LACK OF EFFECT OF LONG TERM PHENOBARBITAL ADMINISTRATION ON VITAMIN D METABOLISM, MINERAL HOMEOSTASIS AND ENDOCHONDRAL BONE GROWTH IN THE RAT

by

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ABSTRACT

This study focussed on mineral homeostasis and vitamin D metabolism in young rats, pair fed a standard diet and subjected to either chronic phenobarbital or saline (control group) injections. Caudal vertebrae and "plaques" of induced endochondral bone were examined histologically and histomorphometrically to determine whether osteomalacia had been induced.

Significant mixed function oxidase induction occured, but mean serum 25-(OH)D concentrations remained unchanged. Mean serum 1,25-$(OH)_2$D concentrations remained within known normal ranges, reflecting an apparently unaltered vitamin D activation pathway. Serum calcium and phosphorus concentrations were not consistent with an osteomalacic syndrome.

Histologically, no adverse effects on the endochondral sequence of development were observed in the induced bone. Histomorphometry of the caudal vertebrae and the induced bone failed to support a diagnosis of osteomalacia.

Although phenobarbital-induced osteomalacia in the human is multifactorial, this study underscores the importance of a diet replete in calcium, phosphorus and vitamin D in preventing the syndrome.

EFFETS D'UNE ADMINISTRATION CHRONIQUE DU PHÉNOBARBITAL SUR L'HOMÉOSTASE PHOSPHOCALCIQUE, LE MÉTABOLISME DE LA VITAMINE D ET LA FORMATION D'OS ENCHONDRAL

RÉSUMÉ

Le métabolisme de la vitamine D et l'homéostase phosphocalcique furent étudiés chez de jeunes rats recevant une diète normale et soumis à des injections à long terme soit de phénobarbital, soit de solution saline physiologique (groupe contrôle). Des coupes histologiques de vertèbres caudales et de "plaques" d'ossification induite selon la séquence endochondrale furent examinées et analysées par des méthodes histomorphométriques, afin de déterminer si des lésions ostéomalaciques avaient été induites.

Quoiqu'une induction significative des oxidases à fonctions mixtes a été induite par le traitement, on n'a pas observé une diminution des concentrations sériques moyennes de la 25-(OH)D. Les taux sériques moyens de la 1,25-$(OH)_2$D se situaient dans l'éventail des valeurs normales chez le rat, indiquant que l'activation de la vitamine D n'a apparemment pas été affectée. Les concentrations sériques de calcium et phosphore ne concordaient pas avec un syndrome d'ostéomalacie.

Au point de vue histologique, aucun effet nocif sur la séquence du développement enchondral de l'os ne fut observé dans l'os induit. La morphométrie osseuse des vertèbres caudales et de l'os induit démontrait l'absence d'ostéomalacie.

Quoique chez l'homme l'étiologie de l'ostéomalacie induite par le phénobarbital demeure énigmatique, cette étude démontre que l'apparition de ce syndrome peut être prévenue par un apport alimentaire adéquat en calcium, phosphore et vitamine D.

ORIGINAL CONTRIBUTION

Young male rats pair fed a standard diet replete on calcium, phosphorus and vitamin D were subjected to either long term phenobarbital or saline (sham) injections.

Mean serum 25-(OH)D concentrations did not differ significantly, indicating an adequate and an unaffected vitamin D pool. Serum $1,25\text{-}(OH)_2D$ remained within accepted normal ranges, thus reflecting an apparently unaltered vitamin D activation pathway.

Sequential serum calcium and phosphorus determinations did not reveal values consistent with an osteomalacic syndrome.

In vertebral metaphyseal bone and in "plaques" of induced endochondral bone, histological and histomorphometric proof of an osteomalacic process was lacking.

In the context of chronic phenobarbital treatment of epileptic patients, this study suggests that the vitamin D activation process is not adversely affected and that osteomalacia does not develop, provided that an adequate dietary content in calcium, phosphorus and vitamin D is ensured.

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INTRODUCTION

SKELETAL EMBRYOLOGY AND GROWTH

Endochondral and intramembranous bone formation are two different processes mediating prenatal and postnatal skeletal growth. Fetal skeletal growth is initiated by the endochondral process in which mesenchymal cells aggregate and differentiate to form cartilage models of the fetal bones. These are subsequently invaded by capillary tufts in the diaphyseal region forming the primary center of ossification, and in the epiphyseal regions at the secondary ossification centers. Immediately following, these cartilage anlages are mineralized, and then resorbed, and finally gradually replaced by bone tissue.

Longitudinal growth of the skeleton, particularly that of the long bones also occurs by an endochondral process: cartilage cells of the epiphyseal growth plates situated at the metaphyseal-epiphyseal junctions produce calcified cartilagenous bars which advance in the metaphyseal zones to be partially eroded by chondroclasts and replaced by young "woven" bone laid down by osteoblasts. This primary bone structure or "primary" spongiosa is then removed by osteoclasts and replaced by lamellar bone ("secondary spongiosa").

Latitudinal bone growth in the diaphyseal zone occurs by intramembranous bone formation, whereby bone matrix produced by cells directly beneath the osteogenic periosteal envelope becomes mineralized to yield woven bone, which gradually is incorporated into a cortical lamellar pattern (Ogden, 1980). Thus, in this process, bone is formed without an intervening cartilage stage, which is an integral part of the endochondral process of bone formation.

Diametric expansion of the physeal plate occurs by interstitial expansion and cell division within the physis, and also by means of endochondral ossification of

cells produced by the ossification groove of Ranvier. Enlargement of the funnel-like shape of the metaphyseal segments takes place by intramembranous ossification, and is a function of the groove of Ranvier (Shapiro et al., 1977).

SKELETAL MODELING AND REMODELING

Skeletal growth during childhood and adolescence and bone structural integrity during adulthood are maintained by means of two different activities. Bone modeling refers to processes by which bone enlarges and changes its shape throughout the growth period to finally assume adult proportions; it therefore alludes to growth in length (mediated exclusively by endochondral bone formation) and in width (produced by intramembranous and endochondral processes). Bone remodeling relates to the intrinsic cellular activities occuring during childhood on adult life whereby bone tissue is removed by osteoclasts and replaced by osteoblasts in the same location so as to maintain the structural as well as the physiological properties at the bone organ level (Frost, 1973; 1979). This cycle of events comprises a balanced or "coupled" process of bone resorption by osteoclasts followed by bone formation by osteoblasts, and is mediated by specialized groups of cells known as Basic Multicellular Units (BMU) (Frost, 1973) acting on the three functional bone tissue envelopes, namely the haversian, the periosteal and the endosteal envelopes. BMUs ultimately modify the biomechanical properties of the adult skeleton; they furthermore participate directly in some of the metabolic functions of the skeleton as an ion reservoir of calcium, phosphorus, magnesium, sodium and carbonate.

CONTROL OF SKELETAL MODELING AND REMODELING

The mechanisms whereby modeling and remodeling are controlled are poorly understood, but include genetic factors influencing cellular metabolic func-

tions, cellular development and cellular interactions; external and internal stimuli such as physical stress, weight bearing and piezoelectric properties of bone crystals; and also hormonal control. The hormones modulating these activities can be classified as systemic hormones and calcium-regulating hormones (Harrison and Harrison, 1979). The systemic hormones affecting cartilage and bone growth are the same ones affecting all cell proliferation and growth: growth hormone-mediated somatomedin; the thyroid hormones; the adrenal androgens; insulin; and, during the pubertal growth spurt, the estrogen and androgen of gonadal origin. Parathyroid hormone (PTH) and vitamin D (vit D) are the two principal calciotropic hormones which influence directly or indirectly bone modeling and remodeling, probably through changes effected in the synthetic activities of either the osteoblast (Meunier, 1979), the osteocyte or the osteoclast.

DRUG-INDUCED METABOLIC BONE DISEASES

A variety of disease states, metabolic stimuli and external agents can affect modeling to the extent of causing major aberrations of skeletal growth. Similarly, these factors can influence or disrupt bone remodeling by producing a disequilibrium between the normally coupled events of osteoclastic bone resorption and osteoblastic bone formation. If the disease is protracted or the offending agent strong enough, the resulting changes produced at the cellular and tissue level will be manifested clinically by symptoms and signs characteristic of one of the different types of metabolic bone diseases of adolescence or of adulthood.

The research carried out in the past on the mechanisms of osteopenia induced by exogenous glucocorticoids stimulated interest in the study of other metabolic bone diseases possibly related to chronic drug intake. The importance of accurately defining and understanding these disorders stemmed from the widespread use of these drugs and by their recognized essential role in controlling many disease states.

Among these drugs commonly used in clinical medical practice, anticonvulsant medication has been found to be susceptible of causing a syndrome characterized by bone pain, fatigue, biochemical and occasionally histological evidence suggestive of an osteomalacic process.

ANTICONVULSANT-INDUCED OSTEOMALACIA

Current anticonvulsant medication effectively controls most seizure disorders despite the fact that epilepsy occurs in association with a variety of metabolic conditions or as a result of different types of organic lesions of the central nervous system.

Among the many types of anticonvulsant agents now in use, over the years and up until now phenobarbital and diphenylhydantoin employed singly or in combination have constituted the mainstay of medical control of most seizure disorders.

With the widespread use of these two different agents, side effects were rapidly recognized and have been amply described. However, it was not until 1967 and 1968 that reports first appeared on the "latent" side effects of either of these two medications on bone and mineral metabolism (Schmid, 1967; Kruse et al., 1968).

These reports and subsequent ones (Genel et al., 1972; Matsuo et al., 1972; Borgstedt et al., 1972; Teotia and Teotia, 1973) called attention to the frequent association of symptoms and signs of osteomalacia in patients receiving these drugs; the term "anticonvulsant induced osteomalacia" was thus coined.

While symptoms were not frequent manifestations of the syndrome, they included fatigue, and in rare cases pain related to pseudofractures. Biochemical parameters which were altered in many cases included hypocalcemia, elevated alkaline phosphatase, and elevated immunoreactive parathyroid hormone (iPTH) concentrations (Bouillon et al., 1975; Kruse et al., 1980). Histological confirmation,

obtained only in a few cases, showed increased amounts of unmineralized osteoid. Radiologically, the bones of some patients appeared osteopenic.

Though the earliest descriptions of anticonvulsant associated osteomalacia were contained in reports from Europe (a continent where mild vitamin D deficiency is not uncommon) (Richens and Rowe, 1970; Dent et al., 1970; Christiansen et al., 1972; Sotaniemi et al. 1972; Murchison et al., 1975; Mosekilde et al., 1977) and though they dealt with epileptics living in Northern European communities (and thus patients likely to receive less sunlight because of geographic reasons), subsequently the same findings were discovered in patients under phenobarbital or diphenylhydantoin treatment living in North America. In addition, even though the condition was initially shown to affect predominantly institutionalized epileptic patients receiving long term anticonvulsants (Richens and Rowe, 1970; Hunter, 1971), later all epileptic in-patients or out-patients of any age group were found to be at risk of developing the condition (Hahn et al., 1972b; Lifshitz and Maclaren, 1973). However, the clinical spectrum of the syndrome of anticonvulsant-associated osteomalacia was soon discovered to be extremely broad:

For example, the reported incidence of hypocalcemia ranged from 4% to 70% depending on the population studied and the sensitivity of the technique employed; on the other hand, the mean reduction in serum calcium relative to matched controls averaged between 0.3 and 0.8 mg/dl, with the incidence of frank hypocalcemia varying from 4 to 30 per cent. Significant elevations in serum alkaline phosphatase levels occured in 24 to 40 per cent of cases with increases in both liver (44%) and bone fractions (55%) (Hahn et al., 1972b). Serum phosphate levels were occasionally reduced but most often remained in the normal range.

The radiographic evidence of osteopenia in this syndrome has remained controversial. Kruse et al. (1968) estimated a 15% incidence of radiologic bone disease in a German juvenile epileptic outpatient population. A 22% rate of serious

bone disease detected by routine radiographic technics was reported by Lifshitz et al. (1973) in one large group of institutionalized children on chronic anticonvulsant drug regimens; in this population, it was pointed out that bone loss occured primarily in the non-ambulatory patients confined indoors. On the other hand, an opposing view was presented in an American study (Livingston et al., 1973) in which no X-ray changes were found on reviewing skull films of 15,000 outpatient juvenile epileptics seen over a 36 year period. Moreover, Lussier-Lazaroff et al. (1971) showed that roentgen examination of 37 children who had received anticonvulsant medications for an average duration of more than four years failed to demonstrate any evidence of rickets. Photon absorption bone-mass measurements (which have an error of 4% approximately; Hahn et al., 1974) have in fact documented more accurately than X-rays the significantly reduced bone mass in anticonvulsant-treated epileptics compared to age-matched controls (Lifshitz and MacLaren, 1973; Christiansen et al., 1973; Hahn et al., 1975b; Dymling et al., 1979; Christiansen et al., 1981).

In contrast to the numerous reports detailing the biochemical changes related to anti-epileptic treatment, only a few descriptions pertaining to the histo-morphological changes have been published. Experimental animal models were developed to study this effect, but the data has been difficult to extrapolate to humans partly because of the "inappropriate" protocols in which osteomalacia and rickets were produced only after massive doses of drugs had been administered to animals often maintained on rachitogenic diets (Harris et al., 1978; Villaraele et al., 1978). Clinical histological data which has been sparse, was obtained from reviews of patients receiving principally diphenylhydantoin, alone or in combination with other anticonvulsants. One of these studies simply described excessive osteoid and increased osteoclastic activity (Dent et al., 1970); others have reported more information in terms of "static" histological measurements of increased osteoid seam thickness, diminished calcification front, and also increased trabecular osteo-

clastic resorption surfaces with increased mean volume of periosteocytic lacunae suggesting secondary hyperparathyroidism (Melsen and Mosekilde, 1976; Johnell et al., 1979; Eastwood et al., 1980). Recently, Melsen and Mosekilde (1980) have presented data revealing normal mineralization lag times in bone biopsies obtained from 20 epileptics receiving diphenylhydantoin alone or in combination with other anticonvulsants for at least 10 years.

RISK FACTORS PREDISPOSING TO INCREASED SEVERITY OF ANTICONVULSANT-INDUCED OSTEOMALACIA

Over the years, careful epidemiological assessments of the different series from various parts of the world helped to identify a number of risk factors shared by most of the affected epileptics. A few of these variables were subsequently shown to correlate well with the production of either biochemical or radiological characteristics of the syndrome, whereas many other observed features were found to be simply associated with, or possibly predisposing to the development of the syndrome.

Multiple anticonvulsant drug regimens and the total daily dose of the drug received were identified as factors likely to produce more severe derangements of mineral metabolism in epileptics (Richens and Rowe, 1970; Hahn et al., 1972b; Hahn et al., 1975b). Additionally, the incidence of these abnormalities was noted to increase with the duration of therapy (Lifshitz and Maclaren, 1973; Rodbro, Christiansen and Lund, 1974; Tolman et al., 1975; Bouillon et al., 1975). It was also suggested that the various types of anticonvulsant drugs differed widely in their ability to produce disordered mineral metabolism (Richens and Rowe, 1970), but this has not yet been specifically confirmed.

It is acknowledge that reduced dietary content in vitamin D_2 or D_3 and reduced sunlight exposure resulting in decreased skin synthesis of vitamin D_3 lead to a state of absolute or relative hypovitaminosis D; it has been argued that, in

patients receiving anticonvulsants for prolonged periods, these factors compounded the chances of developing the biochemical picture of rickets or osteomalacia. These two risk factors were found to be critically important in anticonvulsant-treated institutionalized epileptics receiving limited sunlight exposure, or in patients living in temperate climates, where a seasonal effect on 25-(OH)D levels was clearly demonstrated, with lower levels prevailing during winter months (Haddad and Hahn, 1973). Furthermore, the variations from country to country in the frequency of anticonvulsant-induced rickets or osteomalacia probably stemmed from the variations in vitamin D intake and amount of sunlight exposure.

Another related parameter studied in these patients was physical activity as it relates to bone mass. In adults, physical stress is the most potent known stimulus for bone formation, and its effect is thought to be mediated by induction of piezoelectric forces in bone, resulting in increased osteoblastic activity. Indeed, in many patients with seizure disorders, osteopenia has been documented repeatedly by means of radiography and of photon absorption densitometry. Decreased physical activity resulting from sedative effects of anticonvulsant drugs and/or the physical limitations imposed by associated neurological disorders probably accounted for the major portion of the osteopenia (Murchison et al., 1975). In fact, a significant positive correlation between bone mineral content and muscle strength was noted by Dymling et al. (1979) in controls and in epileptics maintained on combination anti-epileptic drugs.

Interactions between nutritional factors and vitamin D biotransformation have also been postulated in the context of anticonvulsant-induced alterations in vitamin D metabolism. Alvares et al. (1976) have demonstrated in man the marked influences of dietary carbohydrate and protein ratios on the hepatic microsomal mixed function oxidase system. Thus, since it is acknowledged that many institutionalized epileptics do not receive balanced nutritious diets, it is conceivable that

the type of nutrition that certain epileptics receive may have an influence on their vitamin D metabolism.

Finally, many drugs such as tranquilizers, oral hypoglycemic agents, etc. induce hepatic microsomal enzyme activity (Conney et al., 1967) which in turn may affect vitamin D metabolism (see below). Thus, in epileptics, the potential for drug-induced modifications of vitamin D metabolism in a wide range of clinical situations is apparent.

PATHOGENESIS OF ANTICONVULSANT-INDUCED OSTEOMALACIA

With the advent of sensitive diagnostic laboratory techniques, attempts were made to shed light on the mechanisms of the drug-induced hypocalcemia in epileptic patients receiving anticonvulsants. The interest was thus concentrated in two main areas: altered vitamin D metabolism and intestinal calcium malabsorption. These investigations yielded data which eventually permitted other related research pertaining to the anticonvulsant-induced modifications of bone and mineral homeostatic mechanisms. The most important scientific research carried out in this domain will be highlighted next, following an overview of current knowledge on vitamin D metabolism.

1- ALTERED VITAMIN D METABOLISM

i) Normal Vitamin D metabolism (Fig. 1)

Vitamin D refers to a group of steroids having important effects on bone and mineral homeostasis. In humans, the two important ones are vitamin D_2 (D_2) and vitamin D_3 (D_3). D_2 is formed by the irradiation of the plant sterol ergosterol. D_3 is produced in the skin from the conversion of 7-dehydrocholesterol to pre-vitamin D_3 under the influence of ultraviolet light, followed by thermal conversion of

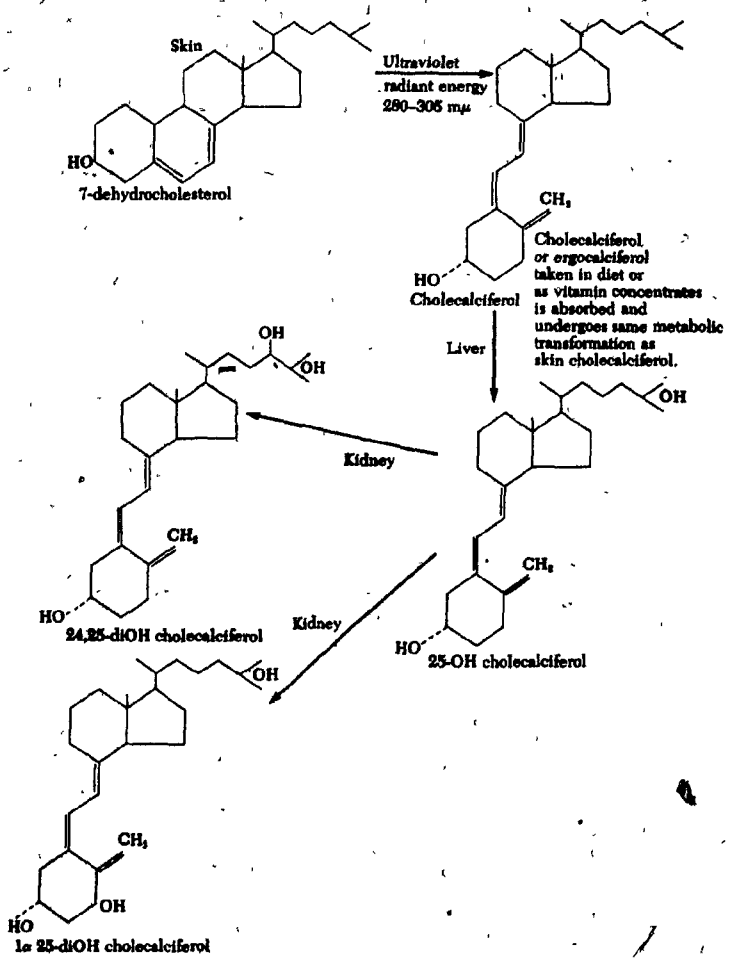

FIGURE 1 - Diagrammatic representation of vitamin D metabolism in man.

previtamin D_3 to vitamin D_3. Although in sunny climates most of the vitamin D requirements can be obtained from skin synthesis, in temperate climates vitamin D must be added to the diet to compensate for limited D_3 skin synthesis. In view of the fact that vitamin D is soluble in fat, the absorption of vitamin D is affected by factors which influence fat absorption. Once absorbed, large amounts of vitamin D are stored in muscle and fat, while the rest is circulated to the liver where it is hydroxylated predominantly in the mitochondria, and also in the microsomes by calciferol-25-hydroxylase to yield 25-hydroxyvitamin D (25-(OH)D) (Bhattacharyya et al., 1974a; Bjorkhem et al., 1978). Although the molecular components of this reaction are not completely understood, it appears that calciferol-25-hydroxylase behaves as a mixed function oxidase enzyme (Madhok et al., 1978) and that the involvement of cytochrome P-450 in this reaction, once considered doubtful, now appears more certain (Bhattacharyya et al., 1974b). 25-OHD is the most abundant circulating form of vitamin D, and its serum concentration (normal range: 25 to 35 ng/ml) is thought to reflect the vitamin D status of an individual. Due to the dual source of vitamin D in humans, the serum concentration of 25-OHD is under the influence of both dietary vitamin D and also the amount of sunlight exposure. After transport to the kidney, it is hydroxylated a second time by an enzyme located in the mitochondrial fraction of renal cortical cells to yield either 1,25-$(OH)_2$D or 24,25-$(OH)_2$D.

It has become widely accepted that 1,25-$(OH)_2$D is the most biologically active form of the vitamin, and that it behaves as a steroid hormone. In particular, a cytoplasmic receptor has been shown to play a role in the uptake of the free hormone; the resulting steroid-receptor complex moves to the nucleus, where it binds to the chromatin (Norman, 1974; DeLuca et al., 1976). Normal serum concentration values of 1,25-$(OH)_2$D range from 30 to 55 pg/ml. Hyperparathyroidism, hypocalcemia and hypophosphatemia tend to increase serum 1,25-$(OH)_2$D values.

1,25-$(OH)_2D$ is considered, at present, the most important vitamin D metabolite regulating bone and mineral metabolism (DeLuca, 1978). It promotes the absorption of calcium and phosphorus from the gastrointestinal tract, acts synergistically with parathyroid hormone in the mobilization of calcium from bone, and stimulates the transfer of phosphate from bone and soft tissue into extracellular fluid. Many studies have tried to implicate it as an agent exerting direct effects on skeletal growth and on cartilage and bone mineralization. However, the evidence in support of this view is lacking. An indirect effect can be surmised by its ability to promote the availability of calcium and phosphate. The possibility of other vitamin D metabolites including metabolites of 1,25-$(OH)_2D$ having vitamin D activity has not yet been eliminated (Schnoes et al., 1980).

The 24,25-$(OH)_2D$ metabolite has been considered an inactive form of vitamin D. However, many investigators suspect that it exerts an effect on cartilage and bone mineralization (Ornoy et al., 1978; Bordier et al., 1978). Some of the observations obtained from current studies on this matter include increased intestinal absorption of calcium in man (Kanis et al., 1978; Brickman, 1979), healing of fracture callus in chicks (Dekel et al., 1979), and healing of rachitic lesions in chicks (Ornoy et al., 1979). Interestingly, production of 24,25-$(OH)_2D$ may be stimulated by 1,25-$(OH)_2D$ (Taylor, 1979). Nevertheless, the regulation of 24,25-$(OH)_2D$ in man remains poorly understood.

In Figure 1 are shown the principal vitamin D metabolites mentioned above. It is important to point out that other metabolites, produced in smaller concentrations, do exist, but their effects on bone and mineral metabolism have yet to be determined.

ii) Effects of anticonvulsants on hepatic metabolism of drugs and hormones

Almost all anticonvulsants are capable of increasing biliary flow (Roberts and Plaa, 1966), and of inducing cytochrome P-450 dependent hepatic microsomal mixed function oxidase activity (Conney et al., 1967). These enzymes as a group are known to be involved in degradative or anabolic reactions of many hormones and of many drugs (Conney et al., 1967). An increased activity of these enzymes results in accelerated biotransformation of drugs in vivo with an increased production of polar, hydroxylated and biologically inactive compounds which are eliminated in the bile and urine (Conney et al., 1967). This phenomenon of hepatic microsomal enzyme induction by drugs resulting in significantly reduced duration and intensity of the activity of drugs or hormones, has been particularly well documented in the case of exogenous estrogen, progesterone or corticosteroids administered concurrently with hepatic microsomal inducing agents such as phenobarbital and diphenylhydantoin (Levin et al., 1967; Jubiz et al., 1970).

iii) Effects of anticonvulsant medication on vitamin D metabolism

A lower circulating level of the 25-(OH)D metabolite was the first observed alteration in vitamin D metabolism in epileptics receiving long term anticonvulsant therapy (Hahn et al., 1972b). The possible mechanisms invoked included anticonvulsant-related malabsorption of vitamin D, drug-altered vitamin D metabolism and catabolism, accelerated excretion and/or tissue disposal of the metabolites, or different combinations of these mechanisms.

In the rat, possible inhibition of vitamin D absorption by chronic phenobarbital intake was ruled out by Schaefer et al. (1972) and later by Hahn et al. (1975a) when they demonstrated normal (^{14}C) cholecalciferol absorption and normal ^{3}H vitamin D_3 absorption. Chronic diphenylhydantoin use was also shown in another study (Hahn and Halstead, 1979) not to interfere with vitamin D absorption.

In man, Matheson et al. (1976) demonstrated that ^{3}H-vitamin D_3 absorption was unaffected by either chronic phenobarbital or chronic diphenylhydantoin therapy.

The bulk of the investigative work dealing with lowered 25-$(OH)_2$D levels in epileptics receiving long term anticonvulsants focussed on altered vitamin D metabolism and excretion. Hahn et al. (1972b) reported that the serum values of 25-(OH)D in adult epileptic outpatients receiving combined phenobarbital and diphenylhydantoin therapy were significantly decreased in 33% of patients compared to untreated controls, with the lowest values seen in patients with lowest vitamin D intake and least exposure to sunlight; however in a group of 13 patients receiving either chronic phenobarbital or diphenylhydantoin therapy, similar but less marked changes were seen. In a study comparing the effects of a three week course of vitamin D (2000 IU/day) on serum 25-OHD levels in a group of institutionalized epileptics receiving anticonvulsants and also in a control group of institutionalized psychiatric patients not receiving anticonvulsants, Bouillon et al. (1975) found a significantly smaller rise in serum 25-OHD in the epileptics maintained on anticonvulsants. The explanation put forth was that though the non-epileptic cohabitant controls appeared to have subclinical nutritional hypovitaminosis D due to limited sunlight exposure and because of dietary reasons, that the anticonvulsant-treated epileptics living in the same conditions developed low serum 25-OHD levels because of the additive influence of anticonvulsant increased vitamin D degradation.

In work carried out by Gascon-Barré (1978), the rat's ability to dispose of pharmacological doses of vitamin D_3 was investigated with special interest on the influence of pretreatment of the animal with either phenobarbital (50 mg/kg) alone or in combination with diphenylhydantoin (14 mg/kg). It was shown that the treated rats exhibited an accelerated disposal of the excessive amounts of vitamin D as measured by lower serum levels of 25-(OH)D, and also by the significantly increased median lethal dose (LD50) of vitamin D_3 as well as the median time to effect (Lt50).

Hahn et al. (1972a, 1973) and Silver et al. (1974) proposed a mechanism of accelerated disappearance of vitamin D in rats and in humans by an anticonvulsant-induced increase in hepatic production of more polar and hydroxylated inactive vitamin D metabolites. However, one must point out the small group of patients studied in the first report, along with the in vitro nature of the results obtained from the animal models used in all these studies. Furthermore, in the light of the more precise current techniques of isolating and identifying the main vitamin D metabolites (see below), one must also object to the interpretation given to the results of these three reports, as the peak V metabolite isolated with the techniques available at that time contained all the dihydroxylated vitamin D compounds, including 1,25-$(OH)_2$D and 24,25-$(OH)_2$D; thus, according to today's improved methods of assaying vitamin D metabolites, the significance of these results is unclear. Finally, as these experiments have not since been repeated elsewhere using current technology, it is difficult at the present time to unequivocably accept this premise.

Increased choleresis in rats and pigs receiving short term phenobarbital was demonstrated by Silver et al. (1974) and Gascon-Barré et al. (1977). Although this might indicate another mechanism of accelerated disposal of vitamin D metabolites in animals receiving phenobarbital, it is nevertheless difficult to extrapolate these results to the human as there exists no data confirming or denying that biliary physiology in the rat and in humans is identical (Hollander, 1981).

However, while many studies generated information on the effects of chronic anticonvulsant use on vitamin D metabolism, investigations on the effects of short term anticonvulsant therapy on vitamin D metabolism also yielded interesting results. Hahn et al. (1972b) noted no change in serum 25-(OH)D levels in epileptic adults who had received for less than six weeks either phenobarbital or diphenylhydantoin, alone or in combination. Following (^{14}C) cholecalciferol administration

to rats and pigs which had received brief courses of phenobarbitone, Silver et al. (1974) noted an enhanced appearance of the 25-(OH)D metabolite. After giving 5 days of phenobarbital treatment (75 mg/kg) to rats, Hahn et al. (1975a) demonstrated an early acceleration of hepatic conversion of ^{3}H vitamin D_3 to ^{3}H-25-(OH)D_3. Similar results were also obtained by Matheson et al. (1976) and Norman et al. (1976). Also, Gascon-Barré et al. (1981, in press) demonstrated in rats given phenobarbital (50 mg/kg) that the typical plasma 25-(OH)D_3 response during the first 48 hours following a single intraportal injection of D_3 proceeded in two distinct phases with an initial transient phase occuring at 24 hours and characterized by a marked increase in plasma 25-(OH)D_3, followed by a second phase typified by an accelerated disappearance of 25-(OH)D_3 in the plasma. Hence, it would appear that the acute effects of anticonvulsant medication on vitamin D metabolism are reflected by an enhanced anabolism of 25-(OH)D, but that the long term effects are an accelerated catabolism and biotransformation into other apparently inactive metabolites.

The work of Jubiz et al. (1977) shed more light on the matter by demonstrating (in many epileptic patients receiving anticonvulsants) normal or slightly elevated levels of 1,25-$(OH)_2$D despite lowered serum 25-(OH)D levels. They concluded that though an anticonvulsant-induced catabolism of 25-(OH)D to inactive metabolites existed in these patients, that sufficient quantities of 25-(OH)D were still available to permit biosynthesis of adequate quantities of 1,25-$(OH)_2$D. They also speculated that since no deficiency in the activated metabolite of vitamin D existed in this syndrome, that either there were no abnormalities of vitamin D metabolism of pathogenetic significance, or, that depressed serum levels of 25-(OH)D possibly contributed to the development of the skeletal abnormalities of anticonvulsant-induced osteomalacia. This timely work thus indirectly suggested possible active involvement of the 25-(OH)D metabolite in

bone and mineral homeostasis, as was suspected by many scientists investigating other forms of metabolic bone disease (Rasmussen and Bordier, 1974; Bordier et al., 1977; Fournier et al., 1977; Ivey et al., 1977; Bell et al., 1979).

At approximately the same time, it was shown by Levison et al. (1977) that long term administration of either diphenylhydantoin or phenobarbital to chicks resulted in an increase in renal mitochondrial 25-(OH)D_3 - 1α hydroxylase. This information, though obtained from animal experiments, provided a possible explanation for normal or slightly elevated serum 1,25-$(OH)_2D_3$ values seen in humans receiving long term anticonvulsants.

On the other hand, it is equally important to cite the results of other experimental and clinical studies which were, in fact, completely opposite to those discussed up to this point:

In 31 ambulatory Israel children and adolescents receiving either phenytoin or phenobarbital for over one year, Weissman et al. (1979) found that serum 25-(OH)D concentrations were similar to those of healthy, age-matched controls, presumably because of the abundant ultraviolet radiation prevalent in Israel. In a group of non-institionalized, non-epileptic, cardiac patients receiving diphenylhydantoin for two years for the control of cardiac anythmias, and with serum concentrations within the therapeutic range for the control of epilepsy, Wark et al. (1979) found normal 25-(OH)D levels, normal serum calcium levels, and elevated hepatic isoenzyme alkaline phosphatase levels, compared to age-matched controls. Moreover, in a double blind series performed on 74 children receiving either phenobarbital or placebo for 5 to 12 months for the control of single febrile seizures, Camfield et al. (1981, unpublished data) did not find any significant alterations in serum 25-(OH)D levels.

In rats, Gascon-Barré (1981; in press) was unable to demonstrate a significant effect on plasma 25-(OH)D concentrations by either phenobarbital,

diphenylhydantoin, or both in combination, in the presence of constant vitamin D_3 intake (100 µg/kg/day). Furthermore, Delvin et al. (1980) showed that a greater phenobarbital inductive influence on microsomal protein synthesis was achieved in rats when vitamin D deficiency was present; they then postulated that, in humans, vitamin D deficiency potentiated anticonvulsant-mediated induction of cholecalciferol-25-hydroxylase resulting in lower serum 25-OHD concentrations.

2- ANTICONVULSANT IMPAIRMENT OF INTESTINAL CALCIUM ABSORPTION

i) Normal mechanisms of calcium absorption

Calcium absorption takes place principally in the duodenal segment of the small intestine. Uptake at the intestinal border occurs by simple diffusion, and transfer to the serosal surface is mediated by carrier proteins including a specific calcium binding protein (CaBP). Vitamin D 1,25-$(OH)_2$D, and 25-(OH)D in pharmacological doses increase the rate of transfer of calcium from luminal fluid into the mucosal cell, and also the rate of elimination of calcium from the mucosal cell into the interstitial fluid bathing the basal and lateral surfaces of the cell. Whether the primary action is to increase the rate of calcium permeation through the luminal surface remains a question. The net effect, however is that, even at high concentrations of calcium in the luminal fluid, calcium moves only slowly into the mucosal cell in the absence of the active vitamin D metabolite.

ii) Anticonvulsant impairment of calcium absorption

The relevant information on this topic was obtained primarily from animal experiments and organ cultures. The latter technique enabled analysis to focus on the direct effects of the drugs at the intestinal level to the exclusion of their indirect effects on intestinal absorption via altered vitamin D metabolism.

In the rat, prolonged intake of diphenylhydantoin, alone or in combination with phenobarbital was shown to impair intestinal calcium absorption, with calcium binding protein activity (and presumably calcium binding protein synthesis) remaining intact (Caspary, 1972; Koch et al., 1972). These observations were supported by the work of Harrison and Harrison (1976), in which they also demonstrated that combined diphenylhydantoin and phenobarbital therapy did not block vitamin D-stimulated passive diffusion of calcium across the intestinal mucosal barrier, nor did it affect vitamin D-stimulated increase of serum calcium in vitamin D deficient rats; a specific inhibitory effect by anticonvulsant medication on an energy dependent calcium transport system within the intestinal mucosa and at the serosal surface was postulated. Prolonged and brief courses of phenobarbital, on the other hand were not found to hinder intestinal calcium absorption in vitamin D repleted rats, in organ cultured rat duodena, or in humans (Koch et al., 1972; Krawitt et al., 1972; Schaefer et al., 1972).

In the chick, Villareale et al. (1974) observed that, regardless of the vitamin D dietary content, that prolonged diphenylhydantoin lead to decreased bone ash, decreased ^{47}Ca absorption, and, in contrast to the reports of Koch et al. (1972) and Caspary (1972), a depressed calcium binding protein activity. These effects were directly related to the diphenylhydantoin dose and inversely proportional to the dietary vitamin D_3 content. Species differences in calcium binding protein responsiveness to diphenylhydantoin was cited as a possible explanation for the differences in the rat and chick models. In a similar model, Corradino (1976) also showed that diphenylhydantoin-treated chicks exhibited both depressed intestinal ^{45}Ca absorption and calcium binding protein synthesis in parallel.

In a clinical study performed on 40 institutionalized and out-patient epileptics receiving combined diphenylhydantoin and phenobarbital therapy, Caspary et al. (1975) demonstrated that 62.5% of the patients exhibited malabsorption of

^{45}Ca despite normal serum calcium and phosphorus levels and normal alkaline phosphatase activity. Similar results were obtained by Shafer and Nuttall (1975) in a clinical study conducted on 28 epileptics receiving diphenylhydantoin and phenobarbital, with a significantly greater ^{45}Ca malabsorption (56%) being observed in patients receiving diphenylhydantoin alone than in patients on both diphenylhydantoin and phenobarbital.

3- END-ORGAN UNRESPONSIVENESS TO THE EFFECTS OF VITAMIN D AND OF PTH

Considerable evidence has accumulated suggesting that anticonvulsants produce resistance to the actions of vitamin D by exerting direct inhibitory effects on mineral metabolism at the tissue level. In the first instance, the finding of normal or elevated 1,25-$(OH)_2D_3$ levels in affected patients (Jubiz et al., 1977) was interpreted by some as a manifestation of end-organ resistance to activated vitamin D (1,25-$(OH)_2D_3$) at the intestinal and bone tissue levels. The inhibition of *in vitro* vitamin D-mediated intestinal calcium transport in the rat by combined *in vivo* diphenylhydantoin-phenobarbital therapy (Harrison and Harrison, 1976) also indicated a blunting of the effects of vitamin D at the intestinal tissue level. Furthermore, it was demonstrated by Hahn et al. (1978) that both phenobarbital and diphenylhydantoin inhibited basal as well as PTH-and 1,25-$(OH)_2D_3$-stimulated release of calcium from cultured fetal rat forelimb rudiments, with diphenylhydantoin exhibiting a several fold more potent inhibitory effect than phenobarbital. Jenkins and co-workers (1974) reported that diphenylhydantoin, but not phenobarbital, inhibited parathyroid extract-stimulated ^{45}Ca release from cultured mouse calvaria; although these results were not consonant with those of Hahn et al. (1978), the differences could be attributable to species differences, or to differences in the drug concentrations employed.

Several clinical studies have demonstrated that chronic diphenylhydantoin can also produce effects that imply end-organ resistance to the effects of PTH. Radiographic surveys of dentition in patients receiving chronic diphenylhydantoin therapy have demonstrated a markedly increased incidence of root abnormalities characteristic of those seen in hyperparathyroidism and pseudohypoparathyroidism (Harris and Goldhaber, 1974). Moreover, calvarial thickening, another feature of chronic hypoparathyroid states, has been observed with increased frequency after prolonged diphenylhydantoin administration (Kattan, 1970).

CHAPTER II

AIMS

AIMS

The clinical entity of anticonvulsant-induced osteomalacia remains a contentious issue. Obstacles to proper interpretation of the available data arise either because of multiple anticonvulsant drug therapy or because of lack of standardization of the content of calcium, phosphate, and vitamin D in the dietary intake. Furthermore, the paucity of adequate histological assessments documenting the events occurring at the bone tissue level under the influence of various anticonvulsants does make it difficult, if not appropriate, to continue labeling the disorder as "osteomalacia".

Anticonvulsants are a heterogeneous group of pharmacological agents. From the review of the literature presented here, it is obvious that they do not all influence metabolism to the same extent. It is thus reasonable to assume that the choice of one anticonvulsant over another might be associated with a higher chance of developing the syndrome.

This project was designed with a view of obtaining a clear understanding of the effects of long term single-drug anticonvulsant treatment on bone and mineral metabolism. Specifically, our aims were:

1. To study the alterations on hepatic and mineral metabolism in young rats receiving long term phenobarbital, with particular attention focussed on the possible effects on vitamin D metabolites.
2. To study concurrently the possible effects of phenobarbital on endochondral bone growth using the rat model of endochondral bone induction developed by Reddi and Huggins (1972).
3. To determine quantitatively the extent of anticonvulsant-induced osteomalacia in the long bones and caudal vertebrae of these animals.

CHAPTER III

METHODS

METHODS

MATRIX-INDUCED ENDOCHONDRAL BONE FORMATION

Endochondral bone formation can be conveniently studied in the rat by means of the matrix-induced endochondral bone-forming system (Reddi and Huggins, 1972; Reddi and Huggins, 1975; Reddi and Anderson, 1976). By means of this model, the discrete stages of endochondral bone differentiation, such as mesenchymal cell proliferation, chondrogenesis, cartilage calcification, osteogenesis and bone mineralization are reproduced in an regular fashion, as shown in Table 1; the many difficulties inherent in the use of the epiphyseal growth plate are thus circumvented. The method consists of subcutaneous implantation of demineralized diaphyseal bone matrix into allogenic rats, and it results in the induction of de novo bone formation by the endochondral process. Type I bone collagen or other extracellular matrix macromolecules contained in the prepared bone matrix powder are presumed to be the substances with specific mitogenic effects which influence the connective tissue mesenchyme to differentiate according to the developmental cascade of endochondral bone formation (Rath and Reddi, 1979; Urist, 1980).

PREPARATION OF BONE INDUCING POWDER

The bone inducing powder was prepared according to the method of Reddi and Huggins (1972), with a few modifications. The femoral, tibial and humeral diaphyseal sections of Sprague Dawley male rats aged 28 days were harvested and cleaned of adherent tissue; the marrow was extracted and the medullary canal was cleaned by means of forceful injections of deionized water. The bones were then broken into small chips measuring 2 x 3 x 3 mm^3, approximately, then washed four times in deionized water over a period of two hours, after which they were defatted

STAGE	EVENT
• DAY 0	• Implantation of cortical bone powder
• DAY 4	• VASCULAR INVASION of bone powder implant
• DAY 7	• Appearance of CHONDROCYTES and onset of cartilage matrix production
• DAY 11	• ↑ CARTILAGE MATRIX production with early CALCIFICATION
• DAY 14	• ↑ Calcification of induced cartilage matrix • Appearance of OSTEOBLASTS and early osteoid formation
• DAY 20	• ↑ OSTEOID FORMATION with early mineralization
• DAY 42	• ↑ Osteoblast number • ↑ MINERALIZATION OF OSTEOID • Appearance of MARROW

TABLE 1 - Developmental sequence of matrix-induced endochondral bone formation.

in alcohol baths (twice in 95% ethyl alcohol for 40 minutes followed by twice in absolute ethyl alcohol for 40 minutes). Finally the bone fragments were washed with anhydrous ethyl ether for 30 minutes after which they were allowed to dry overnight at room temperature.

Next, the dehydrated bone chips were milled in a Spex Freezer Mill set at maximum impact frequency and for a period of 1 minute. The coarse powder thus produced was then demineralized for three hours using 0.5 N HCl (25 mEq/g). The solution was centrifuged at 10,000 rpm for 30 minutes in the SS-34 rotor of a Sorvall RC-2B centrifuge, the supernatant discarded and the precipitate demineralized another 30 minutes in 0.5 N HCl (25 mEq/g); the supernatant was again discarded and the precipitate washed three times in deionized water, 40 minutes each wash, with centrifugation (10,000 rpm for a 30 minute period) being done between each wash. Dehydration of the precipitated powder was done twice in 95% ethyl alcohol (30 minutes each time) and once in absolute ethyl alcohol (30 minutes) with centrifugation (10,000 rpm/for a 30 minute period) carried out between each alcohol bath. The precipitated powder was finally stirred in anhydrous ethyl ether and then allowed to dry overnight in a petri dish.

Three samples of the bone powder preparation were incinerated in an oven at 600°C for 18 hours until no measurable inorganic Ca or Pi could be extracted from the bone powder.

Twenty five milligram aliquots of the sieved bone powder of particle size measuring 180-425 μ were then placed in no. 5 gelatine gelules ready for implantation.

EXPERIMENTAL DESIGN

A schematic representation of the experimental design is shown in Figure 2.

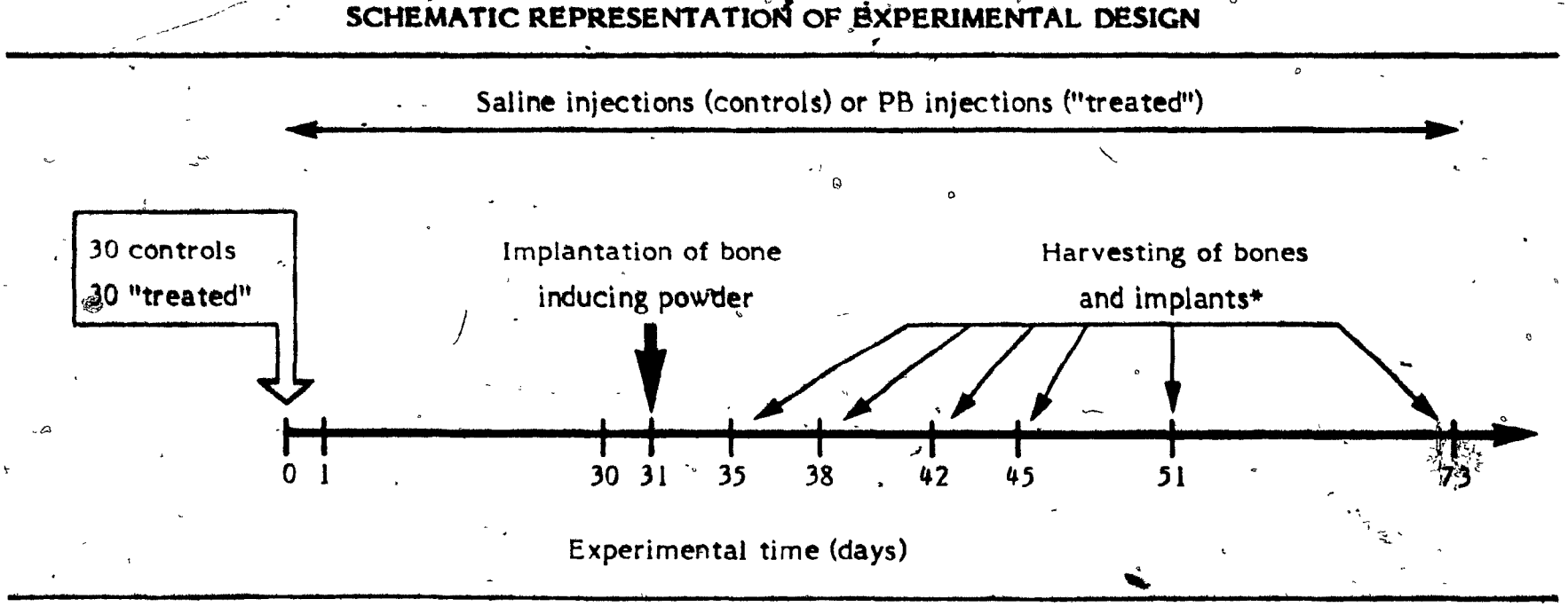

FIGURE 2 - Schematic representation of experimental design.

Sixty 21 day old male Sprague Dawley weanling rats (Canadian Breeding Farms and Laboratories, St.Constant, Quebec, Canada) were equally divided at random into a control group and a phenobarbital (PB)-"treated" group. All animals were housed in individual hanging cages in a windowless room with automatically controlled temperature and lighting, and pair fed 15 g/day of a standard rat chow diet (no. 5012, Ralston-Purina Canada, Woodstock, Ontario, Canada) containing:

Calcium:	0.68%
Phosphorus:	0.61%
Vitamin D:	2.5 IU/gm
Protein, not less than	22.0%
Fat, not less than	4.0%
Fiber, not more than	5.0%

Demineralized water was given ad libitum.

The PB treated animals were administered sodium phenobarbital ("Luminal Sodium", a generous gift of Winthrop Laboratories, Division of Sterling Drug Ltd., Dorval, Quebec, Canada), 50 mg/kg/day, intraperitoneally for 30 days, whereas the controls were given the vehicle only (physiological saline) according to the same schedule.

At this point, all animals were anesthetized with phenobarbital, 45 mg/kg ("Nembutal Sodium"; Abbott Laboratories, Dorval, Quebec, Canada); using aseptic technique, gelules containing 25 mg aliquots of the demineralized cortical bone powder were subcutaneously implanted bilaterally in the anterolateral thoracic region through a midline dorsal interscapular incision which was subsequently closed with 2 stainless steel clips. Following this procedure, the rats continued to receive daily injections of phenobarbital (PB) or saline in the treated and control groups respectively.

Five control and five PB-treated rats were killed at 4,7,11,14,20 and 42 days after implantation of the gellules, each of these times corresponding to key events in the context of induced endochondral bone formation (Table 1). At these same stages were carried out the assays described in the following sections.

PREPARATION OF HEPATIC MICROSOMAL FRACTION

Liver microsomes were isolated according to the method of Schenkman and Cinti (1972). Following decapitation of control and treated animals at the specified intervals, the livers were excised at once, blotted and weighed and then carefully perfused with cold 0.154 M KCl. They were homogenized with a Potter-Elvehjem homogenizer fitted with a Teflon pestel in ice-cold 0.25 M sucrose containing 1 mM NaH_2PO_4 buffer adjusted to pH 7.4 with 1 M NaOH. The nuclei and cell debris were sedimented at an integrated field-time of 8300 g-min at $r_{av.}$ 7.3 cm in the SS-34 rotor of a Sorvall RC-2B centrifuge equilibrated at 4°C. The supernatant was centrifuged at an integrated field-time of 196,200 g-min in the same apparatus and the supernatant, consisting of the microsomal and cytosolic fractions, was retained for aminopyrine demethylase and cytochrome P-450 assays.

AMINOPYRINE DEMETHYLASE ASSAY

The microsomal-cytosolic fraction was resuspended in 0.154 M KCl buffered with 0.1 M NaH_2PO_4 adjusted to pH 7.4 with 0.1 M NaOH. Aminopyrine demethylase was assayed according to the method of La Du et al. (1955) with the following modifications. To a 1.5 ml aliquot of the oxygenated cofactor (10 min with O_2) solution consisting of 1 mM NADPH, 16.7 mM glucose-6-phosphate and 15 mM semicarbazide in 0.5 M NaH_2PO_4, adjusted to pH 7.4 with 0.1 M NaOH, were added: 0.25 ml of glucose-6-phosphate dehydrogenase (3 units) in 25 mM $MgCl_2$, 0.25 ml of 2.0 mM aminopyrine, in 25 mM $MgCl_2$. The mixtures were incubated for

exactly 5 min at 37°C after which time, at fixed intervals, 1 ml of the microsomal suspension containing approximately 1.5 nmoles cytochrome P-450 was added to each flask. The incubations continued for 5 min, and the reaction was stopped by the addition of 1 ml of 0.52 M $ZnSO_4$ followed by 1 ml of saturated $Ba(OH)_2$. After centrifugation at 1000 g for 20 min at 4°c, 2.5 ml of the clear supernatant fluid were used for the formaldehyde measurement by the method of Nash (1953). The units reported are nmoles formaldehyde produced/min/mg of microsomal protein.

CYTOCHROME P-450 AND PROTEIN MEASUREMENTS

Cytochrome P-450 was measured spectrophotometrically using the microsomal fraction resuspended in buffered 0.154 M KCl solution according to the method described by Mazel (1972). The proteins were measured by the method of Lowry et al. (1975) using human serum albumin (Connaught Medical Research Laboratories, Toronto, Ontario, Canada) as standard.

SERUM ASSAYS OF CALCIUM, PHOSPHORUS AND OF VITAMIN D METABOLITES

At the time of sacrifice, blood was extracted from the animals by means of intracardiac puncture and centrifuged. The supernatant serum was then stored frozen at -20°C until the time of assaying.

Serum calcium (Ca) and phosphorus (Pi) were determined by Auto Analyzer procedures (Technicon Instruments, Tarrytown, NY).

The concentration of 25-hydroxyvitamin D (25-OHD) was measured by radioligand assay by the method of Haddad and Chyu (1971) and 1,25-dihydroxyvitamin D $(1,25\text{-}(OH)_2D)$ by the method of Eisman et al. (1976) modified by separating the bound from the free hormone with a Dextran T-20 coated charcoal suspension instead of a polyethylene glycol solution. The circulating 24,25-dihydroxyvitamin D $(24,25\text{-}(OH)_2D)$ was also measured by a competitive binding assay

after careful purification by high liquid chromatography on a 5 µm particle microporasil column (Beckman Instruments, Paolo Alto, CA, USA) equilibrated with 6.5% propan-2-ol/n-hexane as mobile phase. The inter- and intra-assay variation coefficients for the three assays were 10 and 8% respectively.

DETERMINATION OF PERCENTAGE BONE ASH AND OF ASH CALCIUM AND PHOSPHORE CONTENTS

The left tibial and femoral diaphyses and metaphyses were used to determine the percentage bone ash and the calcium and phosphorus content in bone. Following removal at autopsy, the bones were placed in an oven at 100°C overnight, weighed ("dry weights"), and subsequently incinerated in an oven at 600°C for 18 hours. The ashes were weighed and this value expressed as a percentage of the dry bone weight. Calcium and phosphorus contents in the ashes were measured by Auto Analyzer (Technicon Instruments, Tarrytown, NY, USA) and the results expressed as mg of Ca or Pi per mg of dry bone weight. All data shown in Tables 5 and 6 are expressed as mean values ($\bar{x}$) ± standard error of the mean (SEM);. The significance (p) of the differences between PB-treated and controls was evaluated by the Student t-test for independent variates.

HISTOLOGY AND BONE MORPHOMETRY

The remaining two third portions of each plaque of induced bone, longitudinal one half portions of the first three tail vertebrae, and the right tibial and femoral diaphyses and metaphyses were fixed in buffered formaldehyde (pH 7.1), embedded undecalcified in methylmethacrylate, and sectioned longitudinally on a microtome (Autocut, Jung Inc., Heidelberg, W.Germany).

Five micron thick sections of the implants of induced bone (harvested from the animals' right thoracic region), of the epiphyseal and metaphyseal regions of the

vertebrae, and of the long bones were stained with toluidine blue at pH 2.8 (Bordier et al., 1972) or by the Von Kossa method (Villaneuva, 1973) and were quantitatively analyzed using a semi-automatic image analyzer coupled to a photomicroscope (MOP3, Carl Zeiss Inc., W.Germany).

By means of in vivo double tetracycline labeling (Achromycin, 40 mg/kg, administered seven and three days prior to sacrifice) and fluorescent microscopy, the following dynamic parameters were measured in the metaphyseal region of the seventh caudal vertebrae and along the surfaces of the induced bone: the mean osteoid seam thickness (MOST) determined by measuring the mean distance (in microns) between midpoints along the osteoid surface; the calcification rate (CR) by dividing the MOST by the interval of time (4 days) between the two labels; the mineralization lag time (MLT), calculated as the ratio of the MOST over the corresponding CR.

AUTORADIOGRAPHY

Five micron thick sections were coated in complete darkness with NTB^3 liquid emulsion (Eastman Kodak, Rochester, NY), dried in a low humidity atmosphere at 20°C and placed in dark boxes at 4°C for five days. At the end of the exposure period, the slides were developed in D19 and fixed (Unifix, Kodak), rinsed 30 min in running cold tap water, then stained with toluidine blue.

RADIOISOTOPE LABELING OF BONE IMPLANTS

Radioisotopes were obtained from New England Nuclear Canada Ltd; Montreal, Quebec, Canada. Four hours prior to sacrifice, each animal of stage 4 and 7 received 100 µCi of $Na^{35}SO_4$ (carrier free, 5.00 mCi/ml) whereas each animal of stage 11,14,20 and 42 received 100 µCi of $^{45}CaCl_2$ (5.00 mCi/ml).

At sacrifice, the plaques of induced endochondral bone were harvested from the thoracic region and cleaned of adherent tissue. Representative one-third-portions of the specimens of stages 4 (day 35) and 7 (day 38) were washed for 30 minutes in 0.1 M Na_2SO_4 (non radioactive) in 0.05 M sodium phosphate buffer (pH 7.4) while those of stages 11,14,20 and 42 (days 42, 45, 51 and 73, respectively) were washed for 30 minutes in 0.1 M $CaCl_2$ (non radioactive); the specimens were then blotted dry, weighed and then solubilized in 2 cc of 88% formic acid at 90°C. Radioactivity was then assessed in a LKB Model 1216 Rackbeta II liquid scintillation spectrometer using a mixture of 0.2 ml of the solubilized samples and Aquasol (New England Nuclear Canada Ltd; Montreal, Quebec, Canada) as scintillation liquid.

CHAPTER IV

RESULTS

RESULTS

1. EFFECTS OF PHENOBARBITAL ON WEIGHT GAIN AND EATING ACTIVITY

Under the stated conditions of pair feeding of a standard rat chow diet, the PB animals gained significantly less weight than their controls, from day 38 until the end of the experiment (Fig. 3). Important behavioural changes noted in the PB rats included hyperkinesis and voracious appetites at the time of feeding, and also a change in the biphasic circadian cycling of food intake, such that eating activity was confined to the first feeding episode. It is important to point out that daily levels of food consumption were equivalent in both groups.

2. BIOCHEMICAL DATA

i) Effect of phenobarbital on liver weight, cytochrome P-450 content and aminopyrine demethylase activity

The data contained in Table 2 indicates that in the PB animal, significant increases in liver weights and cytochrome P-450 content and hepatic aminopyrine demethylase levels (as an index of mixed function oxidase levels) were obtained after prolonged treatment with phenobarbital, thereby attesting to the effectiveness of the experiment's protocol in stimulating hepatic metabolism in the rat.

ii) Serum calcium and phosphorus determinations

The averages of the sequential serum calcium and phosphorus determinations are shown in Table 3. Mild serum calcium and phosphorus elevations in the PB animals were observed at many stages, but while these values were statistically significant in these instances, nevertheless they were not in keeping with true states

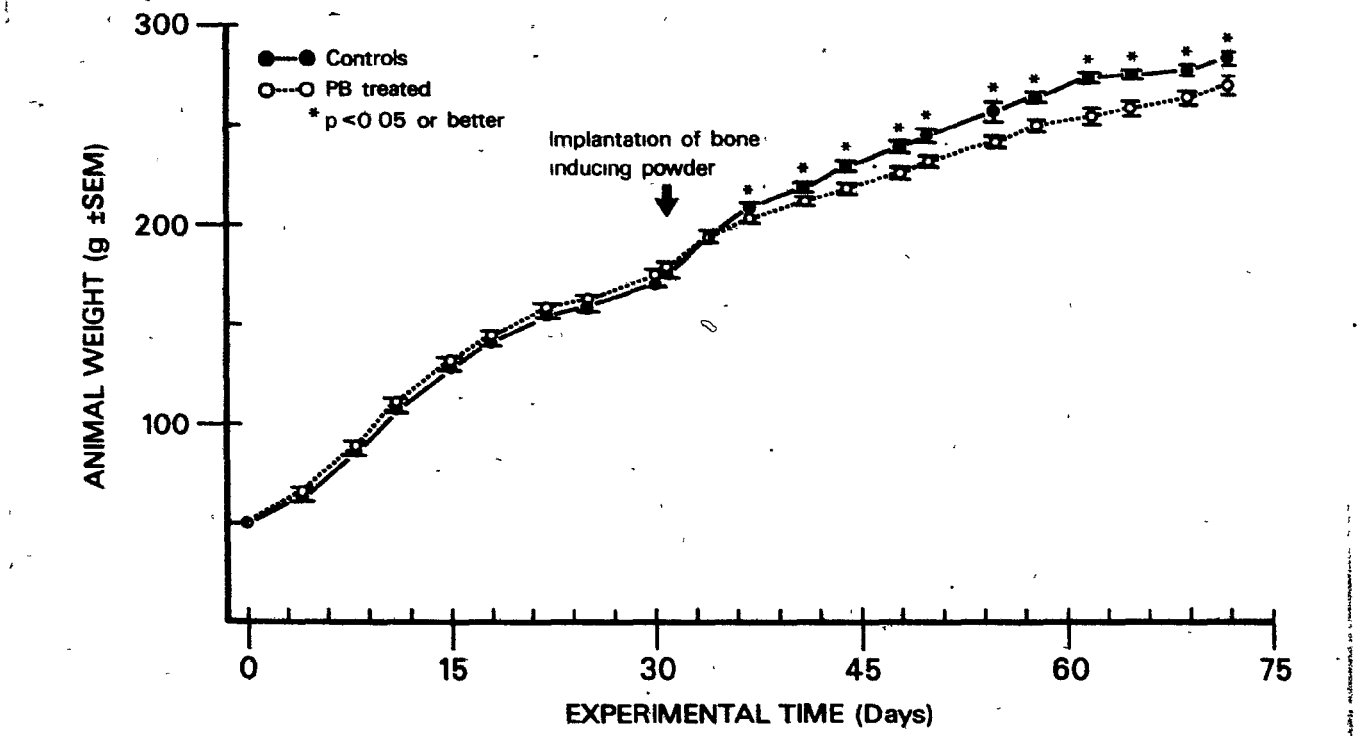

FIGURE 3 - Effects of phenobarbital treatment on animal weight.

TABLE 2 - EFFECT OF PHENOBARBITAL TREATMENT ON LIVER WEIGHT, CYTOCHROME P-450 CONTENT AND AMINOPYRINE DEMETHYLASE ACTIVITY

	CONTROLS	n	PB/TREATED	n	p *
LIVER WEIGHTS ($\bar{x}$ ± SEM**)	7.6 ±0.3	5	9.9 ± 0.5	5	< 0.005
CYTOCHROME P-450 (nmoles/mg microsomal protein)	0.87 ± 0.5	9	1.33 ± 1.3	10	< 0.01
AMINOPYRINE DEMETHYLASE (nmoles/ mg microsomal protein/min)	5.5 ± 0.6	4	11.4 ± 1.9	4	< 0.025

* significance of difference was calculated by the Student's t-test for independent variates

** $\bar{x}$ SEM : mean ± standard error of the mean

n = sample size

TABLE 3 - SERUM CALCIUM (Ca) AND PHOSPHORUS (Pi) MEASUREMENTS

Experimental time (days)	Ca					Pi				
	C	n	PB	n	p*	C	n	PB	n	p*
35	9.7 ± 0.2	3	10.5 ± 0.2	5	< 0.025	7.2 ± 0.1	3	8.1 ± 0.1	5	<0.005
38	n.d.		n.d.		n.d.	n.d.		n.d.		n.d.
42	9.6 ±0.1	4	10.3 ± 0.3	4	<0.05	7.8 ± 0.3	4	8.4 ± 0.2	4	<0.05
45	10.0 ± 0.2	4	10.0 ± 0.1	4	N.S.	7.8 ± 0.2	4	8.0 ± 0.4	4	N.S.
51	9.7 ±0.2	5	10.2 ± 0.1	4	<0.025	7.3 ± 0.3	5	7.5 ± 0.2	4	N.S.
73	9.5 ±0.1	3	10.0 ± 0.0	5	< 0.0005	5.8 ± 0.2	3	6.8 ± 0.2	5	< 0.005

* significance of difference was calculated by the Student's t-test for independent variates

n = sample size

n.d.: not done

of hypercalcemia or hyperphosphatemia. The data for day 7 could not be determined due to insufficient quantities of sera.

iii) Sequential measurements of vitamin D metabolites

In Table 4 are shown the mean serum values of the vitamin D metabolites obtained sequentially at the key developmental stages of the matrix-induced endochondral bone forming system (Reddi and Huggins, 1972; c.f. below).

Mean serum 25-(OH)D levels did not differ significantly in the PB animals from controls. Mean values for PB and control animals ranged from 14.0 ng/ml to 28.1 ng/ml.

Serum 1,25-$(OH)_2$D levels in the PB animals were similar to controls at days 38, 42, 45 and 51. Although the levels of this metabolite were significantly lower in the PB animals at days 35 and 73, these lower values were still within the normal range (Shepard and DeLuca, 1980). The elevated mean value of 1,25-$(OH)_2$D in controls at day 51 was swayed upward by two high measurements (113 and 117 pg/ml); the physiological significance of these particular results is unknown.

At days 35 and 73, the levels of 24,25-$(OH)_2$D were significantly increased in the PB animals compared to controls. However, caution is advised in interpreting the 24,25-$(OH)_2$D values, as the fractions measured possibly contain other vitamin D metabolites such as 25,26-dihydroxyvitamin D and the vitamin D 3,26-lactone; nevertheless, we believe our 24,25-$(OH)_2$D fractions to be pure as a result of the technique employed here consisting of two separations with two different solvents, as opposed to a single separation with one solvent, which is the technique most frequently employed by most investigators.

TABLE 4 - SEQUENTIAL MEASUREMENTS OF VITAMIN D METABOLITES

Experimental time (days)	25-OHD (ng/ml)				24-25$(OH)_2$D (ng/ml)				1,25$(OH)_2$D (pg/ml)			
	Controls	n	PB animals	n	Controls	n	PB animals	n	Controls	n	PB animals	n
35	14.0 ± 1.0	5	14.0 ± 2.0	5	4.1 ± 0.2	5	8.1 ± 0.9[1]	5	55.0 ± 6.0	5	36.0 ± 5.0[2]	5
38	21.0 ± 2.2	4	19.0 ± 1.9	4	10.6 ± 0.8	4	10.0 ± 0.4	4	58.7 ± 8.4	4	51.8 ± 4.6	4
42	24.8 ± 2.9	4	18.9 ± 1.6	4	10.0 ± 0.5	4	8.9 ± 0.9	4	29.8 ± 3.9	4	26.4 ± 5.8	4
45	25.2 ± 4.0	4	28.1 ± 0.7	4	16.3 ± 1.0	4	17.2 ± 0.9	4	25.2 ± 4.0	4	28.1 ± 0.7	4
51	21.8 ± 3.7	5	25.4 ± 0.8	4	13.8 ± 0.6	3	17.6 ± 1.9	3	80.6 ± 14.8	5	50.8 ± 5.8	4
73	19.9 ± 1.4	3	22.3 ± 0.6	5	12.3 ± 0.5	3	15.2 ± 0.7[2]	5	53.7 ± 2.5	3	36.9 ± 4.0[2]	5

* Significance of difference was calculated by the Student's t-test for independent variates

1 $p < 0.005$

2 $p < 0.025$

n = sample size

iv) Bone ash weight and ash calcium and phosphorus contents

In Tables 5 and 6 respectively, are shown the mean values of the long bone ash content, and of calcium and phosphorus contents (μg) in the bone ashes expressed in relation to the dry weight (mg) of the long bones. Again, despite statistically significant results obtained at some of the intervals, the values must be interpreted as not being trully different, as the calculated differences were due to the high precision of the techniques employed, the small values for errors of the mean, and also due to the small number of samples involved.

3. HISTOLOGY

i) Effects of phenobarbital on induced endochondral bone

The protocol for the matrix-induced endochondral bone-forming system yielded plaques of bone formed by an endochondral sequence, both in control and PB-treated animals, in approximately 60% of animals. In the remaining 40%, the bone powder had either been completely resorbed and none remained at autopsy, or else the bone-inducing powder had been completely encapsuled such that it could not incite any bone-forming response in the organism. The "success rate" of bone induced by this protocol was deemed statisfactory and concorded with the rate of bone induction obtained in other experiments using this system (Reddi, 1981, personal communication).

At day 35, the plaques which measured approximately 1 cm were covered with a thick, firm, fibrous capsule and were surrounded by edema fluid. The cut sections revealed loose bone particles and the histological sections showed a vascular response invading the implant and surrounding the bone particles. The intensity of the reaction was equivalent in both controls and PB animals. With polarizing light, the bone matrix was noted to be entirely lamellar in structure and thus of cortical origin. A few foci of cartilage growth plate matrix were seen in the

TABLE 5 - SEQUENTIAL DETERMINATION OF PERCENTAGE ASH CONTENT IN LONG BONES[1]

Experimental time	Day 35	Day 38	Day 42	Day 45	Day 51	Day 73
Controls	74.6 ±0.4	70.7 ±0.3	70.3 ±0.3	70.4 ± 0.2	70.2 ± 0.2	71.1 ± 0.1
PB animals	76.2 ± 0.5	70.4 ± 0.1	69.3 ± 0.4	69.9 ± 0.2	70.5 ± 0.1	70.7 ± 0.3
"p" value[2]	$p < 0.025$	p = N.S.	$p < 0.05$	$p < 0.05$	p = N.S.	p = N.S.

1) i.e. Weight of bone ashes÷ dry bone weight x 100%

2) Significance of difference was calculated by the Student's t-test for independent variates

N.S. = not significant

TABLE 6 - BONE ASH CALCIUM AND PHOSPHORUS CONTENT[1]

Experimental time		Day 35	Day 38	Day 42	Day 45	Day 51	Day 79
Ca	Controls	282 ± 3	284 ± 4	274 ±8	278 ± 5	280 ± 4	285 ±2
	PB animals	294 ± 4[2]	284 ±2	266 ± 9	280 ±4	278 ± 4	280± 3
Pi	Controls	142 ±3	142 ± 2	138 ± 3	144 ±2	140 ± 0	140 ± 0
	PB animals	148 ± 3	148 ± 2[2]	136 ± 0	144 ± 2	138 ± 2	140 ± 0

1) Expressed in µg/mg dry weight of bones. Significance of difference was calculated by the Student's t-test for independent variates.

2) $p < 0.05$

homologous bone powder and undoubtedly represented unresorbed, long cartilage columns which had penetrated deeply into the diaphyseal sections of the long bones of the donor animals, and which had been retained throughout the preparation of the bone powder.

At day 38, the implants were more firm and opaque, and were more adherent to underlying muscle. The cut surface was more homogenous and solid, yet still slightly granular in consistency. Histological examination of toluidine blue sections revealed foci of early cartilage cell differentiation with production of ground substance as exemplified by increased uptake of the dye at these locations. Similar changes were seen in both groups of animals, and to the same extent.

At day 42, the gross appearance of the implants was pearly white. Microscopically, cartilage cell proliferation was abundant on the surfaces of the powder particles, and greater amounts of ground substance were being produced (Fig. 4) and mineralized, as witnessed by the von Kossa staining. An interesting and new observation made at this point was the finding of similar chondrocyte cellular activity within old Haversian canals of the donor bone powder, thus showing that bone induction was not restricted to the surface of the bone particles but that it could also take place inside of old Haversian systems of the homologous bone matrix. Moreover, it was observed that the focus of these activities was not at the center of the plaque, as advocated by Reddi and Huggins (1972), but that it was more or less diffuse throughout the button-like plaque. Again, at day 42, these cellular responses to the presence of the inducing powder were seen in both control and PB animals.

At day 45, the presence of regularly arranged rows of osteoblasts producing unmineralized osteoid within old haversian systems and along the surfaces of the inductive powder was noted in both control and PB animals. Active osteoclasts were also present. The intensity of these events at this stage was identical in both groups of animals.

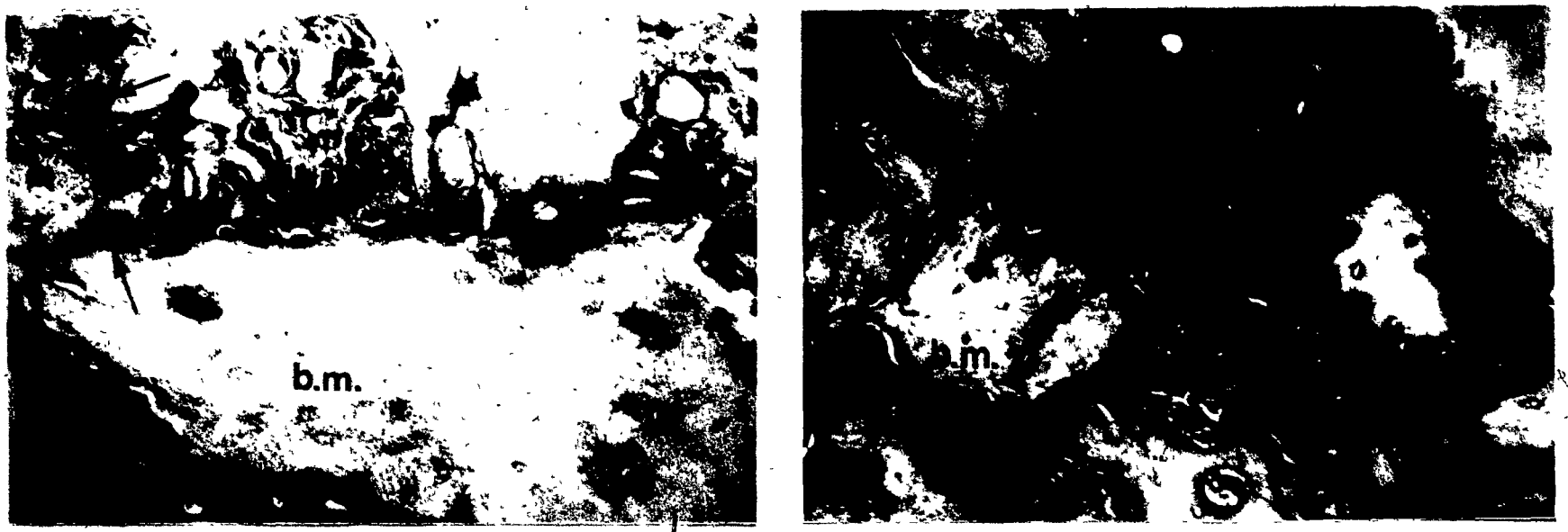

FIGURE 4 - Representative histologic sections of implants of induced bone harversted at 11 days after implantation from control (left) and PB-treated (right) rats showing in both the osteogenic bone matrix (b.m.) surrounded by cartilagenous matrix produced by chondroblasts (arrows). Note the presence of cells in old osteocytic lacunae.

(5 µm thick toluidine blue stained section, 250 X)

At day 51, the osteoid produced was almost all mineralized in both groups of animals (Fig. 5).

At day 73, the plaques felt hard to touch and looked blood-tinged on their cut surfaces. Histological evaluation revealed mainly calcified matrix, active osteoblasts, marrow elements, and remaining decalcified bone powder. The extent of the development and the sizes of the resulting bone implants were identical in both groups of animals.

The regularity of the time of appearance of these developmental stages of matrix-induced bone was uniform and predictable in most circumstances, in both animal groups. However, occasionally it was observed that an implant from a control or a PB animal contained histological features typical of the next most advanced stage of growth. For example, a section from an 11 day old implant harvested from a control animal (Fig. 6) clearly demonstrated active osteoblasts producing osteoid, a feature usually seen only in a 14 day old implant. Similarly, in one 20 day old implant were found marrow precursor cells in the form of loose cells mixed with fibrous connective tissue elements. Thus, this particular model of bone induction faithfully recapitulated the events of endochondral bone formation, with variables of plus or minus three days observed in the early stages.

ii) Histomorphometry

Histomorphometric data for day 73 is reported in Table 7. This stage alone was chosen for measurements and interpretation of implant and vertebral bone development as it was considered likely to yield more meaningful information than would be possible at the other stages. Not only were attempted measurements of induced bone at earlier intervals difficult to obtain because of the heterogenous character of the induced bone (see above), but also because an insufficient number of possible readings (on account of immaturity of the new bone) rendered any

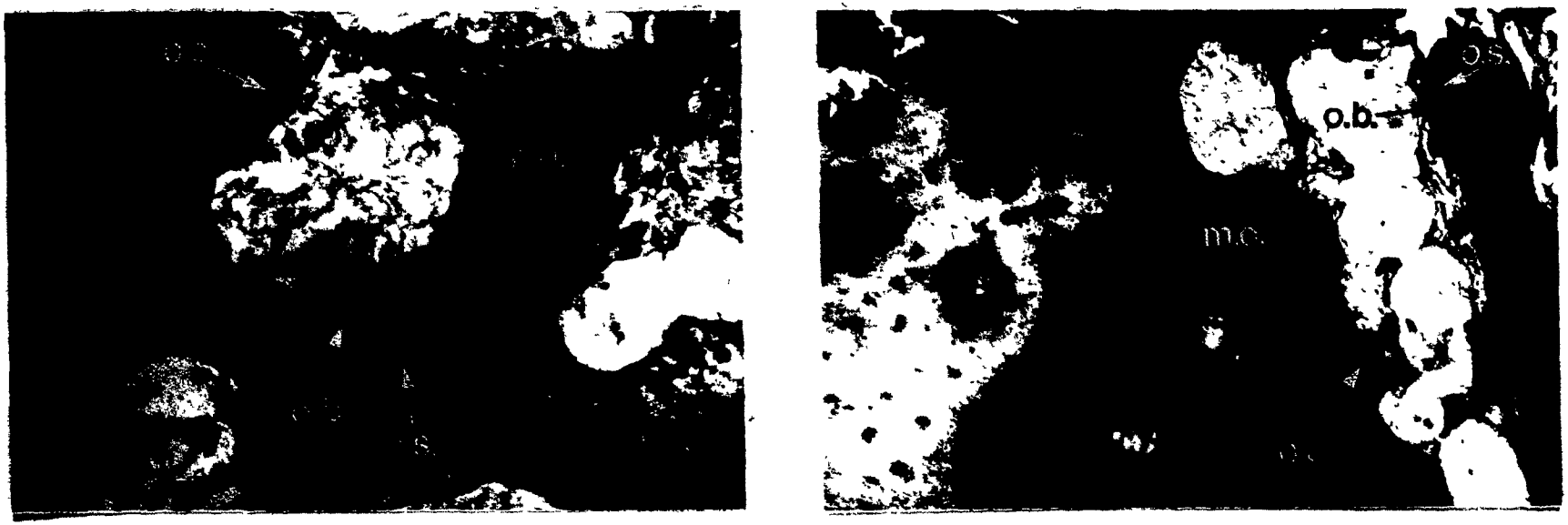

FIGURE 5 - Histologic sections of implants of induced bone harvested 20 days post implantation (control on left; PB-treated on right). Mineralized (m.o.) and unmineralized osteoid (o.s.) are seen under rows of regularly arranged osteoblasts (o.b.). Osteoclastic activity (o.c.) is also visible. (5 µm thick Von Kossa stained sections, 250 X)

FIGURE 6 - Histologic section of 11 day old implants of induced bone in a control rat showing events usually seen in a 14 day old implant. Osteoblasts (o.b.) are seen producing osteoid (o.s.) on the surface of the bone-inducing matrix (b.m.)
(5 μm thick toluidine blue stained section, 250 X)

TABLE 7 - QUANTITATIVE HISTOLOGY OF BONE[1]

Day 42

	Mean osteoid seam thickness (µm) (Mean ± S.D.)		Calcification rate (µm/day) (Mean ± S.D.)		Mineralization lag time (days) (Mean ± S.D.)	
	Implant	Vertebra	Implant	Vertebra	Implant	Vertebra
Controls n = 4	5.71 ±0.48	6.00 ±0.19	2.45 ±0.32	2.41 ± 0.18	2.36 ± 0.39	2.50 ± 0.18
Treated n = 5	6.09 ±0.40	5.98 ±0.09	2.82 ±0.35	2.21 ±0.16*	2.19 ±0.38**	2.70 ±0.20

1 Significance of difference was calculated by the Student's t-test for independent variates

* $p < 0.01$ compared to implant in same animals

** $p < 0.02$ compared to vertebra of same animal

n = sample size

measurement at these earlier stages imprecise and misleading. The information presented in Table 7 represents mean values of the measurements ± standard error of the means.

The quantitative measurements of the plaques of induced bone and of the vertebrae of one group were compared to each other, and then compared to the values obtained in animals belonging to the other group. No significant differences were noted in mean osteoid seam thickness in implants of controls compared to those of PB animals, or between implants from one group compared to the vertebra in the same group, or between vertebrae from either group. Double fluorescent uptake with tetracycline was seen in the induced bone as well as the vertebral metaphyseal bone (Figs. 7 and 8). No differences were found in the mean calcification rate of the implant compared to that of the vertebra. However, in PB animals, the mean calcification rate of the implant was found to be significantly increased compared to that of the vertebra; on the other hand, it is apparent that the differences shown are not large, and that they probably do not have biological significance.

Mineralization lag time was essentially the same in control implants and vertebrae, and between vertebrae from control and PB animals. Due to the difference in calcification rate of PB implants and vertebrae stated above, there was a significant difference calculated in the mineralization lag time of the PB implants and vertebrae, with implant mineralization lag time being slightly shorter in the implants. Again, the difference is small, and probably does not constitute a biologically significant observation.

iii) Autoradiography

Autoradiographs obtained from sections of vertebrae at day 73 revealed essentially the same extent of ^{45}Ca incorporation in both PB animals and controls

FIGURE 7 - Sections of control (left) and PB-treated (right) implants of induced bone harvested 42 days post implantation (day 73). Uptake of the double labels was evidenced by the two layers (arrows) of fluorescence at the sites of active bone formation on the surface of the bone-inducing matrix (b.m.).

15 µm unstained section, fluorescent light, 250 X)

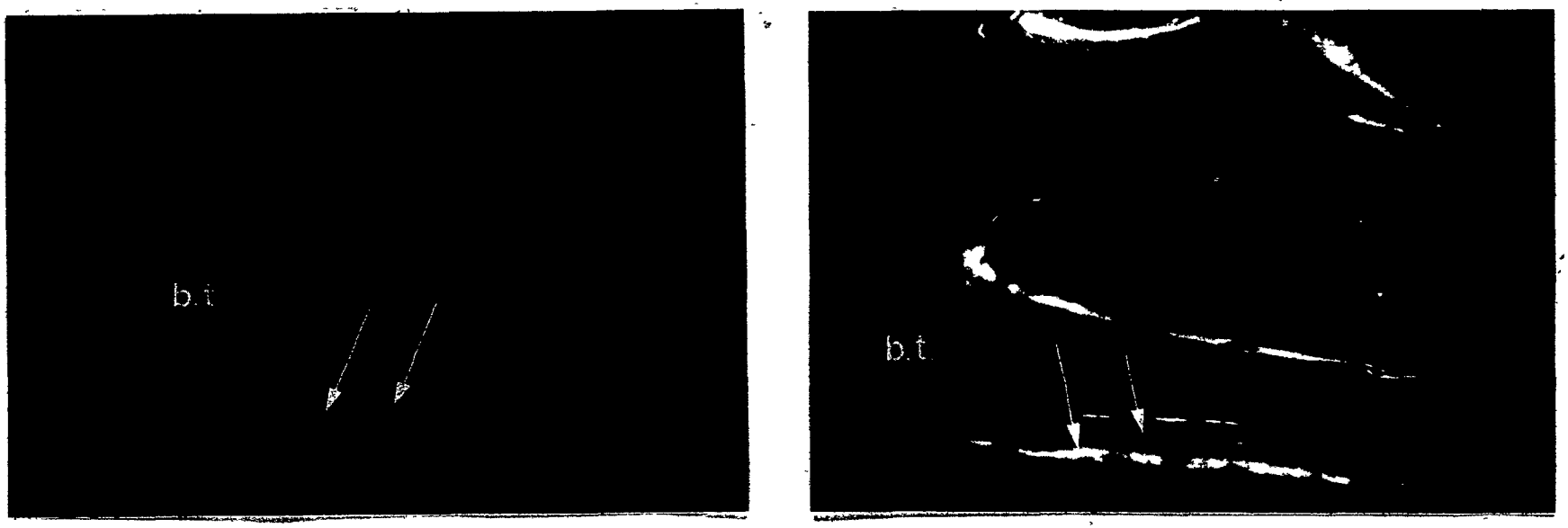

FIGURE 8 - Sections through metaphyseal region of control (left) and PB-treated vertebrae (right) harvested at day 73, showing uptake of both fluorescent labels (arrows) (b.t. = bone trabecula). (15 µm unstained section, fluorescent light, 250 X)

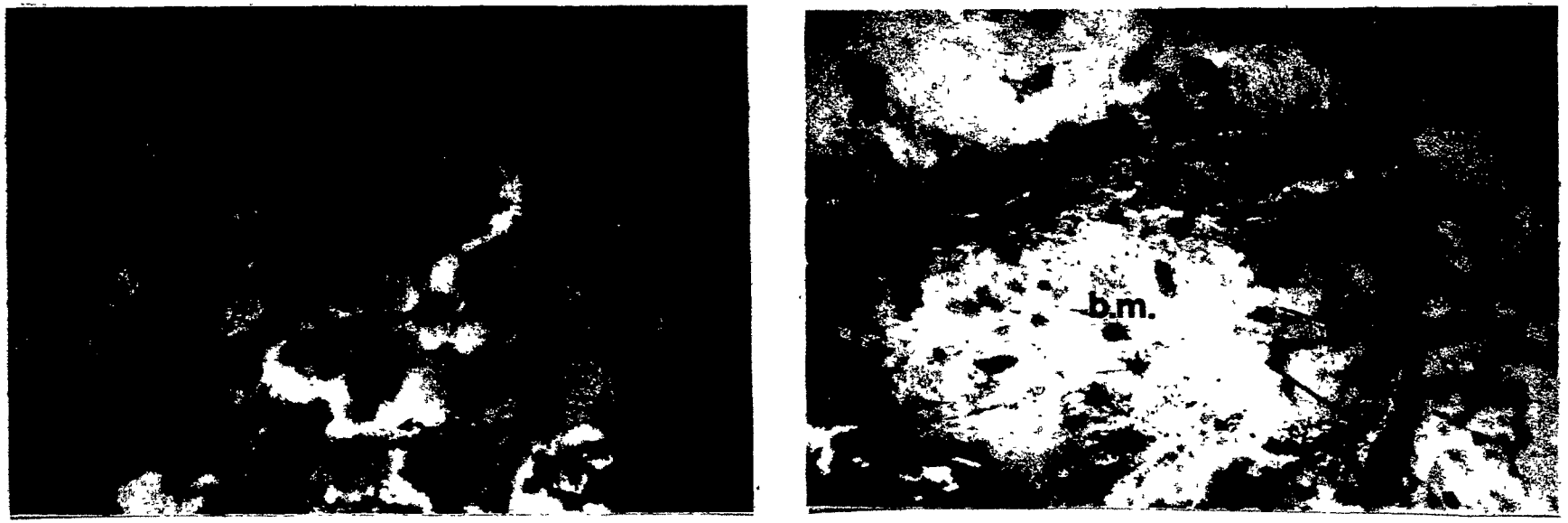

FIGURE 9 - ^{45}Ca autoradiography of control (left) and PB-treated (right) implants harvested 42 days after implantation showing active sites of mineralization (arrows) of the matrix (b.m.) in both animal groups.

(5 µm sections, toluidine blue stained sections, 250 X)

TABLE 8 - RADIOISOTOPE INCORPORATION IN IMPLANTS MATRIX-INDUCED ENDOCHONDRAL BONE[1]

Experimental time	^{35}S Incorporation			^{45}Ca Incorporation		
	Day 35	Day 38	Day 42	Day 45	Day 51	Day 73
Controls	279 ±37	280 ± 21	1803 ± 603	2102 ± 624	1596 ± 244	2653 ± 374
PB animals	225 ±71[3]	251 ± 51[3]	185 ±85[2]	1164 ±464[3]	1327 ±513[3]	1349 ± 353[2]

1) Mean of the measurement (dpm/mg tissue) ± standard error of the mean. Significance of difference was calculated by the Student's t-test for independent variates.

2) $p < 0.01$

3) p: not significant

(Fig. 9). ^{45}Ca deposition in the newly produced osteoid was evidenced both at the surface of the matrix powder and also within newly "colonized" Haversian canals of the matrix powder. A few osteoblasts also showed intracellular radioactivity, reflecting incipient nucleation of mineral on the osteoid. These results thus corroborated the histological observations reported above.

iv) Radioisotope labeling of bone implants

The results of radioisotope labeling of the implants done at the key developmental stages of matrix-induced endochondral bone formation are shown in Table 8. Significant differences in mean values of dpm of isotope/mg of tissue were noted at days 42 and 73, with PB implants receiving less isotope incorporation. However, it was necessary to interpret this data otherwise, in the light of the following information:

As the experiment was being performed, it became apparent that the radioisotope labeling results depended very much on which part of the plaque was being submitted for this assay. Since the bone induced in the implants tended to be heterogenous in structure, often a small portion of the specimen was used for ^{35}S or ^{45}Ca counting in order to retain a larger portion for histological evaluation. Therefore, due to these sampling techniques, which were used in an effort to obtain the most material for descriptive and quantitative histological information, it is impossible to lend credence to the differences in the results of implant radioisotope labeling.

CHAPTER V

DISCUSSION

DISCUSSION

Selection of the rat as an experimental animal this work was considered appropriate since the results obtained could then readily be compared to those of other related studies undertaken with the same model, and also because the matrix-induced endochondral bone forming system was shown to be more reliable in the rat than in other rodents (Huggins et al., 1970; Reddi, 1981, personal communication).

The results of this experiment support the contention that, under normal nutritional circumstances, long term anticonvulsant treatment induces liver microsomal enzyme production in humans and in the experimental animal (Conney, 1967), but does not affect vitamin D activation, nor does it induce histologic or biochemical features of osteomalacia or of rickets (Livingston et al., 1973: Balazs et al., 1974; Ray et al., 1974; Villareale et al., 1978; Wark et al., 1979; Weisman et al., 1979; Melsen et al., 1980; Camfield et al., 1981, unpublished data; Gascon-Barré et al., 1981, in press). The differences between our results and most reports dealing with anticonvulsant-induced osteomalacia may stem from our rigid conditions of experimentation, namely pair feeding of a standard rat diet replete in calcium, phosphorus and vitamin D, a condition which mimicks the clinical setting of most patients receiving anticonvulsants. As opposed to other clinical surveys or animal experiments dealing with this syndrome, the protocol of this experiment was designed to monitor simultaneously the main biochemical parameters and also the histologic events during long term single drug anticonvulsant therapy, in order to provide a global view of the effects and possibly a better understanding of the mechanisms involved. An attempt was thus made to reduce to a minimum the additive influence of the risk factors predisposing to the development of the

syndrome. On the other hand, one could argue that the vitamin D content (2.5 IU/g) in the "standard" rat chow diet was large enough to afford a good safeguard against the development of signs of vitamin D depletion. Nevertheless, this amount of vitamin D in the diet was not excessive; it complied with the minimum requirement of 1 IU/g diet/day (Subcommittee on Laboratory Animal Nutrition, National Research Council, Washington, DC) necessary to prevent nutritional rickets in the rat, and it compared with the vitamin D content in other commercial diets.

General effects of long term phenobarbital treatment which included slower weight gain and a change in the circadian cycling of food intake, are in agreement with the observations of Peraino et al. (1980).

Although the normal ranges of serum vitamin D metabolites have yet to be established in the young rat, it can still be stated with confidence that the serum vitamin D metabolite levels measured in the PB animals remained within ranges observed in control animals and are therefore likely to have provided sufficient tissue levels of the hormone. The levels were furthermore comparable to the concentrations obtained elsewhere using diets providing approximately the same daily quantity of vitamin D (Shepard and De Luca, 1980).

With our protocol, hepatic microsomal mixed function oxidase induction was obtained as evidenced by the increase in aminopyrine demethylase activity and in cytochrome P-450 content.

Serum 25-(OH)D levels in the PB-treated rats being similar to those of controls, reflected the adequate bioavailability of the parent vitamin. It has been shown that acute treatment of rats with phenobarbital alters the in vitro hepatic cholecalciferol 25-hydroxylase kinetics (Delvin et al., 1980) in such a way that at low substrate concentration there is a decreased production of 25-(OH)D while at high substrate concentration the hydroxylation is increased. It could therefore be postulated that the unchanged 25-(OH)D levels reflect a balance between an

increased liver hydroxylation rate and an increased choleresis (Silver et al., 1974; Gascon-Barré et al., 1977) of 25-(OH)D.

Normal serum 1,25-$(OH)_2$D values in the PB animals maintained throughout the experiment are a consequence of an adequate 25-(OH)D pool and an intact renal hydroxylating mechanisms, and also correspond to the serum concentrations obtained elsewhere (Shepard and De Luca, 1980) in normal rats fed diets providing approximately the same daily quantity of vitamin D. As there exist three orders of magnitude between 1,25-$(OH)_2$D and 24,25-$(OH)_2$D serum concentrations, it was difficult to establish a definite link between these two metabolites. As mentioned previously, comigration of other dihydroxylated vitamin D metabolites (such as 25,26-dihydroxyvitamin D and the vitamin D 3,26-lactone) may be responsible for the elevated 24,25-$(OH)_2$D concentrations at days 35 and 73. This possibility is unlikely since with two different solvents used for purification, these other dihydroxylated metabolites do not comigrate with 24,25-$(OH)_2$D. The 24,25-$(OH)_2$D values in both animal groups furthermore fall in the range of values obtained by Danan et al. (1980) in normal rats of the same age.

In keeping with the lack of effect of long term PB administration on serum levels of vitamin D metabolites in the rat, no effect of biological significance on serum calcium and phosphorus values was observed. Sufficient dietary calcium and phosphorus intake and adequate calcium intestinal absorption (Koch et al., 1972; Krawitt et al., 1972; Schaefer et al., 1972) are responsible in part for assuring the maintenance of mineral homeostasis in the PB animals, along with adequate concentrations and activity of the 1,25-$(OH)_2$D metabolite. Furthermore, normocalcemia was not linked to a mild secondary hyperparathyroid state, on account of the normophosphatemia in the PB animals, and as witnessed by the appropriate bone resorption-formation equilibrium observed histologically (see below).

There may be several explanations for the discrepancies between these biochemical results and those of other investigators. In the first place, most clinical studies have been difficult to interpret because of the difficulties in clearly defining the dietary intake of calcium, phosphorus and vitamin D in anticonvulsant-treated and in control patients, and because of the impossibility of measuring endogenous vitamin D synthesis or of quantifying sunlight exposure. In the present study both animal groups were equal in terms of dietary content and of lack of exposure to ultra violet rays.

Furthermore, in contrast to the protocols of the present study and those of other related animal experiments (Gascon-Barré, 1981, in press; Hahn et al., 1978), it has not been possible in most clinical investigations to discriminate between the biochemical effects induced by one anticonvulsant over those produced by another, mainly because most epileptics studied were controlled with multi-drug as opposed to single-drug regimens. Since the individual types of anticonvulsant medications control epilepsy by different mechanisms of action, and since they consequently have different side effects, one would suspect that these pharmacologically related but distinct medications would not necessarily exert the same side effects on bone and mineral metabolism. Yet, it seems that the majority of the clinical reviews have ignored this fundamental objection, and have ascribed the observed biochemical abnormalities in treated epileptics to "anticonvulsant medication", although many different types of anticonvulsants were administered singly or in combination. Furthermore, since a wide variety of drugs (including aspirin) are known to be hepatic mixed function oxidase inducers, it is not unreasonable to suspect that part of the observed alterations in vitamin D metabolism in this group of patients (who often received other medication for the treatment of concurrent illnesses) could have as well been effected by other drugs taken concomitantly.

Finally, one must point out the often overlooked influence of dietary macronutrient composition on hepatic microsomal enzyme activity (Alvares et al., 1976), a factor which applies to many institutionalized and many out-patient epileptics receiving anticonvulsant treatment. Indeed, since many of these epileptics are unable or unwilling to ingest a balanced diet on account of debility, associated chronic illnesses, impairment of deglutition or because of associated organic brain lesions causing behavioural changes, or since many epileptics unfortunately are not fed a balanced nutritious diet, one must take into account in these individuals the marked influence of an unbalanced low protein/high carbohydrate diet on hepatic microsomal enzyme activity and thus on the plasma elimination rates of vitamin D metabolites. In this respect, it would be possible to observe lower 25-(OH)D levels in these individuals, especially in vitamin D-deficient states, by virtue of the different kinetics of the anticonvulsant-induced calciferol-25-hydroxylase system (Delvin et al., 1980).

In contrast to most of the published clinical and experimental studies in which a tissue diagnosis of osteomalacia was made, in this animal model osteomalacia was not observed in either the long bones or in the plaques of induced endochondral bone of the PB rats. Once again, one can account for the present results by the adequate vitamin D, calcium and phosphorus dietary contents which were effective in preventing this problem from developing.

In comparing these histologic results with those of other studies, it is important to draw attention to the current histological definition of osteomalacia which relies very much on histomorphometric data (Frame and Parfitt, 1978). In the context of metabolic bone diseases, histomorphometric analysis of bone samples following <u>in vivo</u> double tetracycline labeling offers a unique opportunity to elucidate the mode of interference by a pathogenetic factor with the remodeling system (Jaworski, 1976). In a steady state situation with regard to osteoid

thickness, the rate of apposition of osteoid equals the rate of mineralization; any systematic change in this proportion will result in a disappearance or an accumulation of unmineralized bone. This assumption makes it possible to calculate the mineralization lag time or the time interval between apposition and subsequent mineralization of osteoid. This measurement is a characteristic feature of different metabolic bone diseases, and the changes thus observed in the mean width of osteoid seams may be explained by variations in calcification rate of osteoid and in the mineralization lag time (Melsen et al., 1980).

Osteomalacia can thus be defined as a metabolic bone disease linked to many disease conditions in which there is a defect in the mineralization of the preosseous matrix of mature lamellar bone, leading to an accumulation of non-mineralized or poorly mineralized osteoid over the surfaces of both compact cortical and spongy trabecular bone. This histological picture results from an uncoupling between the rates of bone matrix synthesis and mineralization, so that osteoid seams increase slowly in thickness, or there is marked delay in the onset of mineralization (mineralization lag time) because of a disturbance of matrix maturation (Frame and Parfitt, 1978).

It is important to stress that the observation of wide osteoid seams is not necessarily synonymous with osteomalacia, as wide osteoid seams are seen in high bone turnover states, such as Paget's disease (Meunier, 1975), uremic osteitis fibrosa (Sherrard et al., 1974), and primary hyperparathyroidism (Melsen and Mosekilde, 1980) which are conditions in which the calcification rates are proportionally prolonged such that mineralization lag times remain normal.

Unfortunately, most of the available histological proof of osteomalacia associated with anticonvulsants has simply consisted of observations of wide unmineralized osteoid seams (Dent et al., 1970; Teotia and Teotia, 1973; Villaraele et al., 1978; Lidgren et al., 1979; Johnell et al., 1979; Eastwood et al., 1980). This

information is thus incomplete, according to current quantitative methods of tissue diagnosis of metabolic bone diseases.

On the other hand, the histological results of this experiment are in agreement with the ones presented by Melsen and Mosekilde (1980) in which a normal mineralization lag time was calculated in bone tissue obtained from 20 epileptics receiving an average of 294 IU/day of vitamin D and maintained on diphenylhydantoin alone or in combination with other anticonvulsants for at least 10 years. Therefore, in this clinical study and in the present animal model, no evidence of uncoupling between bone matrix synthesis and mineralization was detected using histomorphometric methods. It is important to stress that normal nutritional circumstances prevailed in both studies, and in particular vitamin D, calcium and phosphorus dietary contents were adequate.

The data obtained here complement results from other investigations pertaining to this illusive syndrome, and also support the few published studies advocating views opposing the mainstream of opinions on this matter. From this work and from the available literature, it is possible to formulate an explanation of the mechanisms involved in producing the biochemical and histological features of phenobarbital-induced "osteomalacia":

The mechanism involved in phenobarbital-induced modifications of vitamin D metabolism in humans at the hepatic level are still poorly defined and of unknown clinical significance. Much of the available information stems from in vitro work (Hahn et al., 1972a; Delvin et al., 1980) and thus is difficult to extrapolate to man. Chronic phenobarbital treatment may be associated with mild lowering of serum calcium or phosphorus levels, but there exists no evidence indicating a direct phenobarbital-induced malabsorption of calcium or of impaired mobilization of mineral at the tissue level (Melsen et al., 1980); it is suggested that other causes of hypocalcemia should be investigated in epileptics with mild or severe hypocalcemia.

However, in individual receiving chronic diphenylhydantoin therapy, hypocalcemia may be the result of impaired intestinal calcium absorption (Shafer and Nuttall, 1975); the in vitro work of Hahn et al. (1978) also suggests a diphenylhydantoin-induced hypocalcemia related to impaired PTH-mediated mobilization of mineral.

The osseous response to chronic phenobarbital treatment might differ from that to long term diphenylhydantoin therapy. If dietary vitamin D and sunlight exposure are adequate, no bone tissue changes of osteomalacia are likely to develop with prolonged use of either anticonvulsant. When, in individuals receiving long term diphenylhydantoin, vitamin D input and sunlight exposure are not sufficient to maintain adequate concentration of active vitamin D metabolite, then histological changes diagnostic of osteomalacia and of secondary hyperparathyroidism (Melsen et al., 1976) might predominate; it is surmised that an analogous situation might take place in patients maintained on long term phenobarbital medication. The severity of these osseous changes, either in the vitamin D depleted or in the vitamin D repleted individuals, are also affected by the presence or absence of any of the risk factors associated with the pathogenesis of the syndrome.

Bone density in epileptics receiving anticonvulsants may be reduced by a variable amount, but this is more likely to be altered as a result of decreased physical activity and strength, or because of concomitant disease such as osteoporosis.

Due to the lack of histological evidence of osteomalacia in the majority of phenobartital-treated epileptics, it does not seen appropriate to label the syndrome as "osteomalacia". Bone tissue changes of osteomalacia are likely to occur only in individuals who are marginally deficient in vitamin D. Finally, vitamin D in the diet should be sufficient or an appropriate supplement should be added (Hahn, 1980) in order to minimize the biochemical changes observed in some epileptics receiving anticonvulsant medication.

CHAPTER VI

CONCLUSIONS

CONCLUSIONS

It was concluded from this study that:

1. Chronic phenobarbital administration does not induce osteomalacia in the vitamin D-repleted rat.

2. Chronic phenobarbital administration has no effect on the processes of induced endochondral bone formaiton and metaphyseal bone remodeling.

3. Long term phenobarbital administration does not appear to alter the vitamin D activation pathway.

4. Osteomalacia observed in humans maintained on chronic phenobarbital therapy is multifactorial and depends largely on dietary calcium, phosphorus and vitamin D availability.

CHAPTER VII

BIBLIOGRAPHY

REFERENCES

- Alvares, A.P., Anderson, K.E. and Conney, A.H. (1976). Proc. Natl. Acad. Sci. USA 73: 2501-2504.
- Balazs, T., Hooper, W., Farber, T.M. et al. (1974). Toxicol. Appl. Pharmacol. 29: 47-52.
- Bell, R.D., Pak, C.Y.C., Zerwekh, J. et al. (1979). Ann. Neurol. 5: 374-378.
- Bhattacharyya, M.H. and De Luca, H.F. (1974a). Arch. Biochem. Biophys. 160: 58-62.
- Bhattacharyya, M.H. and De Luca, H.F. (1974b). Biochem. Biophys. Res. Commun. 59: 734-741.
- Bjorkhem, I. and Holmberg, I. (1978). J. Biol. Chem. 253: 842.
- Bordier, P. Ryckwaert, A., Marie, P. et al. (1977). In: Norman, A.W., Schaefer, K., Coburn, J.W. et al. (eds): "Vitamin D: Biochemical, Chemical, and Clinical Aspects Related to Calcium Metabolism." Walter de Gruyter, Berlin and New York, pp 897-911,
- Bordier, P.J. and Tun Chot, S. (1972). J. Clin. Endocrinol. Metab. 1: 197-215.-
- Borgstedt, A.D., Bryson, M.F., Young, L.W. et al. (1972). J. Pediat. 81: 9-15.
- Bouillon, R., Reynaert, J., Claes, J.H. et al. (1975). J. Clin. Endocrinol. Metab. 41: 1130-1135.
- Brickman, A.S. (1979). In: Norman, A.W., Schaefer, K., Coburn, J.W. et al (eds): "Vitamin D, Basic Research and its Clinical Application." Walter de Gruyter & Co., Berlin and New York, pp. 1085-1090.
- Camfield, C.S., Delvin, E.E., Glorieux, F.H. et al. Unpublished data. Normal Serum 25-Hydroxyvitamin D Levels in Phenobarbital-Treated Toddlers.
- Caspary, W.F. (1972). Naunyn Schmiedebergs Arch. Pharmacol. 274: 146-153.

- Caspary, W.F., Hesch, R.D., Matte, R. et al. (1975). In: Norman, A.W., Schaefer, K., Coburn, J.W. et al (eds): "Vitamin D and Problems Related to Uremic Bone Disease." Walter de Gruyter, Berlin and New York, pp. 737-742.
- Christiansen, C., Kristensen, M., Rodbro, P. (1972). Br. Med. J. 3: 738-739.
- Christiansen, C.K., Bi. Lund, B.J., Lund, O.H. et al. (1981). Metab. Bone Dis. & Rel. Res. 3: 17-22.
- Christiansen, C., Rødbro, P., Lund, M. (1973) Br. Med. J. 2: 208-209.
- Conney, A.H. (1967). Pharmacol. Rev. 19: 317-366.
- Corradino, R.A. (1976). Biochem. Pharmacol. 25: 863-864.
- Danan, J.L., Delorme, A.C., Cuisinier-Gleizes, P. et al. (1980). Calcif. Tissue Int. 31: Supp.
- Dekel, S., Ornoy, A., Sekeles, E. et al. (1979). Calcif. Tiss. Int. 28:245-251.
- DeLuca, H.F. (1978). In: DeLuca, H.F. (ed): The Fat-Soluble Vitamins, Vol. II of Handbook of Lipid Research. Plenum Press, New York, pp. 69-132.
- DeLuca, H.F. and Schnoes, H.K. (1976). Ann. Rev. Biochem. 45: 631-666.
- Delvin, E.E., Arabian, A. and Glorieux, F.H. (1980). Biochem. Pharmacol. 29: 441-445.
- Dent, C.E., Richens, A., Rowe, D.J.F. et al (1970). Brit. Med. J. 4: 69-72.
- Dymling, J.F., Johnell, O., Lidgren, L. et al (1979). In: Norman, A.W., Schaefer, K., Coburn, J.W. et al (eds): "Vitamin D, Basic Research and Its Clinical Application." Walter de Gruyter, Berlin and New York, pp. 1193-97.
- Eastwood, J.B., Phillips, M.E. and De Wardener, H.E. (1980). Metab. Bone Dis. & Rel. Res. 2: 83-86.
- Eisman, J.A., Hamstra. A.J., Kream, B.E. et al. (1976). Arch. Biochem. Biophys. 176: 235.

- Fournier, A., Bordier, P., Gueris, J. et al. (1977). In: Norman, A.W., Schaefer, K., Coburn, J.W. et al (eds): "Vitamin D: Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism ". Walter De Gruyter, Berlin and New York, pp. 667-669.
- Frame, B. and Parfitt, A.M. (1978). Ann. Intern. Med. 89: 966-982.
- Frost, H.W. (1973). "Bone Remodeling and Its Relation to Metabolic Bone Disease." Springfield, Charles C. Thomas.
- Frost, H.W. (1979). Calcif. Tissue Res. 28: 181-200.
- Gascon-Barré, M. and Côté, M.G. (1978). Toxicol. and Appl. Pharmacol. 43, 125-135.
- Gascon-Barré, M. and Côté, M.G., Glorieux, F.H. et al. (1981). Biochem. Pharmacol. In Press.
- Gascon-Barré, M. and Glorieux, F.H. (1977). In: Norman, A.W., Schaefer, K., Coburn, J.W. et al (eds): "Vitamin D: Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism." Walter De Gruyter, Berlin and New York, pp. 781-783.
- Genel, M. et al. (1972). Pediatr. Res. 6: 395.
- Haddad, J.G., Jr., and Chyu, J.C. (1971). J. Clin. Endocrinol. 33: 992-995.
- Haddad, J.G. and Hahn, T.J. (1973). Nature 244: 515-517.
- Hahn, T.J. (1980). Clin. Endocrinol. Metabol. 9: 107-129.
- Hahn, T.J., Birge, S.J. and Scharp, C.R. (1972a). J. Clin. Invest. 51: 741-748.
- Hahn, T.J., Boisseau, V.C. and Avidi, L.V. (1974). J. Clin. Endocrinol. Metab. 39, 274-282.
- Hahn, T.J., Haid, S.D. and Halstead, L.R. (1973). Clin. Res. 21: 626.
- Hahn, T.J., Halstead, L.R., Scharp, C.R. (1975a). Clin. Res. 23: 111A.
- Hahn, T.J., Hendin, B.A., Scharp, C.R. et al (1975b). New Engl. J. Med. 292: 550-554.

- Hahn, T.J., Hendin, B.A., Scharp, C.R. et al. (1972b). New Engl. J. Med. 287: 900-904.
- Hahn, T.J., Scharp, C.R., Richardson, C.A. (1978). J. Clin. Invest. 62: 406-414.
- Harris, M., Rowe, D.J.F. and Darby, A.J. (1978). Calcif. Tiss. Res. 25: 13-17.
- Harris, M. and Goldhaber, P. (1974). Arch. Oral Biol. 19: 981-984.
- Harrison, H.C. and Harrison, H.E. (1976). Proc. Soc. Exp. Biol. Med. 153: 220-224.
- Harrison, H.E. and Harrison, H.C. (1979). "Calcium and Phosphate Homeostasis in Disorders of Calcium and Phosphate Metabolism in Childhood and Adolescence." W.B. Saunders, Toronto, pp. 18-23.
- Hollander, D. (1981). J. Lab. Clin. Med. 97: 449-462.
- Huggins, C., Wiseman, S., Reddi, A.H. (1970). J. Exp. Med. 132: 1250-1258.
- Hunter, J., Maxwell, J.D., Stewart, D.A. et al. (1971). Brit. Med. J. 4: 202-204.
- Ivey, J.L., Mory, E.R., Liu, C-C. et al. (1977). In: Norman, A.W., Schaefer, K., Coburn, J.W. et al (eds): "Vitamin D: Biochemical, Chemical, and Clinical Aspects Related to Calcium Metabolism." Walter de Gruyter, Berlin and New York, pp. 349-359.
- Jaworski, Z.F.G. (1976). In: Meunier, P.J. (ed): "Bone Histomorphometry, Proceedings of the Second International Workshop", Armour Montague, Lyon, pp. 193-235.
- Jenkins, M.V., Harris, M. and Wills, M.R. (1974). Calc. Tiss. Res. 16: 163-167.
- Johnell, O., Nilsson, B.E., Wallöe, A. et al. (1979). Calcif. Tissue Int. 28: 93-97.
- Jubiz, W., Hauassler, M.R. and McCain, T.A. (1977). J. Clin. Endocrinol. Metab. 44: 617-621.
- Jubiz, W., Meikle, A.W. and Levinson, R.A. (1970). N. Engl. J. Med. 283: 11-14.
- Kanis, J.A., Adams, N.D., Earnshaw, M. et al. (1977). In: Norman, A.W., Schaefer, K., Coburn, J.W. et al. (eds): "Vitamin D: Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism." Walter De Gruyter, Berlin and New York, pp 671-673.

- Kanis, J.A., Cundy, T., Bartlett, M. et al. (1978). Brit. Med. J. 1: 1382-1386.
- Kattan, K.R. (1970). Am. J. Roentgenol. Radium Ther. Nucl. Med. 110: 102-105.
- Koch, H.U., Kraft, D., von Herrath, D. et al. (1972). Epilepsia 13: 829-834.
- Kraft, D., von Herrath, D. and Schaeffer, K. (1974). Epilepsia 15: 81-90.
- Krawitt, E.L. and Stubbert, P.A. (1972). Proc. Soc. Exp. Biol. Med. 140: 420.
- Krüse, R. (1968). Monatschrift für klinische Kinderheilkunde 116: 378-381.
- Kruse, K., Bartels, H., Ziegler, R., et al (1980). Eur. J. Pediatr. 133: 151-156.
- La Du, B.N., Gaudett, L. Trousof, N. et al (1955). J. Biol. Chem. 214: 741.
- Levin, W., Welch, R.M. and Conney, A.H. (1967). Endocrinology 80: 135-140.
- Levison, J.C., Kent, G.N., Worth, G.K. et al (1977). Endocrinology 101: 1898.
- Lidgren, L., Nilsson, B.E. and Wallöe, A. (1979). Calcif. Tissue Int. 28: 99-102.
- Lifshitz, F. and Maclaren, N.K. (1973). J. Pediatr. 83: 612-620.
- Livingston, S., Berman, W. and Pauli, L.L. (1973). JAMA 224: 1634-1635.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. et al (1951). J. Biol. Chem. 193: 265.
- Lussier-Lazaroff, J. and Fletcher, B.D. (1971). J. Assoc. Can. Radiol. 22: 144-147.
- Madhok, T.C., Schnoes, H.K. and DeLuca, H.F. (1978). Biochem. J. 175: 479-482.
- Matheson, R.T., Herbst, J.J., Jubiz, W. et al (1976). J. Clin. Pharmac. 16: 426.
- Matsuo, N. et al (1972). Pediatr. Res. 6: 400.
- Mazel, P. (1972). In: La Du, B.N., Mandel, H.G. and Way, E.L. (eds): "Fundamentals of Drug Metabolism and Drug Disposition." Williams & Wilkins, Baltimore, p. 546.
- Melsen, F and Mosekilde, L. (1976). Calc. Tiss. Res. 21(5): 247.
- Melsen, F. and Mosekilde, L. (1980). Acta Pathol. Microbiol Scand. 88: 83-88.
- Meunier, P. (1975). Lyon Med. 233: 839-853.
- Meunier, P.J., Edouard, C., Arlot, M. et al (1979). In: MacIntyre, I., Szecke, M. (eds): "Molecular Endocrinology." Elsevier/North Holland Biomedical Press, Amsterdam, pp. 283-292.

- Mosekilde, L., Christensen, M.S., Lund, B. et al (1977). Acta Endocrinol. 84: 559-565.
- Murchison, L.E., Bewsher, P.D., Chesters, M. et al (1975). Postgrad. Med. J. 51: 18-21.
- Nash, T. (1953). Biochem. J. 55: 416.
- Norman, A.W. (1974). Vitam. Horm. 32: 326-384.
- Norman, A.W., Bayless, J.D. and Tsai, H.C. (1976). Biochem. Pharmac. 25: 163.
- Ogden, J.A. (1980). In: Urist, M.R. (ed): "Fundamental and Clinical Bone Physiology." J.B. Lippincott Co., Toronto, pp. 108-171.
- Ornoy, A., Goodwin, D., Noff, D. et al (1978). Nature: 276, 517-519.
- Ornoy, A., Sekeles, E., Cohen, R. et al (1979). In: Norman, A.W., Schaefer, K., Coburn, J.W. et al. (eds.): "Vitamin D, Basic Research and Its Chemical Application". Walter de Gruyter, Berlin and New York, pp. 363-367.
- Peraino, C., Ehret, C.F., Groh, K.R. et al (1980). Proc. Soc. Exp. Biol. Med. 165: 473-479.
- Rasmussen, H. and Bordier, P. (1974). "The Physiological and Cellular Basis of Metabolic Bone Disease." Williams and Wilkins, Baltimore, pp. 211-221.
- Rath, N.C. and Reddi, A.H. (1979). Nature 278: 855-857.
- Ray, A.K. and Rao, D.B. (1974). J. Amer. Geriat. Soc. 22: 222-225.
- Reddi, A.H. and Huggins, C.B. (1972). Proc. Natl. Acad. Sci. 69: 1601-1605.
- Reddi, A.H. and Huggins, C.B. (1975). Proc. Natl. Acad. Sci. 72: 2212-2216.
- Reddi, A.H. and Anderson, W.A. (1976). J. Cell. Biol. 69: 557-572.
- Richens, A. and Rowe, D.J.F. (1970). Brit. Med. J. 4: 73-76.
- Roberts, R.J. and Plaa, G.L. (1966). Biochem. Pharmacol. 15: 333-341.
- Rodbro, P. Christiansen, C. and Lund, M. (1974). Acta. Neurol. Scand. 50: 527-532.
- Schenkman, J.B. and Cinti, D.L. (1972). Life Sci. 11:247.

- Schaefer K., Kraft, D., von Herrath, D. et al (1972). Epilepsia 13: 509-519.
- Schmid, F. (1967). Fortschr. Med. 85: 381-382.
- Schnoes, H.K. and De Luca, H.F. (1980). Fed. Proc. 39:
- Shafer, R.B. and Nuttall, F.Q. (1975). J. Clin. Endocrinol. Metab. 41: 1125-1129.
- Shapiro, F., Holtrop, M.E. and Glincher, M.J. (1977). J. Bone Joint Surg. 59A: 703.
- Shepard, R.M. and De Luca, H.F. (1980). Arch. Biochem. Biophys. 202: 43-53.
- Sherrard, D.J. and Baylink, D.J. (1974). Clin. Endocrinol. 39: 119-135.
- Silver, J., Neale, G., and Thompson, G.R. (1974). Clin. Sci. Mol. Med. 46: 433-448.
- Sotaniemi, E.A., Kakarainen, H.K., Puranem, J.A. et al (1972). Ann. Intern. Med. 77, 389-394.
- Taylor, C.M. (1979). In: Norman, A.W., Schaefer, K., Coburn, J.W. et al (eds): "Vitamin D, Basic Research and its Clinical Application." Walter de Gruyter, Berlin and New York, pp. 197-203.
- Teotia, M. and Teotia, P.S. (1973). Amer. J. Dis. Child 125: 850-852.
- Tolman, K.G., Jubiz, W., Sannella, J.J. et al (1975). Pediatrics 56: 45-51.
- Urist, M.R. (1980). " Fundamental and Clinical Bone Physiology." J.B. Lippincott Company, Philadelphia, pp. 331-368.
- Villanueva, A.R. (1973). In: Jaworski, Z.F.G. (ed): "Proceedings of the First Workshop on Bone Morphometry." University of Ottawa Press, Ottawa, pp. 341-353.
- Villareal, M.E., Chiroff, R.T., Bergstrom, W.H. et al (1978). J. Bone Joint Surg. (Am.) 60: 911-916.
- Villareal, M., Gould, C.V., Wasserman, R.H. et al (1974). Science 183: 671-673.
- Wark, J.D., Larkins, R.G., Perry-Keene, D. (1979). Clin. Endocrinol. 11, 267-274.
- Weisman, Y., Fartal, A., Eisenberg, Z. et al (1979). Br. Med. J. : 521-523.