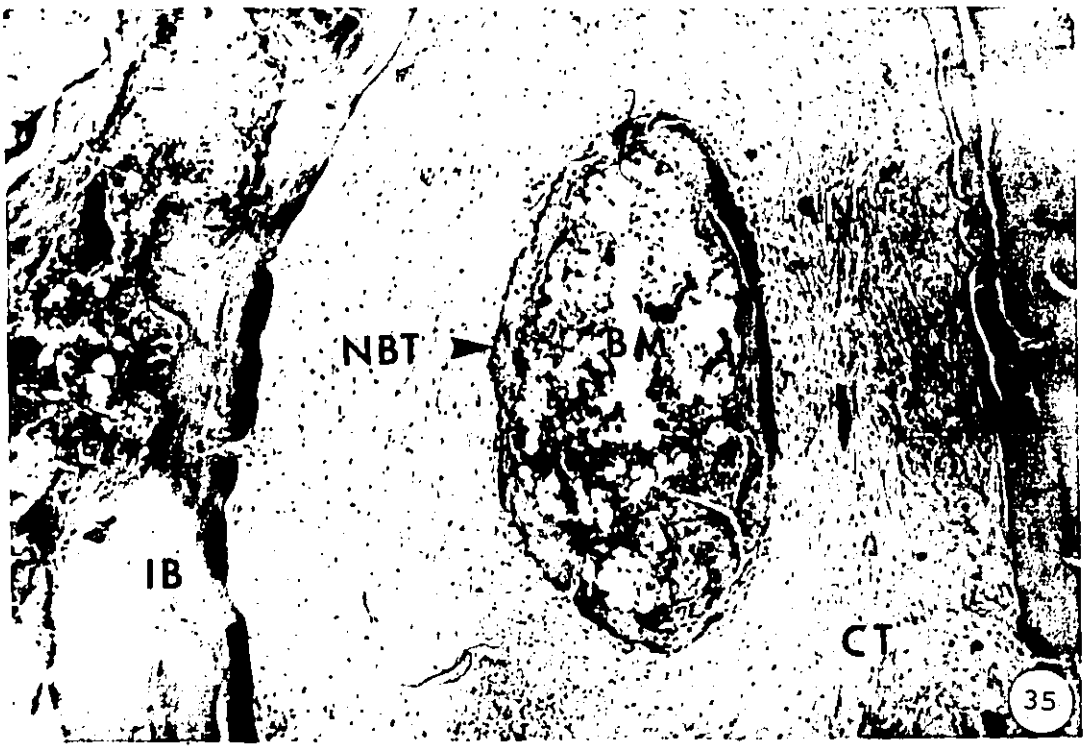

NBT
BM
IB
CT
35

Figure 36 Is a section of marrow tissue from implant of magnesium deficient bone into normal rat. A megakaryocyte-like cell can be seen in the middle of the figure. Abundant rounded cells are also present. The histology of this bone marrow is similar in all the cases in which it was present.
H.P.O. Stain 400X

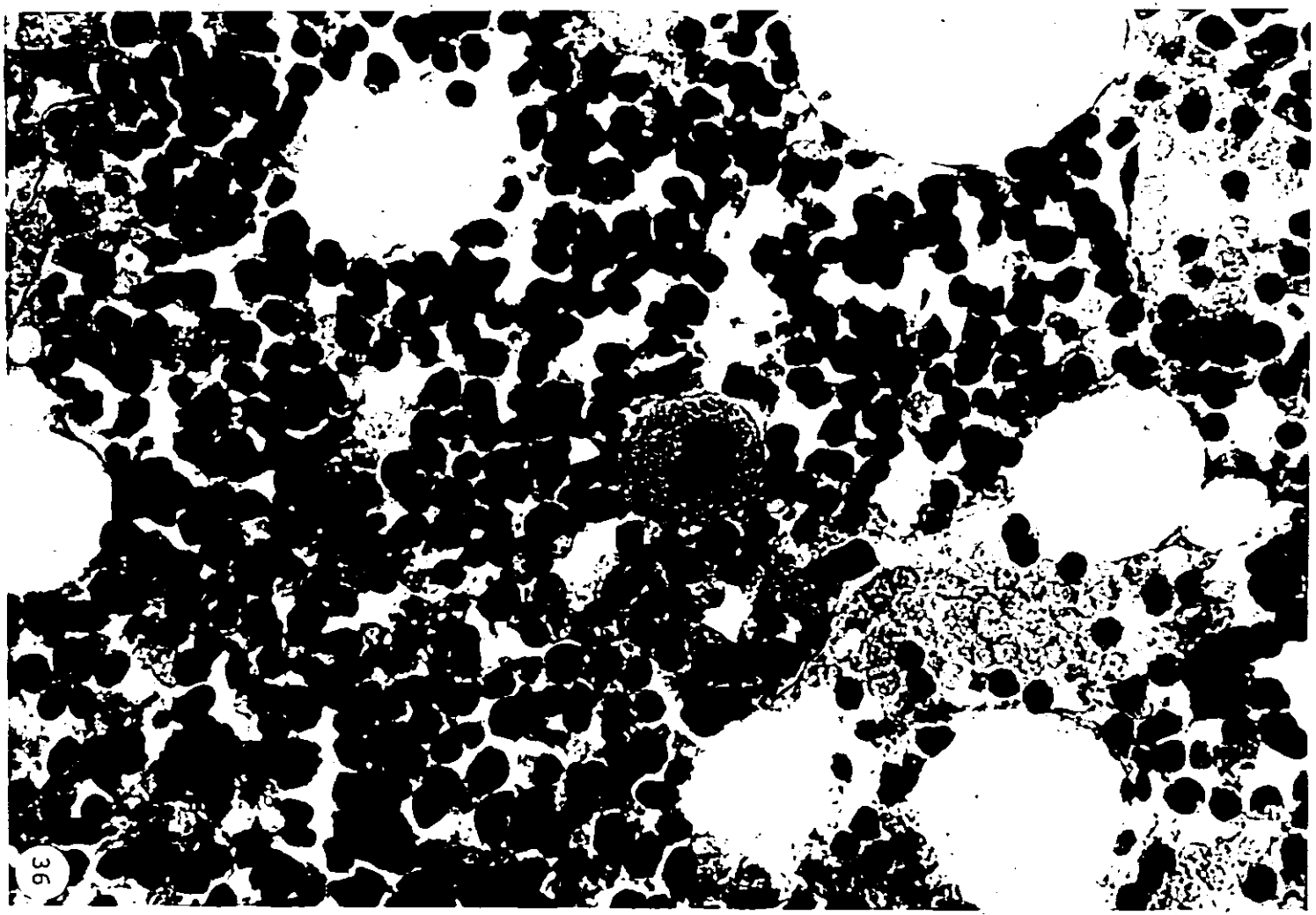
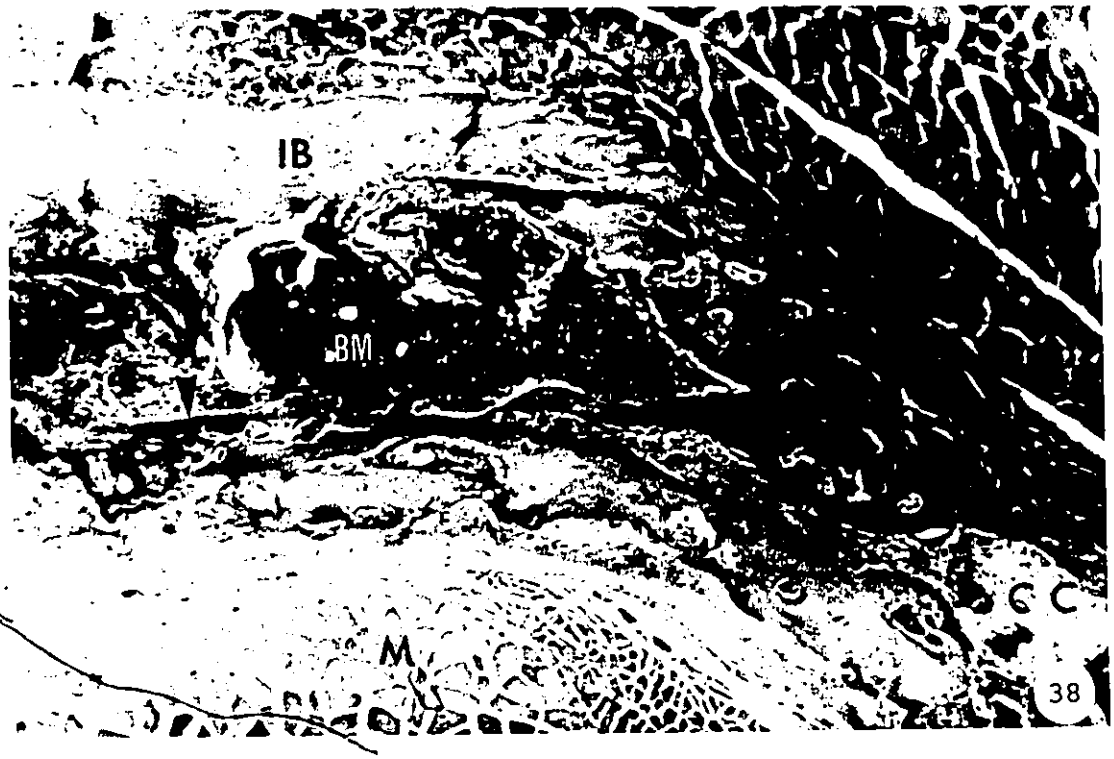

36

Figure 37 From implant of calcium deficient bone into normal host. Shows new bone trabeculae (arrows) in the inner surface of the graft. A mass of new bone can be observed in the central cavity (NB) surrounded by connective tissue. H.P.O. Stain 100X

Figure 38 From implant of calcium deficient bone into normal host. Bone marrow tissue type is present in the central cavity (BM). The new bone (arrows) is present in a thin layer covering the inner surface of the graft (IB). Connective tissue is also present in the central cavity (CC).

37

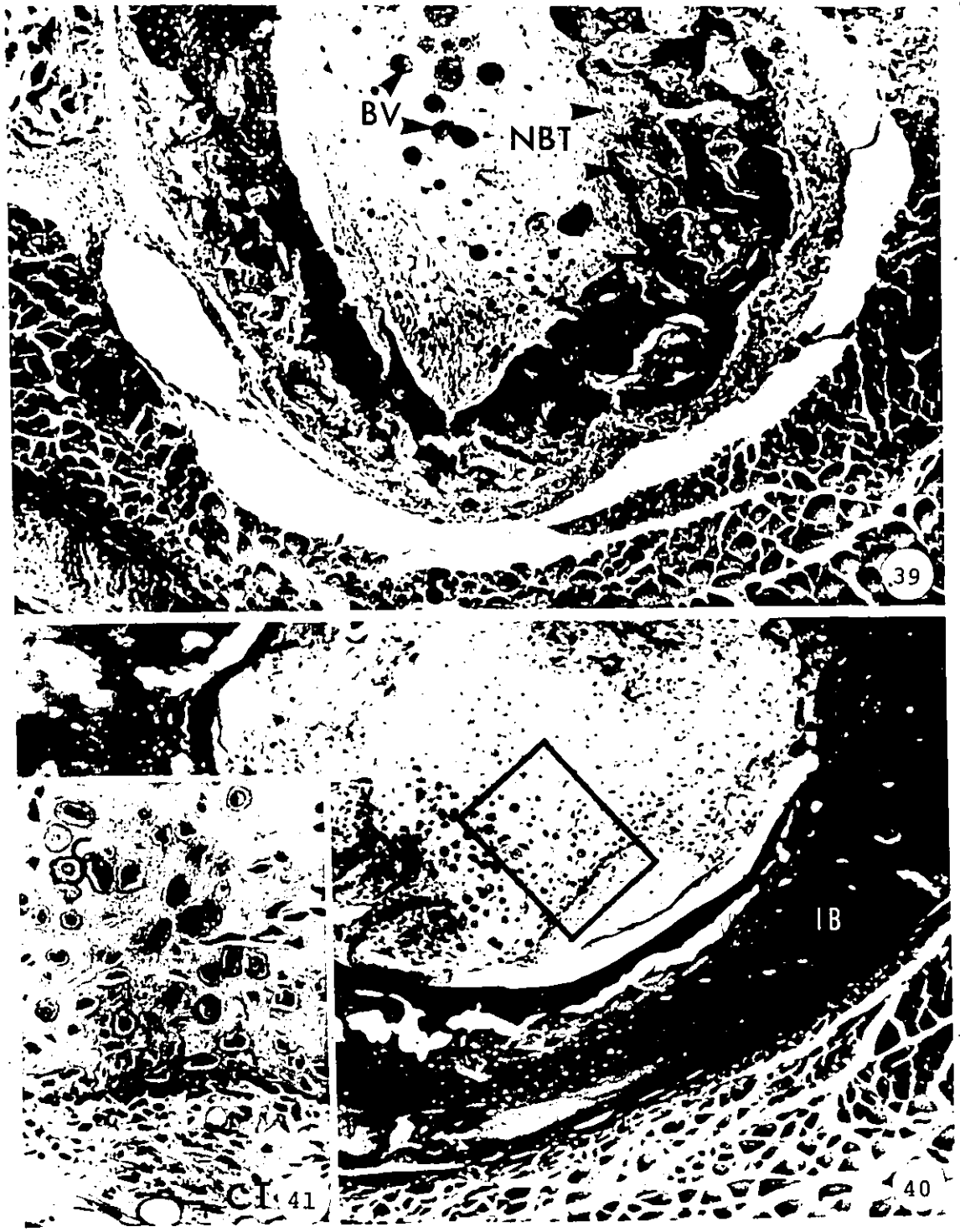


38

Figure 39 Corresponds to implant of bone from gonadectomized and magnesium deficient rat into normal host. Small trabeculae of new bone (NBT) are present in the inner edge of the graft (arrows). The central cavity is filled with connective tissue (CT) and blood vessels (BV).
H.P.O. Stain 100X

Figure 40 From implant of bone from gonadectomized rat into normal host. The central cavity of the graft is almost filled with new cartilage (C) and new bone (NB).
H.P.O. Stain 100X

Figure 41 Is a high magnification of the zone of new cartilage (C) from Fig. 40. Notice the area of cartilage surrounded by small cells probably chondrocytes.
H.P.O. Stain 250X


BV
NBT
39
IB
40
CM
CI
41

CHAPTER IV

DISCUSSION

A. Characteristics of the response of normal hosts to grafts of normal devitalized bone.

The evolution of devitalized rat long bone grafted into the abdominal muscles of young rats has been analyzed in detail by Urist (1965, 1971). In order to serve as a background for the present study a first series of experiments was conducted in which Urist's basic technique was repeated. The only difference with Urist's procedure consisted in the selection of the lumbar paravertebral muscles (according to Bélanger, Robichon and Urist, 1975), instead of the abdominal ones as implantation site.

The lumbar muscles are of course much bulkier and facilitate the implantation of large grafts without technical difficulties; the site appears to provide a better vascularization and produces less mechanical stress (Bélanger, Robichon, Urist, 1975). Since the evolution of the connective tissue surrounding the graft was to be analyzed, it was necessary to take extreme precautions in order to avoid contamination of the graft and the implantation wound. In addition to a careful sterilization of all the materials and

instruments, the utilization of a horizontal laminar flow bench as operating table was found to be of special value. This type of bench has been widely accepted by tissue culture laboratories in which a thoroughly sterilized environment is required, but is not generally used for surgical procedures. The fact that septic contamination was never found demonstrates the usefulness of the gadget in this type of experiment. The hosts subjected to intramuscular implants of devitalized normal bone showed tolerance of the grafts as indicated by the characteristics of the mesenchyme tissue surrounding and infiltrating the graft.

At the same time, the differentiation of bone, cartilage and bone marrow from the above mentioned mesenchyme tissue is obviously the result of an induction process produced by the graft. These observations confirm the formation of heterotopic bone by induction process already described in numerous works by Urist et al, (1952, 1965, 1967, 1971) which are analyzed in detail in the introduction. Bone differentiation, however, was found in only 61% of the grafts. This percentage of positive response is smaller than that found by Urist , (1965) who obtained bone formation in

90% of the cases, but larger than that found in older works (Chalmer and Urist, 1952; Selle and Urist, 1961; Zaccalini and Urist, 1964) in which scanty deposits of bone were induced in about 30% of the grafts. In the present experiments, decalcification (0.6 N. HCl) and freeze drying techniques similar to those used by Urist (1965) have been followed. The differences in yield may be explained by differences in the type of bone used (femur instead of the tibia as usually grafted by Urist). It is also possible that the difference in yield is due to the strain of rats utilized in the present experiment. Despite these differences however, the histological reaction to the graft in this experiment (Series I) was entirely comparable with the observations of Urist (1965). As in this author's experiments, the new bone grew outside and inside the graft, in the form of trabeculae similar to those that appear in fetal osteogenesis. The new bone was also very well mineralized as described by Urist (1971). According to Urist (1965, 1971) the sequence of events is the following: the area of bone graft stimulates the migration of mesenchymal cells from the muscle which then surrounds and infiltrates the

graft; in a second step, the bone graft induces the transformation of those mesenchymal cells into bone and cartilage forming cells. The observations in the present experiment agree with this description. Also in agreement with Urist (1971), multinucleated giant cells (matrix clasts) were observed in the connective tissue surrounding the graft, and in excavation cavities of the implanted matrix. They may play an important role in the resorption of the matrix of the graft and thus facilitate the solubilization of possible "inducing" agents. Small isolated cartilage cells were usually present and had been described previously (Urist, 1965, 1971). The meaning of the cartilage induction shall be discussed below (page 110).

Also confirming Urist's descriptions (1965, 1971) bone marrow was found in connection with the new bone trabeculae. This kind of relationship may indicate that the trabeculae induce the formation of bone marrow. Further elaboration on this point follows in a subsequent section (page 136).

B. Response of magnesium-deficient host to a normal graft.

The animals used as hosts in this experiment showed a symptomalogy characterized with edema hyperemia in the extremities, and obvious irritability. This syndrome is typical of magnesium-deficient rats as described by Watchorn and McCance, (1937) and confirmed by numerous other authors: Bélanger (1957), Heggtveit, Herman and Mishra (1954); Clark and Bélanger (1967); Hunt and Bélanger (1972); Bogoroch and Bélanger (1975); Bélanger et al, (1975). The presence of this symptomology in the present rats confirms that they really became magnesium-deficient. The response of magnesium-deficient hosts to the implantation of devitalized bone grafts was clearly different from the previous description for normal hosts. The main difference consisted in the induction of cartilage in all cases. The induction of bone was contrariwise, very poor; only a few trabeculae were present in some cases. Thus, while in normal hosts, bone differentiated in 61% of the cases and cartilage in only 32%, in magnesium-deficient hosts only 50% of the cases had small amounts of bone and 100% presented significant amounts of cartilage.

It has been recognized that poor vascularization favours chondrogenesis in vivo (Van de Putte, K.A., and M.R. Urist, 1966) and in vitro, (Urist, et al, 1974). It is possible that this happened in these experiments because the new cartilage grew only in areas with relatively poor irrigation. However, Bélanger, Robichon and Urist, (1975) have challenged this argument on the basis of the fact that magnesium deficiency through the release of histamine from the very numerous mast cells actually increases and not decreases the vascularization.

It is believed (Urist, 1965) that previous to the bone and cartilage induction process, the implanted matrix must be partially resolved; sub-products of the digestion of the matrix may play a role as inductors.

The magnesium deficiency in altering the function of parathyroid glands (McManus and Heaton, 1970; McManus, Heaton and Lucas, 1971) may modify indirectly this process of resorption and secondarily the nature of the induction process. It is also possible, according to Bélanger and Robichon (1975) that magnesium deficiency may modify the host's immune response to the implant. This suggestion has some support from the fact that modifications in the lymphatic system (Bois, P., 1968

1969) produced by magnesium deficiency have been described).

Finally, in addition, one should not discard the possibility that the deficiency of magnesium acts directly at a local level. It is known that magnesium is a co-factor in numerous important enzymatic reactions including alkaline and acid phosphatases, A.T. Pases, pyrophosphatases, etc. (See review by Lehninger, 1975). A deficiency of the ion could thus interfere with the process of matrix digestion and/or with deposition of new bone.

That magnesium ions are involved in the synthesis of mucopolysaccharides has been demonstrated by studies using S^{35} incorporation (Bélanger, 1958) and metachromatic staining techniques (Clark and Bélanger, 1967). These studies demonstrated a decreased chondroitin sulfate production in magnesium deficiency.

On the basis of the previously mentioned literature one could postulate that the reduced yield of bone in magnesium-deficient animals could be related to an alteration in the synthesis of matrix of the new bone. This assumption receives support from results by Hunt and Bélanger (1972), Bogoroch and Bélanger (1975),

and Bélanger and Robichon (1975), all of whom demonstrated retardation of bone growth and osteoporosis in magnesium-deficient rats.

C. Response of calcium-deficient host to a normal graft.

While magnesium deficiency of the host was shown to alter the process of bone induction in the sense that cartilage, instead of bone, was more frequently induced in the case of calcium deficiency, the inductive process appeared to proceed normally.

However, once induced, the bone was unable to complete its differentiation and remained as immature, non-mineralized bone (osteoid). It is generally known that osteoid appears in animals fed on diets deficient in vitamin D and/or calcium.

In the present experiment, the diet used was the one described by Kenny and Munson (1959) which is deficient in calcium but contains 3.0000 IU of vitamin D per kg, an amount that supplies, with excess, the needs of growing rats. The lack of mineralization of the induced bone must, in this case, be attributed to the deficiency of calcium. Calcium is distributed

in most tissues and plays an important role in numerous biological phenomenon (cell permeability, nerve conduction, muscle contraction, blood clotting, etc). The concentration of calcium in all these tissues is, however, very low. Calcium salts, contrariwise, reach very high concentrations in normal bone where they are present as a complex of calcium phosphate and carbonate known as apatite (see review by Isadore Zipkin). When bone is formed, it first appears as a bone matrix containing densely arranged collagen fibers, soon it is embedded in amorphous ground substance rich in mucopolysaccharides (see review Bélanger in Histology, Roy D. Greep and Leon Weiss, 1973). Deposit of minerals on this matrix occurs in a second step. The mechanisms by which this calcification is induced are not completely clarified (see review H. Bourne, in The Biochemistry & Physiology of Bone).

It is accepted, however, that an adequate level of calcium in the serum is one of the preconditions for this process to occur. Thus, in the present experiments, one can postulate that the lack of mineralization of the induced bone was produced by an inadequate level of calcium in the serum resultant from the deficient diet. It is known that diets deficient in calcium, by lowering

the concentration of ionic calcium in the serum, stimulated the parathyroid gland (Kalu et al, 1974). The increased parathyroid hormone level usually induces intense bone resorption. Bélanger et al, 1967, Bélanger, Rasmussen, 1968; Jande, Bélanger, 1973, have demonstrated that bone resorption in this case takes place mainly through activation of osteocytic osteolysis, (Bélanger, 1969). It was also shown that only the older more mature osteocytes are involved in this resorption process (Jande, 1971; Jande and Bélanger, 1973). Thus, it is not surprising that the new immature bone in our grafts do not show signs of osteocytic osteolysis. It is of interest to note that an increase of osteoblasts was not found in our material. This fact again, supports Bélanger's (1969) concept, indicating that it is resorptive osteocytes and not osteoblasts which are activated when additional calcium is needed in order to maintain homeostasis.

D. Response of zinc-deficient host to a normal graft.

Most of the zinc-deficient rats responded to the implantation of devitalized bone with formation of bone. However, in all cases, most of this bone did not undergo mineralization and remained as typical osteoid. Similar results have been obtained by Calhoun, Smith and Becker (1974). These authors postulate that the zinc-deficiency affects the early stage of the calcification process, but they do not suggest any mechanism to explain this phenomenon.

One could suggest that zinc forms either part of several metalloenzymes, or acts as a co-factor in some enzymatic reaction. Thus, a deficiency could exert its effect on the mineralization of bone by interfering with the action of those enzymes. Zinc forms part of the molecule of carbonic anhydrase (Moren, 1967), an enzyme which plays a role in the deposit and resorption of the carbonate fraction of hydroxyapatite crystals. Similarly, alkaline phosphatase in the intestine and kidney decreases its activity in rats with zinc deficiency (Hove, Elvehjem and Hart, 1940;

Luecke, Olman and Baltzer, 1963; Reynolds, 1968). In addition, one must recognize the possibility that the lack of mineralization may be a secondary effect of a more general action of the zinc deficiency. Thus, this deficiency interferes with many general metabolic pathways. For example, it interferes with the mobilization of vitamin A from the liver (Barnicot, 1951). This fact may be relevant since vitamin A produces abnormal activity of the osteoblast in the skull bone of the dog (Mellanby, 1950) under vitamin A deficiency. On the basis of Mellanby (1950), Barnicot and Datta, 1972, suggest that vitamin A acts as a specific chemical control in the activity of the osteoblast and osteoclast, but they did not explain the control. It was also observed by Fell, Mellanby and Pelc (1956) and Dziewiatkowsky (1954) that the incorporation of sulphate into the cartilage matrix in vitamin A deficiency was lower than in the normal rats.

E. <u>The response of normal host to grafts from magnesium-deficient rats</u>.

The reaction of the mesenchymal tissue to the implant was different from the normal grafts. In fact, the connective tissue surrounding the implant was always

invaded by multinucleated giant cells foreign body-like. They appear to represent a rejection reaction. It seems that the rejection reaction is the result of the changes in the biochemical composition of the bone matrix after magnesium deficiency. As reported by Hunt and Bélanger (1972); Bélanger, Robichon and Urist (1975); Bogoroch and Bélanger (1975), magnesium deficiency in rats causes hyperplasia in the linea aspera of the femur. It is possible that these changes produce synthesis of proteins which may act as an antigen in the implanted bone. This possibility is supported by the fact that the rejection reaction is evident in the area facing the linea aspera, which is the site where the histological changes took place according to Hunt and Bélanger (1975).

F. Bone marrow.

In the experimental series (I, IV, V, VI) in which new bone grew in trabecular form, bone marrow was present in large or small amounts. The new bone marrow differentiated in the vicinity of the new bone trabeculae. Although the amount of bone was not quantified, it appears that the differentiation of

bone marrow occurred only in cases in which a substantial amount of new bone had formed. This is supported by the observation that in cases which showed a small amount of new bone trabeculae, bone marrow did not appear.

These observations could indicate that the presence of new bone trabeculae may be necessary for the growth of the bone marrow. It is possible that the formation of bone marrow tissue occurs by induction of the new bone trabeculae. Of course, these observations would require further research in order to verify them.

G. Induction Process.

The induction in cation-deficient animals showed a typical result which deserves detailed discussion.

In the grafts evolved in Mg-deficient animals, the induction process took place, but an unusual amount of cartilage cells differentiated. I will try to interpret this result on the basis of the previous scheme of induction mechanism (page 30).

Since numerous mesenchymal cells modified their differentiation into cartilage cells, one must suppose that the inductor substance in these cases, reached their nuclei. One must then conclude that the changes

occurred only at the level of the transcription mechanisms of the induced cells (see Scheme page 30), so that the equipment of enzymes proper of cartilage cells developed instead of those which are characteristic of osteoblasts. It is known that important biochemical differences exist between the matrix produced by both cell types. Thus, the collagen produced in both cases, is different and the cartilage matrix contains large amounts of chondroitin sulfuric acid (See Bélanger in Histology, Greep and Weiss, eds., 1973).

Magnesium is known to act as a co-factor in numerous enzymatic reactions which directly or indirectly affect the synthesis of proteins and/or mucopolysaccharides. It is clear in our experiments, that the deficiency of Mg has altered the pattern of protein and/or mucopolysaccharide synthesis of the induced cells, but it is not possible to pinpoint with precision which modifications are more relevant to the changes observed. Very precise biochemical studies would be needed in order to clarify this point.

In the case of grafts developed in calcium-deficient animals, it was observed that normal amounts of bone were formed but this was atypical and characteristically of the osteoid type. Since calcium is also an important co-factor, numerous enzymatic reactions and for

several of the steps in energy production and protein synthesis, the fact that in our experiments a normal amount of bone was formed, appears to indicate that the whole induction process was not affected by the deficiency. Bone matrix was not normally mineralized, but this can be explained as a direct effect of the low availability of calcium. It cannot be excluded, of course, that the matrix could have been also inadequate for the process of mineralization, but this seems to be improbable.

The results obtained with grafts implanted into zinc deficient animals, are more difficult to interpret. In the first place, the bone was produced, but it was very atypical and large irregular masses instead of the typical trabeculae, were formed. It is difficult to decide if this morphological change represents a real alteration in the induction process. The presence of isolated cartilage cells intermingled with the bone masses, phenomenon never observed in other experimental groups, is also difficult to interpret. Most of the bone induced in Zinc-deficient animals, was of the osteoid type. The calcification of bone matrix requires several enzymes, two of the main ones being the alkaline phosphatase and carbonic anhydrase. It has been shown that the activity of alkaline phosphatase

decreases with Zinc deficiency. It is also known that carbonic anhydrase is a metallo-protein with a nucleus constituted by Zinc. It is thus possible that Zinc deficiency produces a lack of mineralization through an inhibition of these and/or other enzymes.

Finally, Urist's results had shown that the induction of bone produced in the adult by devitalized bone grafts is comparable to the embryonic induction and showed that the inductor agent is also in this case, a diffusible factor of proteinic nature. Our present results, and especially those obtained in Magnesium deficient hosts, support Urist's hypothesis showing that the same as in embryonic induction, the final result of the induction depends not only of the nature of the inducing agent but, very importantly, of the state of the target tissue.

CONCLUSION

The host response to normal devitalized bone graft is modified in magnesium-,calcium-, and zinc-deficient hosts.

1) The magnesium-deficient host response is characterized by predominant differentiation of cartilaginous tissue.

2) Calcium deficiency in the host decreases the capacity to respond to the inductor and impedes

the mineralization of the new bone matrix resulting in the formation of osteoid.

3) The zinc deficiency did not alter the capacity of the host to respond to the induction stimulus. However, some of the masses of new induced tissue appeared to be multiform, cartilage and bone cells appearing intermingled. Induced bone appeared qualitatively different than the normal and, in addition, it did not undergo mineralization.

4) Bone marrow only differentiated in the vicinity of new bone trabeculae, this occurred only in cases in which a substantial amount of new bone had formed.

5) The induction produced by devitalized bone grafts depends not only of the nature of the inducing agent, but very importantly, of the state of the target tissue.

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