UR-3490-1468

STUDIES ON THE MECHANISMS UNDERLYING THE TRANSFER OF CALCIUM AND PHOSPHATE FROM BONE TO BLOOD

by

Robert J. Brommage, Jr.

Submitted in Partial Fulfillment
of the
Requirements for the Degree

DOCTOR OF PHILOSOPHY

Supervised by: William F. Neuman

Department of Radiation Biology and Biophysics
School of Medicine and Dentistry
The University of Rochester
Rochester, New York

1978

DISCLAIMER

STUDIES ON THE MECHANISMS UNDERLYING THE TRANSFER OF CALCIUM AND PHOSPHATE FROM BONE TO BLOOD

by

Robert J. Brommage, Jr.

Submitted in Partial Fulfillment
of the
Requirements for the Degree

DOCTOR OF PHILOSOPHY

Supervised by: William F. Neuman

Department of Radiation Biology and Biophysics
School of Medicine and Dentistry
The University of Rochester
Rochester, New York

1978

VITAE

The author was born on [redacted]. Upon graduation from St. Anthony's High School in 1969, he entered Manhattan College and graduated magnum cum laude with a B.S. in Physics in June of 1973. The summers of 1972 and 1973 were spent as a Summer Research Student at Brookhaven National Laboratory. In September, 1973 he entered the Department of Radiation Biology and Biophysics at the University of Rochester and received an M.S. in 1976. Part of one semester was spent as a teaching assistant in an introductory biology course. His thesis project was supervised by Dr. William F. Neuman.

ACKNOWLEDGEMENTS

I would like to acknowledge the assistance of many people who have helped me throughout the course of this work:

Dr. William F. Neuman, my thesis advisor, for providing countless hours of encouragement, advice and thought-provoking ideas;

The members of my thesis committee, Drs. William Au, John Brand, James Coleman and Daniel Kimberg for their numerous helpful suggestions;

Betty Jane Bareham and Dr. Philip Scarpace for assistance during my initial exposure to many biochemical techniques, and Drs. Margaret Neuman, Paula Barrett and Sue Nielsen for expanding my laboratory expertise;

The members of the "Third Floor" for continued support and encouragement;

Dr. Milan Uskoković, whose generous gift of 1,25-$(OH)_2D_3$ made many of the studies in this thesis possible;

Dr. Martha Sommerman for taking over the injections of rats during a week of my absence;

Kevin Campbell, Linda Harris, Gary Norton, Barry Peterson and other members of the Department of Radiation Biology and Biophysics whose discussions helped to expand my scientific interest and background;

Mary Wallman for cheerfully typing the manuscript and preparing the illustrations

My parents, whose support and encouragement made the going easier.

The research was supported by the following: NIH Grant No. AM-17074, NIH Grant No. DE-0175 and the Department of Energy.

ABSTRACT

The skeleton is recognized as a crucial organ in the minute-to-minute regulation of the blood levels of calcium and phosphate. The fluxes of calcium and phosphate to and from bone greatly exceed the entry and exit of these ions occurring in the intestine and kidneys. Parathyroid hormone, calcitonin and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) are known to influence the transfer of calcium and phosphate from bone to blood.

Three mechanisms have been proposed to explain the hormonal control of the calcium and phosphate effluxes from bone. The concept of a "bone membrane" maintaining a distinct bone extracellular fluid composition has led to the "pump" and "pH gradient" theories. Under these proposals, calcium and phosphate are either actively transported from bone to blood or dissolved from the bone mineral crystal surface by the action of a decreased pH in the bone extracellular fluid. An alternate "solubilizer" theory proposes that bone cells secrete a substance which increases the solubility of the bone mineral.

The "bone membrane" concept was originally proposed to explain the presence of the apparent anomalously high concentrations of potassium in the bone extracellular fluid. These elevated potassium levels were found to result, in part, from the zeta potential-induced concentration of polarizable ions. Non-vital chick calvaria were shown to concentrate the sodium and potassium in bone water by a factor of approximately two

over the buffer levels of these ions. At the present time, estimates of the volume of skeletal water occupied by cells are sufficiently imprecise to preclude the proper assignment of skeletal potassium between cellular and extracellular compartments. Thus, the available evidence does not allow an unambiguous decision concerning the presence of a bone membrane.

Incubated calvaria from neonatal mice previously injected with 1,25-$(OH)_2D_3$ were used to explore the mechanism of action of this hormone. Calvarial lactate production was unaltered by 1,25-$(OH)_2D_3$ treatment and consequently 1,25-$(OH)_2D_3$ does not appear to promote the mobilization of bone mineral through a lactate-mediated "pH gradient" mechanism. 1,25-$(OH)_2D_3$ did increase the solubility of non-vital bone, clearly demonstrating that the "solubilizer" mechanism is at least partially responsible for the mobilization of bone mineral and the regulation of blood levels of calcium and phosphate.

Studies were undertaken to develop a system of vitamin D-deficient bone suitable for in vitro examinations of the skeletal actions of various vitamin D metabolites. Vitamin D-deficient female rats fed a 0.2% calcium, 0.4% phosphorous diet and supplemented with daily injections of 0.75 pmole of 1,25-$(OH)_2D_3$ were shown to be capable of bearing young. When the injections of 1,25-$(OH)_2D_3$ were terminated at delivery, the dams and pups showed signs of vitamin D deficiency approximately one week later. Vitamin D-deficient rat pups were also produced by placing normal impregnated rats on a vitamin D-deficient diet soon after mating.

TABLE OF CONTENTS

LIST OF TABLES

page

CHAPTER FIVE: An Investigation of the Requirements for Vitamin D in the Maternal and Neonatal Rat.

LIST OF FIGURES

INTRODUCTION

The skeleton functions both as an architectural support structure and as a storage depot involved in electrolyte homeostasis. In many respects, a calcium phosphate mineral phase interwoven among collagen fibers is a system ideally suited to carry out these two functions. Deposition of a collagen matrix at the proper locations determines the unique three-dimensional shape of each bone while bone strength results from the non-compressibility of the mineral phase. The exceedingly small size of the hydroxyapatite crystals, by permitting a reasonable degree of diffusion and providing a large surface area, allows exchange of mineral components with the ions of the general extracellular fluids.

Bone growth and shape are influenced by a combination of local and systemic factors. At the tissue level, pressure appears to play a major role in determining bone growth. Moss and his colleagues have concluded that under normal conditions, physical pressure applied to the craniofacial bones by the expanding neural mass is the dominant factor in the growth of these bones. Osseous deposition and resorption ("transformative" growth), although important in endochondral calcification, are not involved in determining the final position and shape of the calvarium. The skeletal atrophy observed in disuse osteoporosis also attests to the importance of local forces in maintaining the integrity of the skeleton.

For proper skeletal growth, humoral agents are also required. Bone cells must obviously be provided with the nutrients necessary for their proper functioning and these nutrients can only arrive with the assistance

of an adequate circulatory system. Somatomedin has dramatic stimulatory effects on cartilage development, and an impure preparation of somatomedin C has been shown to triple the synthetic rate of collagen in fetal rat calvaria. At the present time, a skeletal factor analogous to the nerve and epidermal growth factors has not been found. The anabolic actions of both parathyroid hormone and vitamin D on the skeleton may result entirely from primary effects on blood electrolytes.

Skeletal remodeling occurs throughout the lifetime of an animal. Even after bone growth has ceased, random osteoclastic resorption of both mineral and organic matrix spontaneously erupts. Following this osteoclastic activity, new bone formation replaces the previously removed bone. Under normal conditions, resorption and formation are in balance and the total skeletal mass remains constant. However, in osteoporosis, new bone formation does not keep up with resorption and the loss of bone, particularly in the vertebrae, greatly decreases the overall load-carrying capacity of the skeleton.

Proper growth, development, shape and remodeling of bone are important for the architectural role of the skeleton in providing both structural support for locomotion and protection of soft tissue. The continual maintenance of these physical aspects of bone structure has been termed skeletal homeostasis.

The skeleton is also involved in mineral homeostasis, that is, the maintenance of a constant "milieu interieur" throughout the general extracellular fluids. Bone, with huge reserves of calcium and phosphate, can

release these ions to the circulation in times of low dietary intake or excessive output, such as that occurring during lactation. In addition to storing calcium and phosphate, bone also contains large quantities of magnesium and sodium and acts to buffer changes in blood pH.

Bone is one of the main organs involved in the regulation of blood levels of calcium and phosphate. Not surprisingly, the three hormones known to be primarily involved in calcium and phosphate homeostasis, parathyroid hormone, calcitonin and 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$], all act upon the skeleton. Parathyroid hormone and 1,25-$(OH)_2D_3$ promote the net transfer of calcium and phosphate from bone to blood while calcitonin decreases the efflux of these ions from bone.

The intestine and kidneys also play important roles in regulating blood levels of calcium and phosphate. Active transport of calcium and phosphate by the gut does not occur in the absence of vitamin D and 1,25-$(OH)_2D_3$ is believed to be the active metabolite of vitamin D_3 responsible for initiating this active transport process. Parathyroid hormone acts on the kidneys to increase the tubular reabsorption of calcium while reducing the reabsorption of phosphate.

Although any attempt to describe the integration of the homeostatic regulatory mechanisms for calcium and phosphate will undoubtedly be incomplete, DeLuca has proposed a relatively simple hypothesis that provides a reasonable framework for further studies. This concept is best understood by considering the responses invoked by hypocalcemia and hypophosphatemia:

A) The increased secretion of parathyroid hormone brought about by hypocalcemia results in:

   i) mobilization of calcium and phosphate from bone mineral,

   ii) increased renal synthesis of $1,25\text{-}(OH)_2D_3$ and the consequent increase in the absorption of calcium and phosphate in the gut,

   iii) increased renal reabsorption of calcium with a concurrent increased secretion of phosphate.

B) The increased synthesis of $1,25\text{-}(OH)_2D_3$ brought about by hypophosphatemia results in:

   i) mobilization of calcium and phosphate from bone mineral,

   ii) increased absorption of calcium and phosphate in the gut.

The key difference between events following hypocalcemia and hypophosphatemia is the renal actions of parathyroid hormone. The enhanced secretion of parathyroid hormone brought about by hypocalcemia removes the tendency towards hyperphosphatemia resulting from the entry of phosphate into blood from bone and intestine by inducing a renal diuresis of this anion. In contrast, since hypophosphatemia does not result in enhanced parathyroid hormone secretion, phosphate is conserved by the kidneys while most of the increased filtered load of calcium is excreted.

There exists almost universal agreement that, among their other actions, parathyroid hormone and $1,25\text{-}(OH)_2D_3$ promote the release of calcium, phosphate and organic matrix from the skeleton while the skeletal actions of calcitonin oppose those of parathyroid hormone and $1,25\text{-}(OH)_2D_3$.

The major purpose behind the experiments carried out as a part of this thesis is the elucidation of the mechanism(s) behind the skeletal actions of these hormones.

The mechanism(s) by which a hormone found at concentrations on the order of 10^{-10} moles/liter can effect the removal of up to gram quantities of bone mineral and matrix is expected to be a multi-step process. It is almost axiomatic that skeletal-active hormones do not act directly upon the extracellular mineral and matrix but invoke a series of specific responses in bone cells which eventually alter extracellular events. Cellular events thought to be involved in the actions of parathyroid hormone, $1,25\text{-}(OH)_2D_3$ and calcitonin include:

1) hormone receptor activation,

2) plasma and mitochondrial membrane ion fluxes,

3) cyclic AMP induced protein phosphorylation,

4) intermediary metabolism of carbohydrates,

5) de novo protein synthesis,

6) release of lysozomal enzymes, and

7) differentiation of precursor cells.

Since the end-result of the actions of parathyroid hormone, $1,25\text{-}(OH)_2D_3$ and calcitonin is a change in the extracellular status of bone, the experiments performed as a part of this thesis were designed with particular attention to extracellular events. Although the organic matrix of bone is

undoubtedly important in skeletal homeostasis, the studies which follow are principally involved with mineral homeostasis and consequently with the mineral phase of bone. In effect, these studies are from the "point of view" of the bone mineral; i.e., if literary license may be taken, what does a calcium or phosphate ion located on the surface of a crystal of bone mineral "see" when the hormonal messenger 1,25-$(OH)_2D_3$ "directs" bone cells to mobilize bone mineral?

The experimental attack on the mechanism(s) involved in the skeletal aspects of mineral homeostasis requires an integration of several approaches. The results of studies on whole animals, perfused bone, incubated bone fragments, isolated cells and sub-cellular systems must be put together to form coherent, physiological explanations. No one technique has more inherent power than another. However, the more "physiologically relevant" approaches, although highly desirable when knowledge of which responses actually take place is needed, are often vague and non-discriminatory when probing the mechanism(s) behind these responses.

While the importance and potential power of the techniques involving isolated bone cells and the preparation of sub-cellular fractions are recognized, studies on intact bone have considerable merit and make up a sizeable fraction of the experiments described in this thesis. Chapters 2, 3, 4 and part of 5 involve in vitro incubations of intact calvaria. Although the majority of Chapter 5 is concerned with chronic animal studies, the development of vitamin D-deficient bone suitable for in vitro experiments provided the impetus behind that investigation.

The initial experiments were performed with the intent of extending the "bone membrane" concept developed to explain the seemingly aberrant concentrations of potassium in bone water. A review of the bone potassium-bone membrane literature is given in Chapter 1. As these experiments progressed, the observed blood/bone extracellular fluid potassium gradient did not appear to be large and, due to errors in determining the cellular potassium content, this gradient might be found to be non-existent.

Since one of the hypotheses arising from the bone membrane concept involved the presence of a blood/bone extracellular fluid pH gradient, experiments were undertaken to determine the bone water pH and the influence of 1,25-$(OH)_2D_3$ upon bone lactic acid production. In unreported experiments utilizing the distribution of the weak acid DMO [5,5-Dimethyl-2,4-oxazolidinedione], no evidence was found to support the proposals that the pH of the bone extracellular fluid differs from that of blood. The data presented in Chapter 4 clearly demonstrate that 1,25-$(OH)_2D_3$ can mobilize bone mineral without altering bone lactate production. The lack of favorable evidence in support of a blood/bone pH gradient coupled with increasing amounts of data indicating the bone membrane is rather permeable, the decrease in the experimental blood/bone potassium gradient and the failure of the bone membrane concept to provide further insights into the mechanisms underlying the skeletal aspects of mineral homeostasis, has led to a decline in enthusiasm for the importance of a bone membrane.

While the bone membrane concept was falling into disfavor, the experiments reported in Chapter 4 gave additional support to work of the

1960's in which the solubility of bone mineral taken from non-vital bone was found to be under hormonal control. The concept of a bone mineral "solubilizer" has been further developed and brought up to date in Chapter 4. Further work is required to determine whether this theory can fully explain the actions of the skeletal-active hormones on the movement of calcium and phosphate between bone and blood.

The concepts developed in this Introduction were obtained from numerous sources. Many references to particular experiments can be found in the five chapters which follow. The somatomedin experiment was performed by E.M. Canalis, R.L. Hintz, J.W. Dietrich, D.M. Marina and L.G. Raisz and appeared under the title, "Effect of Somatomedin and Growth Hormone on Bone Collagen Synthesis In Vitro" in Metabolism 26, 1079-1087 (1977). Excellent review articles on the material presented in this section include:

DeLuca, H.F.: Vitamin D: The vitamin and the hormone. Fed. Proc. 33, 2211-2219 (1974).

DeLuca, H.F. and H.K. Schnoes: Metabolism and mechanism of action of vitamin D. Ann. Rev. Biochem. 45, 631-666 (1976).

Moss, M.L., M.-A. Meehan and L. Salentijn: Transformative and translative growth processes in neurocranial development of the rat. Acta Anat. 81, 161-182 (1972).

Neuman, W.F.: The mileau interieur of bone. Claude Bernard revisited. Fed. Proc. 28, 1846-1850 (1969).

Parfitt, A.M.: The actions of parathyriod hormone on bone: Relation to bone remodeling and turnover, calcium hoemostasis, and metabolic bone disease. Parts I, II, III, IV. Metabolism 25, 809-844, 909-955, 1033-1069, 1157-1188 (1976).

Potts, J.T., Jr. and L.F. Deftos: Parathyroid hormone, calcitonin, vitamin D, bone and bone mineral metabolism. In, Duncan's Diseases of Metabolism (Endocrinology), 7th edition; ed. by P.K. Bondy and L.E. Rosenberg. W.B. Saunders Co., Philadelphia, pp. 1225-1430, 1974.

Raisz, L.G.: Bone metabolism and calcium regulation. In, Metabolic Bone Disease, vol. I, ed. by L.V. Avioli and S.M. Krane. Academic Press, Inc., New York, pp. 1-48, 1977.

Rasmussen, H. and P. Bordier: The Physiological and Cellular Basis of Metabolic Bone Disease. The Williams & Wilkins Co., Baltimore, 1974.

CHAPTER ONE

THE STATUS OF POTASSIUM IN BONE

Studies on the role of the skeleton in buffering the blood levels of pH, sodium and potassium provided the initial impetus for measuring potassium levels in bone (2,3,4,8,14,45,46,47,65,66). The prevalent thinking of the time held that most of the skeletal potassium could not be ionic and therefore bone potassium levels were typically expressed in terms of dry weight. In 1955 Bergstrom (2) concluded that bone potassium values were too high to "be reasonably assigned to the tissue fluids present" and that potassium was "probably associated with (hydroxyapatite crystal) surface carbonate". In their 1959 review of the literature, Edelman and Leibman (17) calculated that when skeletal potassium was expressed in terms of bone water, potassium concentrations were 90 mM or greater and predicted that the "bulk" of bone potassium was nonexchangeable and associated with the mineral phase.

Subsequent studies have shown that the vast majority of potassium in bone is exchangeable over a time course of approximately one hour. Norman, studying bone potassium under a variety of physiological states (45,46,47), first demonstrated that skeletal potassium was nearly completely exchangeable. Complete exchange of skeletal potassium was also found by Triffitt et al. (65) in rat tibia and Scarpace and Neuman (59) in embryonic chick calvaria. In a follow-up to the study of Triffitt et al. (65), Neuman et al. (40) found that only 80% of the potassium in rat tibia exchanged within two hours and the remaining 20% of the potassium exchanged much more slowly. In contrast to potassium, chloride exchange

was complete in slightly over 10 minutes (40). In studies as a part of this investigation, potassium in the rat pup calvarium appeared to be fully exchangeable (Chapter 3) while typically a few per cent of the total potassium in young chick calvaria was nonexchangeable (Chapter 2).

The limited studies undertaken with synthetic hydroxyapatite fully support the data on potassium exchange in bone mineral. Neuman et al. (39) showed that when potassium was present during the precipitation of hydroxyapatite from solutions of calcium and phosphate at neutral pH, 93 to 94% of the potassium associated with these crystals and their hydration water exchanged within one hour. Pak and Bartter (51) showed that increasing the solution potassium concentration increased the amount of exchangeable calcium in the hydroxyapatite hydration water and concluded that small quantities of potassium can substitute for calcium on the hydroxyapatite crystal surface. Such a substitution would make the net charge on the crystal surface more negative and thereby promote additional calcium "counter-ion" accumulation in the hydration water as a result of charge neutralization. To further support their claims of potassium binding by hydroxyapatite, Pak and Bartter (51) studied the release of ^{42}K from hydroxyapatite prelabeled with the isotope for 24 to 65 hours and found that a small amount of potassium was displaced from the crystals only after several hours of incubation.

The only study indicating the presence of large quantities of non-exchangeable skeletal potassium is that of Hartsuck et al. (22). These investigators found that roughly one-half of the potassium in dog and

human bone was nonexchangeable but apparently worked with extremely low count rates at times relatively long compared to the 12.4 hour half-life of ^{42}K. Lacking any confirmation of this data, the evidence presently available must be interpreted to support the view that skeletal potassium is almost completely exchangeable.

Since the exchange studies have shown that roughly 90% or more of the potassium in bone is rapidly exchangeable and therefore, presumably ionic, some explanation is necessary to account for the relatively high concentrations of potassium in bone water. This point was first raised by Triffitt et al. (65) in 1968 and led to the studies of Canas et al. (7) on the levels of skeletal potassium during growth, potassium deficiency, hypophysectomy and vitamin D-deficiency. The conclusion drawn (7) was that a "bone membrane" separated the bone extracellular fluid from the general extracellular fluids and that the bone cells enveloping the mineral were responsible for maintaining a potassium gradient between these two fluid compartments.

Although the concept of a separate extracellular fluid compartment in the skeleton may appear unique, several other tissues are known to contain a highly specialized and individual extracellular fluid. The endolymph of the inner ear has high levels of potassium but low concentrations of sodium, calcium and magnesium (5,12). Carbonic anhydrase appears to play a role in the maintenance of this potassium gradient (19). Avian uterine fluids have high concentrations of potassium and reciprocally low concentrations of sodium during the process of egg shell formation (10,18). The concentrations of potassium in the extracellular

fluid in the brain and the cerebral spinal fluid have been known for many years to be regulated independently of blood potassium levels. Recent microelectrode experiments have confirmed earlier estimates of 3 mM potassium in the brain extracellular fluid (29). The concept of a blood-testis barrier is receiving recognition as the result of ongoing histological studies (57).

Obviously, the concept of a separate bone fluid compartment where composition is regulated by bone cells has numerous implications throughout skeletal physiology. Indeed, Talmage (64), Ramp (54) and Robertson (56) have all proposed mechanisms involving the cellular pumping of calcium from bone to blood in order to explain the actions of parathyroid hormone, vitamin D and calcitonin on the skeleton. However, at the present time there is no direct evidence that an electrochemical potential exists against which calcium must be pumped. There have also been several proposals of a pH gradient between blood and bone extracellular fluid (36,42,60), but again, no direct evidence for such a gradient has been presented. Potassium is the only substance for which there exists any evidence suggesting an active blood/bone extracellular fluid concentration gradient.

Implicit in the initial discussions of the bone membrane was the concept that this membrane provided some type of barrier to restrict the flow of substances to and from the skeleton. To maintain a potassium gradient by a "pump-leak" mechanism such as the one proposed by Scarpace and Neuman (59), bone cells must pump potassium into the bone extracellular fluid as fast as potassium is effluxing out of bone. Naturally, the

greater the potassium "leak", the greater the amount of energy that must be expended by bone cells to maintain the potassium gradient. This membrane barrier is expected to be present at the endosteal and periosteal cells lining bone and at the capillary endothelial cells (41).

Morphological studies have attempted to provide insight into the actual structural features of the bone membrane. Findings such as "The surfaces of all bone tissue examined were covered by cells" by Dudley and Spiro (16) in 1961 have been extended by the groups of Talmage and Matthews to the concept of "bone lining cells". These cells, supposedly osteoblasts, appear to form a "functional syncytium" involving gap junctions with osteocytes and been shown to contract following calcitonin treatment and form "blebs" in response to parathyroid hormone (13). There have been claims both for (1,34,67,69) and against (37,55) the presence of tight junctions between these bone lining cells.

Another approach to the study of the bone membrane is the Ussing chamber investigations of Neuman and associates (43). These studies are designed to measure the fluxes of ions through the periosteum of calvaria under various treatments. The present interpretations of these experiments indicate that this bone membrane is rather permeable and nonselective.

Studies on the permeability of bone capillaries have also been undertaken. Kelly and associates have demonstrated that the rates of sucrose and strontium efflux from the tibial nutrient artery circulation in the dog are in the proportion expected from their aqueous diffusion constants and concluded that both of these substances transversed the

capillaries into the bone interstitial fluid by free diffusion (11). Studies of a similar nature using different sized molecules (30) also led to the same conclusion, namely, there does not appear to be a specific capillary "pore" which discriminates against molecules on the basis of molecular size. Although lanthanum was shown to inhibit the skeletal uptake of calcium from blood (53), no mention was made of the strong tendency of lanthanum to polymerize by a process of olation. A colloidal lanthanum hydroxide aggregate could conceivably block large openings and slow down the transfer of all substances from the capillaries. Following an intravenous injection, lead localizes at the surfaces of bone mineral within 30 seconds (28).

The penetration of proteins from blood into bone has provided additional information on the permeability of bone capillaries. Horseradish peroxidase (MW = 40,000), albumin (MW = 69,000) and the synthetic polymer polyvinyl-pyrrolidone (MW = 35,000) all appear to have little difficulty in entering the bone extracellular fluid (15,49,50). Again, the available data do not indicate the presence of a membrane barrier between blood and the bone extracellular fluid.

The apparently high concentration of potassium in the bone extracellular fluid is difficult to reconcile with the lack of any well-defined barrier restricting the flow of substances through the interfaces separating the bone extracellular fluid from the general extracellular fluids. Although Howard (25) first proposed the existence of a bone membrane from general principles, the finding of Triffitt et al. (65) that the total skeletal

potassium is much higher than expected led to the resurrection of the bone membrane concept and still provides the only direct evidence for the compartmentalization of extracellular fluids by a bone membrane.

Since the "role" of the elevated levels of potassium in bone has not been established, there are no clues associating the bone membrane with any physiological "function". Some metabolic process in bone might be influenced by the concentration of potassium or, possibly potassium might be a counter-ion for the transport of some other substance out of the bone extracellular fluid. Presumably, by searching the literature and recording any values of bone potassium that are given with a simultaneous value of bone water, a review of the effects of various physiological treatments on the concentration of bone potassium can be obtained. In practice however, this approach is not so straightforward since the techniques used to remove marrow and other soft tissue from the bone can have a profound influence upon the measured levels of skeletal potassium.

An experiment was undertaken in an attempt to better understand the effects of sample handling on the values obtained for bone potassium concentrations. Seven rat pups from the same litter were sacrificed at four days of age and their calvaria dissected out. Four calvaria were carefully and firmly wiped with tissue paper before taking a wet weight while three calvaria were weighed immediately after dissection. Following an overnight drying at 110° C, all seven calvaria were reweighed, extracted overnight in 2 N HNO_3 and then the bone extracts were analyzed for sodium and potassium by flame photometry. The sodium, potassium and water contents of these calvaria after each treatment are given in Table 1.

TABLE 1

The Effect of "Wiping" on the Calvarial Contents of Water, Sodium and Potassium

	"Wiped"	"Not Wiped"	Ratio (Wiped/Not Wiped)
Water (% wet weight)	64.5 ±1.7	75.5 ±0.8	0.85
Water ($\frac{\text{water weight}}{\text{dry weight}}$)	1.84 ±0.15	3.09 ±0.14	0.60
Sodium (μmoles/100 mg dry wt.)	26.5 ±1.7	38.0 ±0.9	0.70
Potassium (μmoles/100 mg dry wt.)	14.7 ±0.6	17.8 ±0.6	0.82
Potassium (mmoles/liter water)	80.7 ±2.9	57.8 ±1.3	1.40
Sodium/Potassium	1.80 ±0.06	2.13 ±0.05	0.85

These calvaria came from a litter of rat pups sacrificed at 4 days of age. Three calvaria were "not wiped" while 4 calvaria were "wiped". All data are expressed as the mean ± SEM except for the "wiped/not wiped" ratios which are simply the ratios of the means.

Naturally, wiped calvaria had a markedly reduced water content. Assuming that only minimal quantities of dry matter were removed by the wiping procedure, relative to non-wiped calvaria, wiped calvaria lost 40% of their water content and 30% of their sodium content but only 18% of their potassium content. The combined effect of these changes due to wiping resulted in a 40% increase in bone potassium, when expressed in terms of bone water. Undoubtedly, the wiping procedure removed considerable quantities of non-skeletal extracellular fluid having a high sodium to potassium ratio but the amount of bone cellular and extracellular water and electrolytes removed is uncertain.

This experiment demonstrates one of the basic experimental problems of bone potassium analyses--"To wipe or not to wipe". Some wiping appears to be necessary but how much is not clear. At what point do "scrupulously cleaned" bones (65) become dried out samples with falsely elevated potassium levels due to the loss of water through evaporation? The option of not wiping or using only minimal pressure in wiping exists for immature calvaria but the diaphysial shafts of long bones must be heavily wiped to remove muscle adjacent to the periosteum and the marrow adjacent to the endosteum. In reporting values of bone potassium, a simultaneous mention of the skeletal water content is a great help when comparisons are made between different studies. For example, in the study of Geisler and Neuman (21) on rat pup calvaria, the reported water contents averaged 19.7 $\pm$0.4% of the wet weight, a value far below the 60 to 75% values obtained in the experiment reported in Table 1.

Despite these experimental uncertainties and the expected variations among different laboratories in determining the concentration of potassium in bone water, these problems are presumably minimized in a given set of experiments through the use of well-chosen controls. Table 2 contains a list of the effects of various physiological treatments on the levels of bone potassium described in the literature. In some of these investigations, potassium was expressed in terms of bone wet or dry weight but in each instance no change in the skeletal water content was observed or expected.

Parathyroid hormone injection had no effect upon rat femoral potassium levels in the study of Johnston et al. (27) but these authors did not present any data to substantiate their claims. The calcitonin experiment will be described below while the experiment involving hypophysectomy and one of the experiments involving vitamin D-deficiency were performed by Canas et al. (7) and alluded to earlier. Wuthier (72) has also presented data showing that vitamin D deficiency lowers the concentration of potassium in bone water and calculations show that this effect was much more pronounced at two weeks of age than four weeks. Adrenalectomy has been reported to decrease (14) and have no effect (4,47) upon skeletal potassium levels. Thyrotoxicosis increased bone potassium expressed in terms of dry weight (26) while the numerous hormonal changes accompanying pregnancy were without effect on bone potassium levels (32). Skeletal potassium was unaltered in acute hypercalcemia (66), acute hyponatremia (71), starvation (33) and during alterations in the levels of dietary sodium (32). Potassium

TABLE 2

The Effect of Various Physiological Factors on Bone Potassium

Physiological Factor	Effect	Reference
Parathyroid hormone	none	Johnston et al. (27)
Calcitonin	none	this report
Vitamin D deficiency	decrease	Canas et al. (7)
Vitamin D deficiency	decrease	Wuthier (72)
Hypophysectomy	decrease	Canas et al. (7)
Adrenalectomy	none	Borle et al. (4)
Adrenalectomy	none	Norman (47)
Adrenalectomy	decrease	Dosekun (14)
Thyrotoxicosis	increase	Humphrey and Heaton (26)
Acute hypercalcemia	none	Wallach et al. (66)
Pregnancy	none	Kirksey et al. (32)
Starvation	none	Komarkova et al. (33)
Acute hyponatremia	none	Woodbury (71)
Dietary sodium changes	none	Kirksey et al. (32)
Potassium deficiency	decrease	Canas et al. (7)
Acute hypercapnia	increase	Clancy and Brown (8)
Chronic hypercapnia	decrease	Willbanks and Seldin (70)
Chronic hypercapnia	increase	Claudon et al. (9)
Acute acidosis	decrease	Bergstrom and Wallace (3)
Chronic acidosis	none	Norman (46)
Chronic acidosis	decrease	Willbanks and Seldin (70)
Chronic alkalosis	decrease	Willbanks and Seldin (70)

deficiency did lower levels of bone potassium and although this decrease was less than that observed in serum or muscle, expressing the data in terms of water weight rather than dry weight would probably bring the decreases in muscle and bone potassium into much closer agreement (7). Although several studies on the effects of respiratory and metabolic acid-base imbalances on skeletal potassium have been performed (3,8,9,70), no unambiguous trends have been observed. Further details on the experimental methodologies for all of these studies can be obtained by consulting the original literature.

The effect of calcitonin on the concentration of potassium in bone water was determined in an acute experiment done in collaboration with Dr. Phil Scarpace. Ten 21-day-old rats were divided into two equal groups with one group receiving 20 mU/kg of salmon calcitonin and the other group receiving an injection of the vehicle. At one hour following injection, calcitonin treatment lowered serum calcium from 9.1 $\pm$0.3 to 7.0 $\pm$0.2 mg.% (means $\pm$ SEM, n = 5, in each case). Calvarial potassium was determined by the usual analytical procedures described elsewhere. Control calvaria had a potassium level of 82.7 $\pm$3.5 mM potassium (n = 4) while calvaria from the calcitonin-treated rats had 79.3 $\pm$2.9 mM potassium (n = 5). Water contents for these calvaria were 45.8% of the wet weight (n = 9). All data are given as means $\pm$ SEM. Calcitonin was concluded to have no acute effect on skeletal potassium concentration.

As observed in 1963 by Norman (46), "The constancy of bone potassium concentration is striking". Most treatments had little, if any, effect

on the levels of skeletal potassium. Care must be exercised in the analysis of chronic experiments such as those involving vitamin D deficiency. Small changes in the levels of total bone potassium might reflect changes in the nonexchangeable pool of potassium or in the fraction of the total skeletal water occupied by cells and not changes in the concentration of potassium in the bone extracellular fluid. In addition, as described more thoroughly in Chapter 2, increased skeletal mineralization presumably increases the bone mineral concentrative factor for potassium. Hypophysectomy caused the most dramatic change in the levels of bone potassium (7) but unfortunately, potassium was expressed in terms of dry weight rather than water weight.

In the late 1960's Neuman and associates presented data putatively demonstrating decreasing concentrations of skeletal potassium, expressed in terms of bone water, with age (7,65). However, additional experiments undertaken to extend these earlier findings as a part of this thesis have not supported the view that bone potassium falls with age and upon a more detailed analysis of the initial literature, several problems were uncovered. Although Triffitt et al. (65) showed that tibial potassium was completely exchangeable in 90 minutes and almost doubled in tibia from 80-100 gram rats compared to 400-500 gram rats, no effort was made to determine the statistical significance of this finding. Moreover, in a repeat of this experiment by the same workers (40), only 80% of the tibial potassium in 200 gram rats was exchangeable within two hours.

The other work of this group claiming to show a loss of bone potassium with increasing age is that of Canas et al. (7). The observed potassium concentration in bone water was 302 mM in tibia from 50 gram rats but declined to approximately 130 mM in rats weighing 100, 175 and 300 grams. Similarly, cortical bone taken from seven-day-old chicks had a potassium concentration greater than 200 mM while corresponding bone from chicks at 0, 14, 19 and 27 days of age had potassium concentrations less than 100 mM. If the data on the 50 gram rats and seven-day-old chicks were excluded, the remaining data would make a strong case for the dissociation of skeletal potassium levels with age. The seven-day-old chick data is particularly bothersome since the bone potassium concentrations before and after this age were not elevated. No comments were made on the apparent hyperosmolality of the bone extracellular fluid when the potassium concentration rose above 150 mM.

Without a doubt, bone potassium expressed in terms of dry weight falls with age as increasing quantities of bone mineral "replace" the water content of the skeleton. The question raised concerns the concentration of potassium expressed in terms of bone water and, in particular, the concentration of potassium in the bone extracellular fluid. During the course of this investigation several studies involving chicks of various ages were undertaken and bone potassium concentrations were not found to decline with age. Table 3 shows data obtained on young chick calvaria and indicates that calvarial potassium may actually increase with age. A similar increase in skeletal potassium with age was found

TABLE 3

Water Content and Potassium Concentration of Chick Calvaria as a Function of Age

Age (days)	1	2	4	8
H_2O (% wet weight)	49.0 ±1.8	46.6 ±0.9	40.3 ±0.8	35.3 ± 1.1
K (mM)	57.3 ±3.2	63.6 ±2.1	72.0 ±2.9	75.4 ±2.8
K (μmoles/100 mg dry wt.)	5.52 ±0.39	5.54 ±0.15	4.84 ±0.06	4.08 ±0.08

Each value is the mean ± SEM for 8 "punch-outs" (4 calvaria).

in metatarsals taken from 4- and 11-day-old chicks while calvarial potassium remained constant (Table 4). A third study, described in Table 5, also indicated that chick metatarsal potassium concentrations have a tendency to rise with increasing age while calvarial potassium concentrations were fairly constant. Additional data on calvaria from the rat (Chapter 3) and mouse (not reported) did not give any indication that bone potassium concentrations decrease with age.

In contrast to the work of Canas et al. (7) and Triffitt et al. (65), the evidence obtained during the course of this investigation does not support the view that the concentration of potassium in bone water decreases with age. Bursaux et al. (6), in their studies on rat femur, also found that bone potassium concentration did not change with age. These findings suggest that the attempts to link bone potassium levels with some function associated with skeletal growth and mineralization must be viewed with skepticism.

At the present time there is no simple explanation for the varied and occasionally contradictory observations made about bone potassium. The question of why bone cells would go through the energy expenditure required to pump potassium into the bone extracellular fluid remains unanswered. The apparent non-responsiveness of bone potassium levels to acute parathyroid hormone and calcitonin treatment and the relatively minor changes observed in vitamin D deficiency are difficult to reconcile with any proposal to link bone potassium levels with the role of the skeleton in the minute-to-minute regulation of blood levels of calcium and phosphate.

TABLE 4

Chick Calvarial and Metatarsal Contents of Water, Sodium and Potassium

Bone	Age (days)	Water (% wet wt.)	Sodium (μmole/100 mg dry weight)	Potassium (μmole/100 mg dry weight)	Potassium (mM)
Calvaria	4	41.2 ±0.8	20.2 ±0.1	4.1 ±0.1	57.8 ±1.2
Calvaria	11	32.8 ±1.4	20.6 ±0.2	3.1 ±0.1	62.9 ±2.9
Metatarsal	4	36.8 ±1.8	19.6 ±0.5	4.5 ±0.2	78.2 ±5.1
Metatarsal	11	25.8 ±0.6	19.3 ±0.2	3.3 ±0.1	93.8 ±1.7

Each value is the mean ± SEM for six samples. Both calvaria and metatarsals were "wiped" before the start of the analysis. Calvarial samples were larger than the "punch-outs" described elsewhere.

TABLE 5

Chick Bone Sodium and Potassium Contents as a Function of Age

Bone	N	Age (days)	Water (% wet wt.)	Sodium (μmole/100 mg dry wt.)	Potassium (μmole/100 mg dry wt.)	Potassium (mM)
Calvaria	4	6	36.9 ±0.8	21.7 ±0.1	4.2 ±0.1	71.2 ±3.4
Calvaria	5	10	32.5 ±1.3	20.6 ±0.5	3.6 ±0.2	74.6 ±2.3
Calvaria	5	14	39.1 ±1.0	21.1 ±0.3	4.1 ±0.1	64.3 ±1.7
Calvaria	5	16	30.2 ±2.7	20.1 ±0.3	3.2 ±0.4	73.1 ±1.7
Metatarsal	4	6	28.1 ±0.6	17.9 ±0.1	3.7 ±0.1	94.4 ±2.6
Metatarsal	4	10	24.3 ±0.7	18.0 ±0.2	3.3 ±0.1	100.0 ±2.2
Metatarsal	5	14	28.3 ±0.6	18.5 ±0.2	3.4 ±0.1	84.9 ±3.1
Metatarsal	5	16	29.6 ±1.3	18.2 ±0.5	3.6 ±0.2	84.7 ±2.8

The mean body weights of the chicks at 6, 10, 14 and 16 days of age were 49, 72, 100 and 117 grams, respectively. Each value is the mean ± SEM and N refers to the number of samples. Both calvaria and metatarsals were "wiped" before the start of the analysis. Calvarial samples were larger than the "punch-outs" described elsewhere.

The experimental procedure for a "state of the art" analysis of bone potassium concentration simply involves a simultaneous determination of potassium and water on a suitably prepared bone sample. After correcting for the nonexchangeable potassium, the concentration of potassium in the bone extracellular fluid is estimated by making assumptions on the concentration of potassium in the cellular water and the fraction of the total bone water present in cells. The validity of the values presently used for bone cell potassium concentration and the fractional water volume occupied by cells is discussed more thoroughly in Chapter 2. No direct determination of the concentration of potassium in bone extracellular fluid has ever been made.

While an indirect estimation of the skeletal extracellular fluid concentration of potassium is the procedure currently used, progress towards a more direct approach would remove much of the ambiguity associated with the presently required assumptions concerning the contribution of the bone cells to the total bone potassium content. Unfortunately, many of the techniques that have been successfully applied to soft tissue are much more difficult to use on bone.

The first approach that comes to mind in discussions of the measurement of potassium concentration involves the use of the rapidly improving microelectrode technology. Potassium microelectrodes can be made with tip diameters of approximately one micron and when used properly, these electrodes have a rapid, Nernstian response. However, all of the current designs employ glass tips that presumably would shatter upon impinging on

the calcified matrix of bone. Although solid pH electrodes have been made from antimony (63) and iridium (52), no such analogous system appears adaptable to potassium analyses.

The electron microprobe is another potentially sensitive technique that might help to clarify some of the confusion concerning bone potassium (35). The elemental composition of the mineralization front in tibia (68) and predentine (44) has been studied with the electron microprobe, but the content of potassium was not analyzed. Preliminary electron microprobe data obtained by Dr. J. Coleman[1] indicated that the concentration of potassium was constant throughout the embryonic chick calvarium. Although the major problem associated with this approach is relocation of potassium during sample dehydration, the recent development of the proton microprobe (24) eliminates this problem since dehydration of the sample is not necessary.

Since the exchange of skeletal potassium is rapid, autoradiography of ^{42}K should be capable of providing information on the distribution of potassium in the skeleton. However, as with the electron microprobe, the major problem with this technique is the translocation of isotope during tissue processing. In addition, the high energy gamma ray associated with the decay of ^{42}K limits the resolution that can be obtained. Olsson et al. (48) have used the slightly less energetic gamma ray from ^{43}K to investigate

---

[1]Personal communication.

the uptake of potassium by different bones using "whole-body" autoradiography. They found potassium at high concentrations in rapidly growing bones and at the junctions between the skeleton and ligaments.

The most abundant isotope of potassium, ^{39}K, has a net nuclear spin and therefore provides a nuclear magnetic resonance signal that can be utilized to study the physical environment surrounding this isotope. As expected, this technique has been used in the hotly debated discussions of whether potassium is "bound" or "free" in intracellular water with both sides claiming victory (61). The environment of potassium in bone could be studied by ^{39}K NMR spectroscopy and the state of potassium in the hydration water of hydorxyapatite crystals would also be of interest.

In the model proposed by Scarpace and Neuman (59), potassium is pumped into the cells comprising the bone membrane from the general extracellular fluid. Potassium is thought to flow from the bone extracellular fluid back to the general extracellular fluid in the channels between cells. Such a "pump-leak" system was proposed to provide a means of generating an extracellular fluid potassium gradient between bone and blood. Since the content of potassium in bone was strongly decreased by the addition of ouabain, the active uptake of potassium into the bone membrane cells was attributed to the actions of a Na^+, K^+-ATPase transport protein (59). If this model were correct, then this ouabain-inhibited transport protein would be expected to be found in the greatest concentrations on the side of the plasma membranes of the bone membrane cells facing away from the bone mineral.

This hypothesis can be tested with the use of the morphological localization of the Na^+,K^+-ATPase protein with tritium-labeled ouabain "inhibitor autoradiography". This technique has been used to localize the Na^+,K^+-ATPase in gall bladder (38) and intestine (62) and the use of tritium-labeled acetazolamide led to the identification of osteoclasts as the source of bone carbonic anhydrase (16a). Since the tritium beta particle has a low energy and the inhibitor binds to a specific protein, the problems discussed earlier concerning resolution and translocation with ^{42}K autoradiography are minimal.

Perhaps the most interesting approach to the study of bone extracellular fluid potassium concentrations involves the culturing of bone cells on artificial capillaries. In this system, isolated bone cells are allowed to grow in layers surrounding capillary fibers as medium is continually perfused through the fibers. Heersche et al. (23) have reported preliminary data in which on one occasion bone cells maintained a potassium concentration in the extracapillary space 34% higher than in the perfusion medium while no such gradient was observed for calcium. The addition of parathyroid hormone induced a three-fold potassium gradient without affecting the distribution of calcium. This technique has obvious potential for a systematic investigation of the mechanism behind the transcellular movement of potassium and the factors which regulate this movement.

A radioactive isotope of rubidium, ^{86}Rb, is commonly used as an analog of potassium in membrane transport studies. In their comparison of the distribution of ^{86}Rb and ^{42}K in rabbit tissues, Kilpatrick et al. (31)

found that, relative to potassium, rubidium was concentrated in liver, kidneys and intestine but excluded in brain, bone and urine. The rubidium and potassium isotopes were found in equal concentrations in erythrocytes and skeletal muscle. Rubidium is also known to be a poor substitute for potassium in the diet (58). These results indicate that rubidium is not a perfect potassium analog and suggest the use of ^{42}K, rather than ^{86}Rb be continued in studies on skeletal potassium.

BIBLIOGRAPHY

1. Baud, C.: Submicroscopic structure and functional aspects of the osteocyte. Clin. Orthoped. and Related Res. 56, 227-236 (1968).

2. Bergstrom, W.H.: The skeleton as an electrolyte reservoir. Metabolism 5, 433-437 (1955).

3. Bergstrom, W.H. and W.M. Wallace: Bone as a sodium and potassium reservoir. J. Clin. Invest. 33, 867-873 (1954).

4. Borle, A.B., G. Nichols, Jr. and M.J. Karnovsky: Some effects of adrenalectomy and prednisolone administration on extracellular fluid and bone composition in rats. Endocrinol. 66, 508-516 (1960).

5. Bosher, S.K. and R.L. Warren: Very low calcium content of cochlear endolymph, an extracellular fluid. Nature 273, 377-378 (1978).

6. Bursaux, C.-D., C.-F. Poyart, A. Freminet, E. Bursaux and C. Dubos: Le CO_2 et les Électrolytes de L' Os. Influence de la croissance sur la composition minérale du fémur de rat. Ann. Biol. Clin. 30, 121-126 (1972).

7. Canas, F., A.R. Terepka and W.F. Neuman: Potassium and the Milieu Intérieur of bone. Am. J. Physiol. 217, 117-120 (1969).

8. Clancy, R.L. and E.B. Brown, Jr.: Changes in bone potassium in response to hypercapnia. Am. J. Physiol. 204, 757-760 (1963).

9. Claudon, F., E. Reichart, J.F. Bolot, S. Berstein and S. Sabliere: Bone electrolytes in experiemntal chronic hypercapnia. Bull. Europ. Physiopath. Resp. 12, 55-67 (1976).

10. Cohen, I. and S. Hurwitz: Intracellular pH and electrolyte composition in the uterine wall of the fowl in relation to shell formation and dietary minerals. Comparative Biochem. and Physiol. 49A, 689-696 (1974).

11. Davies, D.R., J.B. Bassingthwaighte and P.J. Kelly: Transcapillary exchange of strontium and sucrose in canine tibia. J. Appl. Physiol. 40, 17-22 (1976).

12. Davis, H.: Biophysics and physiology of the inner ear. Physiol. Rev. 37, 1-49 (1957).

13. Davis, W.L., J.L. Matthews, J.H. Martin, J.W. Kennedy III and R.V. Talmage: The endosteum as a functional membrane. In, Calcium-Regulating Hormones, ed. by R.V. Talmage, M.Owen and J.A. Parsons. Excerpta Medica, Amsterdam, pp. 275-283, 1975.

14. Dosekun, F.O.: The effect of alterations of plasma sodium on the sodium and potassium content of bone in the rat. J. Physiol. 147, 115-123 (1959).

15. Doty, S.B. and B.H. Schonfield: Metabolic and structural changes within osteocytes of rat bone. In, Calcium, Parathyroid Hormone and the Calcitonins. Excerpta Medica, Amsterdam, pp. 353-364, 1972.

16. Dudley, H.R. and D. Spiro: The fine structure of bone cells. J. Biophys. Biochem. Cytol. 11, 627-649 (1961).

17. Edelman, I.S. and J. Leibman: Anatomy of body water and electrolytes. Am. J. Med. 27, 256-277 (1959).

18. El Jack, M.H. and P.E. Lake: The content of the principal inorganic ions and carbon dioxide in the uterine fluids of the domestic hen. J. Reproductive Fertility 13, 127-132 (1967).

19. Erulkar, S.D. and T.H. Maren: Carbonic anhydrase and the inner ear. Nature 189, 459-460 (1961).

20. Gay, C.V. and W.J. Mueller: Carbonic anhydrase and osteoclasts: Localization by labeled inhibitor autoradiography. Science 183, 432-434 (1974).

21. Geisler, J.Z. and W.F. Neuman: The membrane control of bone potassium. Proc. Soc. Exptl. Biol. Med. 130, 608-612 (1969).

22. Hartsuck, J.M., J.E. Johnson and F.D. Moore: Potassium in bone: Evidence for a nonexchangeable fraction. Metabolism 18, 33-37 (1969).

23. Heersche, J.N.M., H.K. Moe, D.M. Brunette, A.V. Rao, S. Riemers and L.G. Rao: Long-term culture of parathyroid hormone and prostaglandin E_1 responsive bone cells in monolayers and on artificial capillaries. Calc. Tiss. Res. 22 (Suppl.) 275-282 (1977).

24. Horowitz, P., M. Aronson, L. Grodzins, W. Ladd, J. Ryan, G. Merriam and C. Lechene: Elemental analysis of biological specimens in air with a proton microprobe. Science 194, 1162-1165 (1976).

25. Howard, J.E.: Present knowledge of parathyroid function with special emphasis upon its limitation. In, Bone Structure and Metabolism, ed. by G.E.W. Wolstenholme and C.M. O'Connor. Little, Brown and Co., Boston, pp. 206-207 (1956).

26. Humphray, H.P. and F.W. Heaton: Relationship between the thyroid hormone and mineral metabolism in the rat. J. Endocrinol. 53, 113-123 (1972).

27. Johnston, C.C., Jr., E.B. Miner, D.M. Smith and W.P. Deiss: Influence of parathyroid activity on the chemical equilibrium of bone and calcium in vitro. J. Lab. Clin. Med. 60, 689-694 (1962).

28. Kato, Y., S. Takimoto and H. Ogura: Mechanism of induction of hypercalcemia and hyperphosphatemia by lead acetate in the rat. Calc. Tissue Res. 24, 41-46 (1977).

29. Katzman, R.: Maintenance of a constant brain extracellular potassium. Fed. Proc. 35, 1244-1247 (1976).

30. Kelly, P.J. and J.B. Bassingthwaighte: Studies on bone ion exchange using multiple-tracer indicatior dilution techniques. Fed. Proc. 36, 2634-2639 (1977).

31. Kilpatrick, R., H.E. Renschler, D.S. Munro and G.M. Wilson: A comparison of the distribution of ^{42}K and ^{86}Rb in rabbit and man. J. Physiol. 133, 194-201 (1956).

32. Kirksey, A., R.L. Pike and J.A. Callahan: Some effects of high and low sodium intakes during pregnancy in the rat. II. Electrolyte concentrations of maternal plasma, muscle, bone and brain and of placenta, amniotic fluid, fetal plasma and total fetus in normal pregnancy.

33. Komárková, A., I. Bilyk, Z. Zahor and V. Czabanova: Effect of short term starvation on bone structure in rats. Physiologia Bohemoslovaca 22, 633-636 (1973).

34. Krempien, B., G. Friedrich, G. Geiger and E. Ritz: Factors influencing the effect of parathyroid hormone on endosteal cell morphology. A scanning electron microscope study. Calc. Tiss. Res. 22 (Suppl.) 164-168 (1977).

35. Lechene, C.P.: Electron probe microanalysis: Its present, its future. Am. J. Physiol. 232, F391-F396 (1977).

36. MacGregor, J.: Blood-bone equilibrium. In, Bone Biodynamics, ed. by H.M. Frost. Little, Brown and Co., Boston, pp. 409-421, 1964.

37. Matthews, J.L., J.H. Martin, J.W. Kennedy, III and E.J. Collins: An ultrastructural study of calcium and phosphate deposition and exchange in tissues. In, Hard Tissue Growth, Repiar and Remineralization, Ciba Foundation Symposium, Associated Scientific Pub., pp. 187-211, Amsterdam, 1973.

38. Mills, J.W. and D.R. Dibona: Distribution of Na^+ pump sites in the frog gall bladder. Nature 271, 273-275 (1978).

39. Neuman, W.F., T.Y. Toribara and B.J. Mulryan: Synthetic hydroxyapatite crystals. I. Sodium and potassium fixation. Arch. Biochem. Biophys. 98, 384-390 (1962).

40. Neuman, W.F., A.R. Terepka, F. Canas and J.T. Triffitt: The cycling concept of exchange in bone. Calc. Tiss. Res. 2, 262-270 (1968).

41. Neuman, W.F. and W.K. Ramp: The concept of a bone membrane: Some implications. In, Cellular Mechanisms for Calcium Transfer and Homeostasis, ed. by G. Nichols, Jr. and R.H. Wasserman. Academic Press, pp. 197-206, 1971.

42. Neuman, W.F. and B.J. Bareham: Evidence for the presence of secondary calcium phosphate in bone and its stabilization by acid production. Calc. Tiss. Res. 18, 161-172 (1975).

43. Neuman, W.F., R. Brommage and C.R. Myers: The measurement of Ca^{2+} effluxes from bone. Calc. Tiss. Res. 24, 113-117 (1977).

44. Nicholson, W.A.P., B.A. Ashton, H.J. Hohling, P. Quint, J. Schrieber, I.K. Ashton and A. Boyde: Electron microprobe investigations into the process of hand tissue formation. Cell and Tissue Res. 177, 331-345 (1977).

45. Norman, N.: The participation of bone in the sodium and potassium metabolism in the rat. I. Simultaneous determination of the exchangeable body sodium and potassium, and the exchangeable and inexchangeable fractions of these ions in bone of the normal rat. Acta Physiol. Scand. 57, 363-372 (1963).

46. Norman, N.: The participation of bone in the sodium and potassium metabolism of the rat. II. The effect of variation of electrolyte intake, acidosis and alkalosis. Acta Physiol. Scand. 57, 373-383 (1963).

47. Norman, N.: The participation of bone in the sodium and potassium metabolism in the rat. III. The effect of adrenalectomy, cortisone, desoxycorticosterone and 2-methyl-9-α-fluorohydrocortisone. Acta Physiol. Scand. 57, 384-396 (1963).

48. Olsson, K.A., R. Soremark and K.R. Wing: Uptake and distribution of rubidium-86 and potassium-43 in mice and rats--an autoradiographic study. Acta Physiol. Scand. 77, 322-332 (1969).

49. Owen, M. and J.T. Triffitt: Extravascular albumin in bone tissue. J. Physiol. 257, 293-307 (1976).

50. Owen, M., C.R. Howlett and J.T. Triffitt: Movement of ^{125}I albumin and ^{125}I polyvinylpyrrolidone through bone tissue fluid. Calc. Tiss. Res. 23, 103-112 (1977).

51. Pak, C.Y.C. and F.C. Bartter: Ionic interaction with bone mineral. II. The control of Ca^{2+} and PO_4^{3-} exchange by univalent cation-Ca^{2+} substitution at the hydroxyapatite crystal surface. Biochim. Biophys. Acta 141, 410-420 (1967).

52. Papeschi, G., J. Bordi, C. Beni and L. Ventura: Use of an iridium electrode for direct measurement of pI of proteins after isoelectric focusing in polyacrylamide gel. Biochim. Biophys. Acta 453, 192-199 (1976).

53. Paradis, G.R., J.B. Bassingthwaighte and P.J. Kelly: Inhibition of transport of ^{47}Ca and ^{85}Sr by lanthanum in canine cortical bone. J. Appl. Physiol. 36, 221-225 (1974).

54. Ramp, W.K.: Cellular control of calcium movements in bone: Interrelationships of the bone membrane, parathyroid hormone and alkaline phosphatase. Clin. Orthoped. Related Res. 106, 311-322 (1975).

55. Rasmussen, H. and P. Bordier: The Physiological and Cellular Basis of Metabolic Bone Disease. The Williams and Wilkins Co., Baltimore, 1974.

56. Robertson, W.G.: Cellular calcium and calcium transport. In, Calcium, Phosphate and Magnesium Metabolism, ed. by B.E.C. Nordin. Churchill Livingstone, New York pp. 230-256, 1976.

57. Russell, L.D.: The blood-testis barrier and its formation relative to spermatocyte maturation in the adult rat: A lanthanum tracer study. Anatomical Record 190, 99-112 (1978).

58. Sasser, L.B., E.W. Kienholz and G.M. Ward: Interaction of rubidium and potassium in chick diets. Poultry Science 48, 114-118 (1969).

59. Scarpace, P.J. and W.F. Neuman: The blood:bone disequilibrium. I. The active accumulation of K^+ into the bone extracellular fluid. Calc. Tiss. Res. 20, 137-149 (1976).

60. Schartum, S. and G. Nichols, Jr.: Concerning pH gradients between the extracellular compartment and fluids bathing the bone mineral surface and their relation to calcium ion distribution. J. Clin. Invest. 41, 1163-1168 (1962).

61. Shporer, M. and M.M. Civan: The state of water and alkali cations within the intracellular fluids: The contribution of NMR spectroscopy. Current Topics in Membrane Transport 9, 1-69 (1977).

62. Stirling, C.E.: Radioautographic localization of sodium pump sites in rabbit intestine. J. Cell Biol. 53, 704-714 (1972).

63. Stock, J.T., W.C. Purdy and L.M. Garcia: The antimony-antimony oxide electrode. Chem. Rev. 58, 611-626 (1958).

64. Talmage, R.V.: Calcium homeostasis--calcium transport--parathyroid action: The effects of parathyroid hormone on the movement of calcium between bone and fluid. Clin. Orthoped. Related Res. 67, 210-224 (1969).

65. Triffitt, J.T., A.R. Terepka and W.F. Neuman: A comparative study of the exchange in vivo of major constituents of bone mineral. Calc. Tiss. Res. 2, 165-176 (1968).

66. Wallach, S., J.V. Bellavia, J. Schorr and D.L. Reizenstein: Tissue distribution of electrolytes, ^{47}Ca and ^{28}Mg in acute hypercalcemia. Am. J. Physiol. 207, 553-560 (1964).

67. Weinger, J.M. and M.E. Holtrop: An ultrastructural study of bone cells: The occurrence of microtubules, microfilaments and tight junctions: Calc. Tiss. Res. 14, 15-29 (1974).

68. Wergedal, J.E. and D.J. Baylink: Electron microprobe studies of bone mineralization rate in vivo. Am. J. Physiol. 226, 345-352 (1974).

69. Whitson, S.W.: Tight junction formation in the osteon. Clin. Orthoped. Related Res. 86, 206-213 (1972).

70. Willbanks, O.L., Jr. and D.W. Seldin: Participation of bone in acid-base balance. Clin. Res. Proc. 4, 36 (1956).

71. Woodbury, D.M.: Effect of acute hyponatremia on distribution of water and electrolytes in various tissues of the rat. Am. J. Physiol. 185, 281-286 (1956).

72. Wuthier, R.E.: Zonal analysis of electrolytes in epiphyseal cartilage and bone of normal and rachitic chickens and pups. Calc. Tiss. Res. 8, 24-35 (1971).

CHAPTER TWO

THE PASSIVE ACCUMULATION OF SODIUM, POTASSIUM AND MAGNESIUM BY CHICK CALVARIA

INTRODUCTION

The principal cations of bone, namely calcium, magnesium, sodium and potassium, exist in a variety of chemical states in the skeleton. The mineral phase (hydroxyapatite and amorphous calcium phosphate) contains the majority of calcium and magnesium but also sodium and some potassium. The hydrated water surrounding this mineral has a composition which depends upon the fluid with which it is in contact. Bone cells contain each of these cations in various compartments and the extracellular fluid of bone has its own composition. In recent years claims have been made that this extracellular fluid is not a simple ultrafiltrate of plasma but rather a separate fluid compartment whose composition is regulated by the layer of cells surrounding bone (23,26,36).

To gain further information on the physiological importance of the concentrations of magnesium, sodium and potassium in bone water, the "passive" concentrations of these cations in non-living bone have been determined and related to previously obtained data from both bone and synthetic hydroxyapatite.

MATERIALS AND METHODS

Four- to five-day-old White Leghorn chicks were sacrificed by decapitation and their calvaria removed by dissecting outside the suture lines. Incubated calvaria were placed in a modification of a buffer solution consisting of 5 mM HEPES, 140 mM NaCl, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 1 mM $MgSO_4$ and 4 mM KCl set to pH 7.4 at 37° C. The specific modifications of this buffer are described in Table 1 and the three figure legends. In general, six to eight calvaria were placed in 100 ml of buffer and swirled with the aid of a magnetic stirring bar in a room maintained at 37° C for periods ranging from one to five hours.

At the conclusion of the incubation the calvaria were removed from the buffer, blotted (wiped) to remove excess water, and each frontal bone was "punched-out" with a cork borer (area of 0.283 cm^2) as described previously (36). The two "punch-outs" from each calvarium were combined and extracted overnight with 1 ml of 2 N HNO_3. Each "punch-out" had a wet weight of approximately 6 mg and contained about 24 μmoles of calcium. Following the extraction, aliquots were removed for analyses of sodium, potassium, calcium and magnesium. The sodium and potassium analyses were done simultaneously on an Instrumentation Laboratories Model 143 flame photometer with 250 microliters of extract added to 10 ml of 15 meq/liter lithium nitrate. Calcium and magnesium were analyzed simultaneously on an Instrumentation Laboratories Model 143 atomic absorption spectrometer using 50 microliters of extract diluted to 10 ml

of 0.1% lanthanum, 0.5% HCl, 32.5 µg/ml $SrCl_2 \cdot 6H_2O$ and 0.2% non-ionic detergent. Preliminary experiments demonstrated that the recoveries of sodium, potassium, calcium and magnesium added to the bone extract were 96, 98, 99 and 99%, respectively.

In each experiment several control calvaria were not incubated but analyzed in the identical fashion as the incubated calvaria. The magnesium data were normalized with respect to the individual calcium content of each calvarium since this procedure tended to decrease the standard errors without influencing the interpretation of the results. A Cahn top-loading digital millibalance (Model DTL 7500-7) was used for the experiments in which water weights of the calvarial "punch-outs" were required (Figure 3). Each "punch-out" was weighed wet and then dried overnight at 110° C before reweighing. These samples were extracted with nitric acid and analyzed individually for sodium and potassium concentrations.

RESULTS

Magnesium

As shown in Figure 1 (parts A and B), incubating chick calvaria in buffers containing 1 mM magnesium and either 2 M urea of 0.1% SDS did not change the bone magnesium content. When magnesium was omitted from the buffer, the bone magnesium content gradually declined (Figure 1C) over a 5-hour period. The bone magnesium content rose slowly when the calvaria were incubated in a buffer containing 5 mM magnesium (Figure 1D).

Several studies have shown that the skeletal content of magnesium is a function of blood levels of magnesium in vivo (1,2,4,35) and buffer magnesium concentration in vitro (1,14,16,30,34). Magnesium is thought to be capable of replacing calcium on a mole for mole basis in bone mineral (25,30,34) although obviously calcium phosphate precipitation is favored over that of magnesium. A discrimination ratio for calcium over magnesium of 45 has been observed with synthetic hydroxyapatite (25).

Alfrey and Miller (1) have shown that approximately 35% of the magnesium in powdered bone can be eluted over a several-hour period and this observation was supported by additional experiments as part of this investigation (data not shown). The exchange of ions in the crystal hydration water is rapid while ions residing on the hydroxyapatite surface exchange with a half-life of approximately 10 hours. Ions in the crystal interior exchange over a period of weeks (18).

FIGURE 1. The Calvarial Content of Magnesium as a Function of Buffer Magnesium Concentration and the Presence of Tissue-Denaturing Agents.

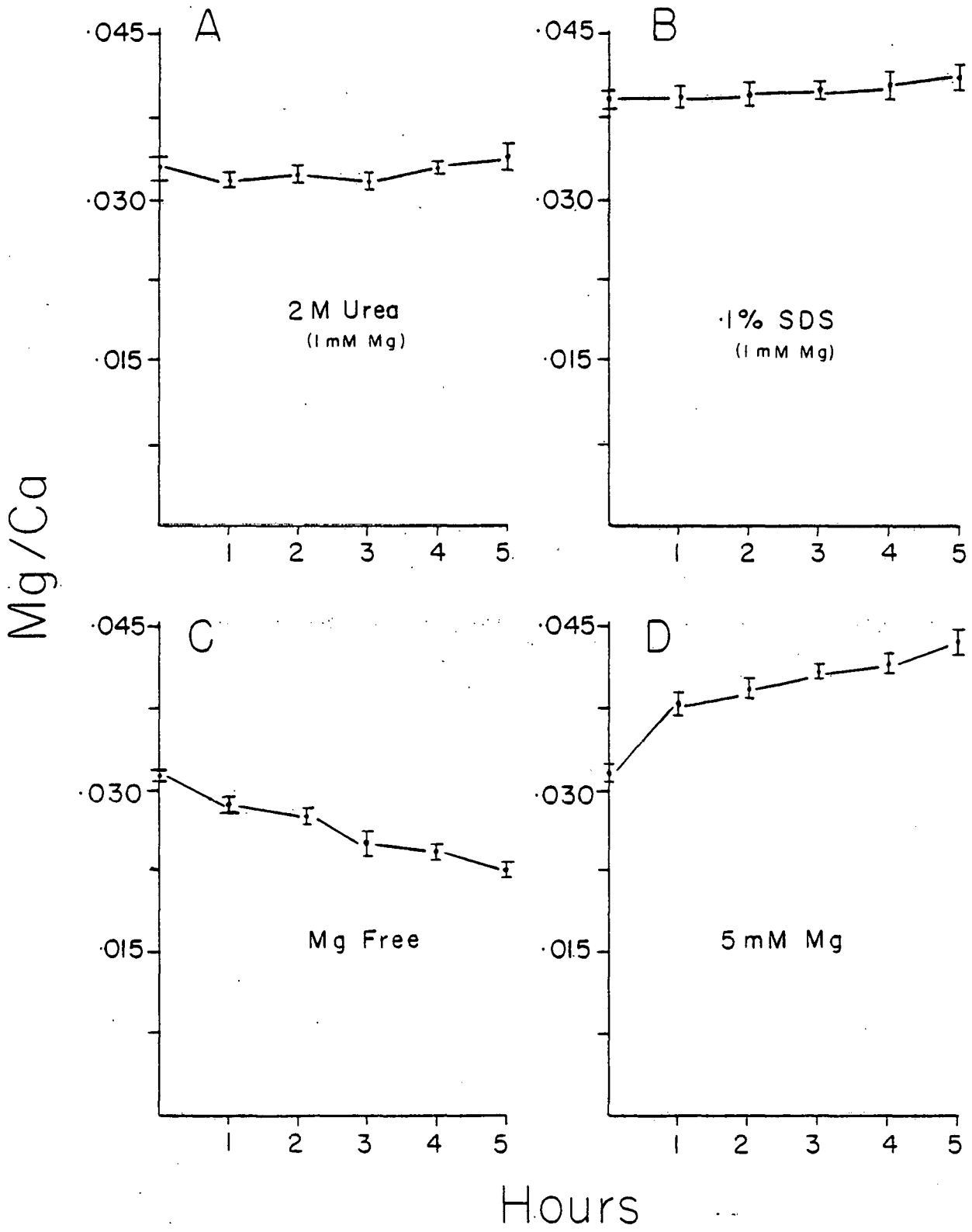
Each graph gives the mean ± SEM of the calvarial Mg/Ca ratio (on a mole basis) for six to nine samples as a function of incubation time. Data from unincubated calvaria are plotted at zero hours; these calvaria contained an average of about 0.8 μmoles of magnesium per two "punched-out" sections. Each experiment was performed with the buffer described in the text modified as follows:

A. The buffer contained 2 M urea.

B. The buffer contained 0.1% SDS.

C. $MgSO_4$ was omitted from the buffer.

D. The buffer contained 5 mM $MgSO_4$.


A
B
C
D
·045
·030
·015
2 M Urea
(1 mM Mg)
·1% SDS
(1 mM Mg)
Mg Free
5 mM Mg
1
2
3
4
5
Mg/Ca
Hours

The best available evidence indicates that roughly two-thirds of the skeletal magnesium is buried within the mineral phase. Since the exchangeability of magnesium in synthetic hydroxyapatite increases with crystal aging (25), bone mineral must exist in a rather "immature" state. From the exchangeability data, most of the remaining one-third of the skeletal magnesium appears to reside of the mineral surface, in the hydration water or in the cells. Although magnesium is no doubt extremely important to the proper functioning of cells, cellular magnesium represents only a small fraction of the total bone magnesium.

Sodium

The data in Figure 2A show that when chick calvaria are incubated in a sodium-free buffer they lose approximately 70% of their sodium content within the first hour of incubation. Further incubation for up to 5 hours did not remove additional sodium from the calvaria. The effect of varying the sodium concentration in the buffer is shown in Figure 2B. The bone sodium content is linearly related to the buffer sodium concentration and, at a buffer concentration of 140 mM, the sodium contents of incubated and unincubated calvaria were identical. The inhibitors used in Table 2 (urea, SDS, iodoacetate and ouabain) had no effect upon the bone sodium content (data not shown). The bone sodium fraction that was lost upon incubation in sodium-free buffer, namely 70% of the total sodium, can clearly be considered exchangeable while the remaining sodium is probably "bound" within the mineral phase. The exchangeable sodium is presumably located in the cellular, interstitial and crystal hydration water.

FIGURE 2. The Calvarial Contents of Sodium and Potassium as a Function of Various Incubation Conditions.

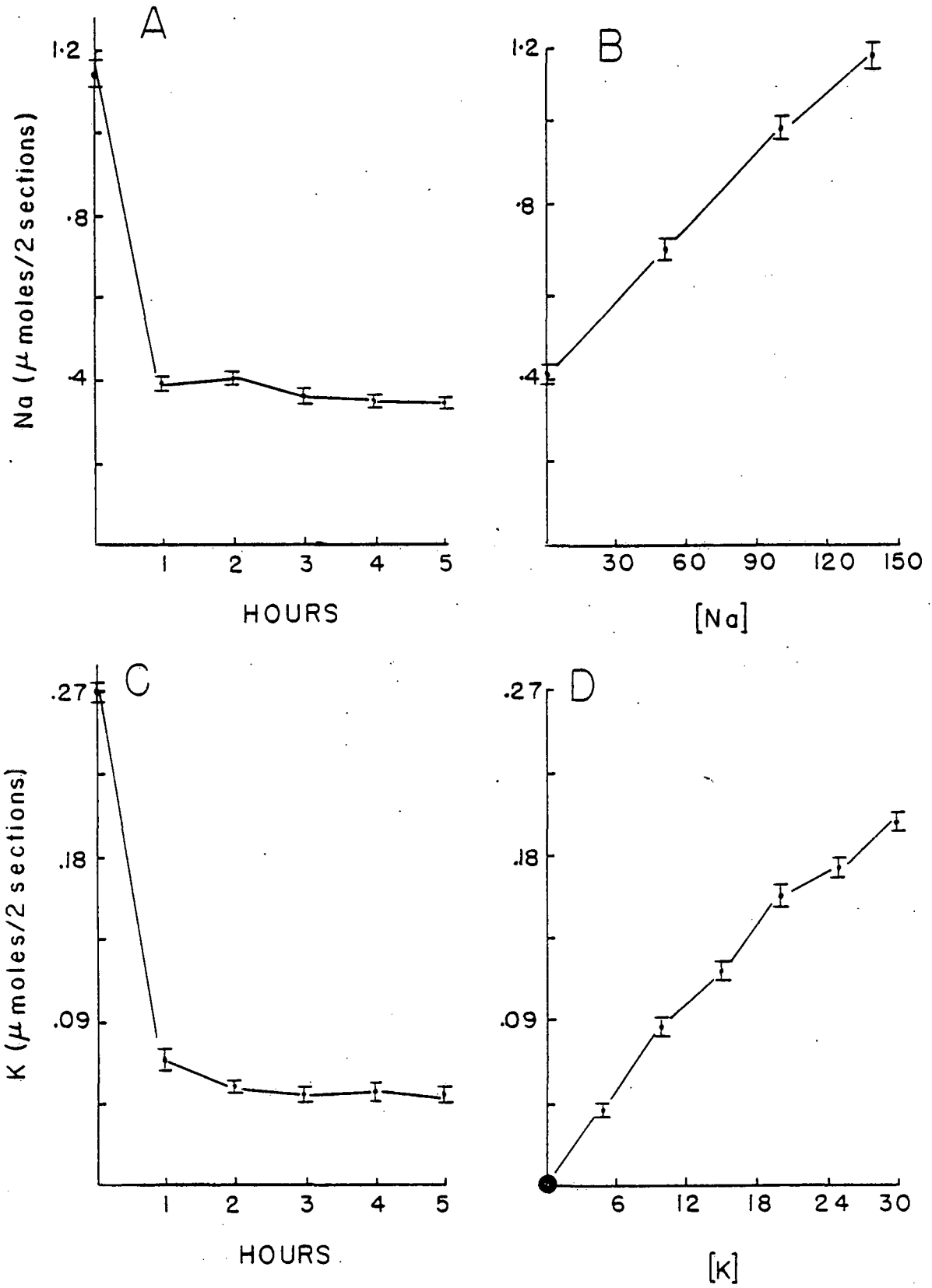
Each graph gives the mean content ± SEM of sodium or potassium for five to eight samples. In parts A and C, data from unincubated calvaria are plotted at zero hours. In parts B and D, the incubation lasted for two hours and the abscissa represents the sodium and potassium buffer concentrations in mmoles/liter. Each experiment was performed with the buffer described in the text modified as follows:

A. NaCl was replaced with choline chloride and NaOH was replaced with KOH.

B. NaCl concentrations were 0, 50, 100 and 140 mM with osmolality maintained with choline chloride.

C. The buffer contained 100 μM ouabain.

D. The buffer contained 100 μM ouabain and various concentrations of KCl.


A
1·2
·8
·4
Na (μ moles/2 sections)
1
2
3
4
5
HOURS
B
1·2
·8
·4
30
60
90
120
150
[Na]
C
.27
.18
.09
K (μ moles/2 sections)
1
2
3
4
5
HOURS
D
.27
.18
.09
6
12
18
24
30
[K]

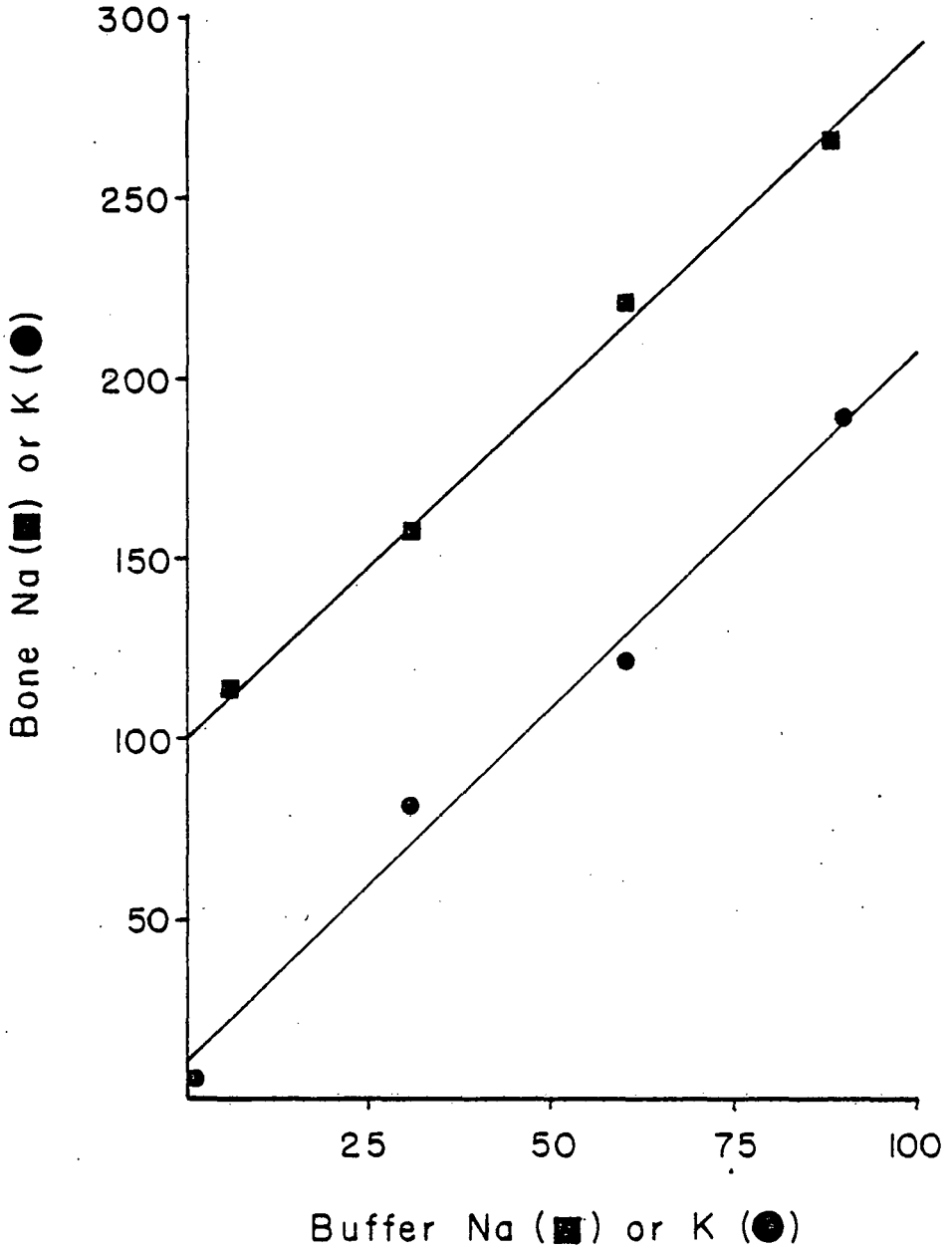
These results are in excellent agreement with prior experience. When sodium is present during the precipitation of calcium and phosphate at neutral pH, as is the case in growing bone, some of this sodium is incorporated in a nonexchangeable fashion into the calcium-phosphate precipitate (19). The fraction of bone sodium that is nonexchangeable depends in large part upon the age of the animal. More mature bone, having a higher mineral to water ratio than younger bone, exhibits a smaller percentage of exchangeable sodium (6,7). In experiments involving ^{24}Na, 73% of the sodium was shown to be exchangeable in embryonic chick calvaria (36), a value very close to the 70% obtained in this study on calvaria from 4- to 5-day-old chicks.

In agreement with this study, the turnover of the exchangeable sodium in synthetic hydroxyapatite (24), rat tibia (39) and embryonic chick calvaria (36) has been shown to be extremely rapid. The concentration of the sodium in the hydroxyapatite hydration water has been found to be directly proportional to the concentration of sodium in the buffer solution to which the hydroxyapatite was exposed (37). Likewise, in this study the exchangeable bone sodium was directly proportional to the buffer sodium concentration.

The actual concentration of sodium in the bone extracellular fluid was estimated from the data presented in Figure 3. This experiment was similar to the one described in Figure 2B except that wet and dry weights were determined on the calvaria "punch-outs". This procedure permitted the bone sodium contents to be expressed in terms of total

FIGURE 3. The Calvarial Concentrations of Sodium and Potassium as a Function of the Buffer Concentrations of these Cations.

All concentrations are given in mmoles/liter. The sum of the sodium and potassium buffer concentrations was maintained between 140 and 150 mM. Each data point is the mean of six to eight calvaria "punch-out" samples. Unincubated calvaria contained 78.2 $\pm$3.7 mM potassium, 381 $\pm$13 mM sodium and 33.6 $\pm$0.9 per cent water (mean $\pm$ SEM, n = 7 in each case) while the water content of incubated calvaria averaged 36.4 per cent.


300
250
200
150
100
50
Bone Na (■) or K (●)
25
50
75
100
Buffer Na (■) or K (●)

bone water. By plotting the "apparent" calvarial sodium concentrations against the buffer concentrations of sodium, the resulting slope gives the "concentrative factor" by which sodium is passively concentrated in the total bone water. The term "passively concentrated" represents the combined concentrative effects of the mineral and organic substances in bone that continue to concentrate sodium following the cessation of cellular activity. The actual mechanism(s) behind this passive concentrative phenomenon will be analyzed in greater detail in the Discussion.

The concentrative factor of chick calvaria for sodium as determined from the slope of the line relating bone and buffer sodium concentrations in Figure 3 is 1.9; a value of 2.0 was obtained in a repeat of the experiment. Thus, the concentration of sodium averaged over the total bone water is approximately double that of the fluid in which the calvaria is exposed. If the cells are assumed not to passively concentrate sodium, then the concentrative factor for sodium in the calvarial extracellular fluid is greater than two. Assuming the cell water is one-quarter of the total calvarial water, the sodium concentrative factor in the extracellular fluid is roughly two and one-third.

The distribution of sodium among the various compartments in the chick calvarium can be accounted for as follows. With an extracellular fluid concentrative factor of two and one-third, a blood sodium concentration of 142 mM, an equivalent of 16 mM sodium present in the total cell water and one-quarter of the calvarial water present in cells, the mean concentration of sodium in bone water is 252.5 mM. This

exchangeable sodium is roughly 70% of the total calvarial content of sodium, the remainder is presumably associated with the mineral phase. Thus, by calculation, chick calvaria contain an equivalent of 360.7 mM sodium, a value less than 6% lower than the observed 382.7 $\pm$11.8 mM (mean $\pm$ SEM, n = 8) in unincubated calvaria.

Potassium

The effect of various inhibitors on calvarial potassium content is shown in Table 1. In sharp contrast to sodium and magnesium, bone potassium levels are strongly influenced by cellular activity. Treatment with sodium dodecyl sulfate (SDS), urea, ouabain and iodoacetate all resulted in the loss of nearly identical amounts of potassium; the final calvarial potassium content consisted of about 17% of the unincubated potassium content. Since ouabain is thought to act specifically upon a membrane-bound Na^+,K^+-ATPase transport protein, the potassium lost in the presence of ouabain (83% of the total) presumably was initially present as a result of the activity of the Na^+,K^+-ATPase. In other words, the entire cellular control of bone potassium levels is apparently expressed through a ouabain-sensitive process. The time course of the potassium loss in ouabain-treated calvaria is shown in Figure 2C; the potassium loss with SDS was as rapid while the loss of potassium in urea- and iodoacetate-treated calvaria was slightly slower than in the ouabain experiment.

The amount of potassium remaining in the calvaria after incubation for two to three hours in potassium-free buffers varied from 0 to 8% of

TABLE 1

Effect of Inhibitors on the Equilibrium Content of Potassium in Chick Calvaria

Inhibitor	Unincubated Potassium Content (μmoles/2 sections)	Final Incubated Potassium Content (μmoles/2 sections)	Incubated / Unincubated Ratio
Urea (2 M)[1]	.256 ±.012 (7)[2]	.032 ±.003 (6)	.13
SDS (.1%)[3]	.260 ±.011 (9)	.045 ±.002 (8)	.17
Iodoacetate (2 mM)	.265 ±.008 (8)	.045 ±.003 (6)	.17
Ouabain (.1 mM)	.272 ±.012 (6)	.049 ±.002 (6)	.18

The potassium content for a single calvarium was determined from two "punched-out" sections as described in the text. Data were taken every hour for 5 hours and in every case there was no change in the K content from 4 to 5 hours. The equilibrium content was taken as the value for the 5th hour.

[1]Concentration of the inhibitor used is given in parentheses.

[2]Values are mean ± SEM with the number of data points given in parentheses.

[3]Sodium dodecyl sulfate.

the unincubated potassium content. Bone potassium has been claimed to be both completely (36,38) and incompletely (22) exchangeable over the course of several hours. Two studies (19,31) have shown that synthetic hydroxyapatite binds potassium to a slight degree. Clearly, a greater fraction of bone sodium is nonexchangeable than bone potassium. The basis for this phenomenon presumably depends upon ionic radius; the ionic radius of sodium is 0.97 Å, very close to the value of 0.99 Å for calcium while potassium has an ionic radius of 1.33 Å.

As is true for sodium, the concentration of potassium in the hydroxyapatite hydration water is directly proportional to the buffer potassium concentration (37). Figures 2D and 3 show that the same phenomenon occurs in chick calvaria. The relationship between bone and buffer potassium concentrations is more linear in Figure 3 than Figure 2D as the result of additional care in maintaining the ionic strength of the buffer constant. In the analysis of the sodium data, the bone water sodium concentration was shown to be approximately a factor of two greater than the buffer sodium concentration. The slope of the line relating bone and buffer potassium concentrations in Figure 2 indicates a calvarial concentrative factor of 2.0 for potassium. Values of 1.5 and 1.7 were obtained in repeats of this experiment.

Given the three experimentally determined values of the calvarial concentrative factor for potassium (1.5, 1.7 and 2.0), and the fact that synthetic hydroxyapatite has a greater concentrative factor for sodium than potassium (37), a calvarial concentrative factor for potassium

of 1.75 will be used for further calculations. This difference in concentrative factors is probably a result of sodium's smaller ionic radius and hence greater quantity of polarizable hydrated water associated with the ion in solution.

Calculations similar to those found in the analysis of the sodium data can be made for potassium. A calvarial concentrative factor of 1.75 for potassium in the total bone water corresponds to a value of 2.0 in the extracellular water of bone, if bone cells are again assumed to contain one-quarter of the total calvarial water. Assuming that an equivalent of 7 mM of potassium is bound to the mineral phase, blood potassium is slightly greater than 5 mM, the cells contain 120 mM potassium and occupy one-quarter of the total bone water, then the total calvarial potassium concentration should be approximately 45 mM.

DISCUSSION

The ionic content of the bone water is of particular interest in light of the evidence suggesting the extracellular fluid of bone has an electrolyte composition differing from that of the general extracellular fluids of the body (23,26,36). The data on magnesium are sufficiently complex to provide any information either for or against the existence of a bone membrane regulating the composition of the bone extracellular fluid. Since the concentration of bone sodium appears to be directly proportional to blood levels of sodium and independent of cellular activity, there is no evidence to suggest that the concentration of sodium in the bone extracellular fluid is controlled by the bone membrane.

The concentration of potassium in chick calvaria has consistently been found to be between 70 and 90 mM, well above the calculated value of 45 mM obtained in the Results Section. There are two explanations that can account for this discrepancy. The cellular contribution to the total potassium content may have been underestimated or, as has been proposed (23,26,36), bone cells might actively pump potassium into the bone extracellular fluid. Although present evidence does not permit an unambiguous resolution of this discrepancy, a discussion of some unresolved problems does provide some direction for future experimentation.

The cellular concentration of potassium and, in particular, the fractional water volume of the calvaria occupied by cells, are both

major factors in accounting for the distribution of potassium in the chick calvaria. A reasonable assumption usually made is that bone cells are similar to other cells in their intracellular potassium concentrations. Published reports on intracellular concentrations of potassium from a variety of tissues typically show values ranging from 100 to 150 mM. The value of 120 mM used for the distribution calculation was meant to represent an average estimate and not an absolute datum from a particular study. The possibility, however slight, that bone cells contain potassium at concentrations much greater than 150 mM has not definitively been ruled out.

The determination of the cellular volume in these and other bones has not proved to be an easy task. Although an estimate of the fraction of the chick calvarial water occupied by cells made by mannitol space measurements has given a value of 20% (28), there is sufficient reason to doubt that figure. Mannitol is known to "slowly" enter cells of several tissues (3,5,8,9) and, in doing so, would underestimate the fractional cell volume. Despite the fact that no such cavities were found in an earlier study (27), recent histological observations (17) have indicated that these chick calvaria contain considerable quantities of marrow in an enodsteal cavity. The existence of a sizeable number of non-osseous (and presumably potassium rich) marrow cells makes the conclusions of the mannitol study (28) appear even more unlikely.

As discussed in Chapter 1, the fundamental problem standing in the way of a resolution of the distribution of potassium in bone is the inaccessibility of the bone extracellular fluid for analysis. The concentration of potassium in the bone extracellular fluid has always

been estimated as the "left-over" potassium unaccounted for by cells. Unless some method for the direct determination of potassium in the bone extracellular fluid is devised, this indirect, "left-over" approach will remain.

The recent observations that chick calvaria contain significant quantities of marrow make this tissue a poor choice for investigations on the distribution of potassium in bone. These marrow cells contribute to the total potassium content of the calvaria and estimates of the bone extracellular fluid space are complicated by the existence of a marrow extracellular fluid space. Obviously, despite the advantages of using chick calvaria as a model system, mainly ease of dissection, relatively small thickness and adaptability to the "punch-out" and Ussing chamber methodologies, some other bone should be used for future studies of the distribution of potassium in bone.

The lack of a decisive solution to the bone potassium dilemma does not mean that no new knowledge was obtained. Although numerous reports in the literature suggest that a passive concentrative mechanism played some role in the distribution of sodium and potassium in bone, this report is the first extensive analysis of this phenomenon. Calvarial extracellular fluid concentrative factors for sodium and potassium of approximately two were calculated. It must be emphasized that the numerical values of these concentrative factors apply only to 4- to 5-day old chick calvaria. Analyses of other bones necessitate quantitation of the concentrative factors for these bones.

This concentrative phenomenon of bone mineral has a bearing upon the energetics of maintaining the proposed blood/bone extracellular fluid potassium gradient. Scarpace and Neuman (36) have suggested that the layer of periosteal cells "enveloping" the calvaria are responsible for this potassium gradient and that these cells actively pump potassium into the bone extracellular fluid. With an extracellular fluid concentrative factor of two for potassium, bone cells would only be required to pump potassium to an effective concentration of 30 mM to maintain an actual potassium concentration of 60 mM in the extracellular fluid compartment. This is a concentration six times greater than the blood levels of potassium. Thus, by energetically maintaining a six to one gradient, the actual measured gradient would in fact be 12 to one. Of course, the presence of a "bone membrane" electrical potential would alter these considerations to some extent, but at the present time no such potential has been measured, nor does existing data give any evidence either for or against such a potential.

Since the concentrations of sodium and potassium in calvarial water increase linearly as a function of the buffer concentrations of these ions up to buffer concentrations of at least 100 mM (Figure 3), the concentrative factor does not appear to result from the "binding" of sodium and potassium. If any significant binding of these ions occurred with a dissociation constant in the millimolar range, then some indication of saturation would be expected in Figure 3. Similar data has been observed in muscle (32), where the cell concentration

of potassium was directly proportional to the buffer potassium concentration up to 350 mM. However, in contrast to to the findings of this study, the slope of the line relating muscle and buffer potassium concentrations was essentially unity. Clearly, some mechanism other than the simple ligand binding of sodium and potassium is required to explain the concentrative phenomenon in bone.

The available evidence points overwhelmingly to the zeta potential present on the surface of bone mineral as the physical basis for the passive concentrative mechanism. This zeta potential is electrostatic in nature and attracts diffusible species (counter-ions) that are polarizable. Anions such as phosphate and sulfate are polarizable due to their multiatom structure while cations such as calcium and potassium are polarizable by nature of their ability to surround themselves with hydrated water molecules. The influence of a zeta potential is greatest on highly polarizable species. Physiologically, calcium and phosphate make the greatest contribution to "discharging" the bone mineral zeta potential. Studies on the exchange of calcium and phosphate with synthetic hydroxyapatite crystals have shown that these ions exist at concentrations close to one molar in the crystal hydration water (18). Sodium and potassium are concentrated by synthetic hydroxyapatite, but to a much smaller degree than calcium and phosphate.

A zeta potential is the result of an electric charge at an interface such as the surface of a crystal. The small size, and hence large surface area to mass ratio, makes the hydroxyapatite zeta potential an

important factor in the analysis of the distribution of ions in bone water. The zeta potential, in addition to attracting ions such as calcium and phosphate, also attracts water molecules and results in the large quantities of hydrated water surrounding hydroxyapatite. Since amorphous calcium phosphate precipitates are larger than hydroxyapatite crystals and consequently have a smaller surface area (11), the cumulative influence of the zeta potential of amorphous calcium phosphate is presumably less than that of hydroxyapatite. Nevertheless, the hydroxyapatite crystal content of potassium appears to decrease with crystal age (20).

The calcium/phosphate ratio of hydroxyapatite crystals can be systematically altered simply by changing the solution calcium/phosphate ratio. In this manner the net charge of the crystal can be altered and the corresponding zeta potential reflects this net crystal charge (12), as do the amounts of calcium and phosphate in the crystal hydration water (29). Even at a net crystal charge of zero, i.e., a calcium/phosphate molar ratio of 1.67, hydroxyapatite concentrates calcium and phosphate to a significant degree. Presumably, the alternating positive (calcium) and negative (phosphate) charges at the surface of the crystals can maintain a zeta potential without the presence of net crystal charge.

When hydroxyapatite is placed in a solution containing citrate, some surface phosphate is replaced by citrate and the crystals become more negatively charged (12). Consequently, citrate-containing crystals can be expected to have increased values of the concentrative factors for

cations such as calcium, sodium and potassium. The partial replacement of mineral calcium by sodium and the binding of carbonate and anionic proteins to the hydroxyapatite surface are other physiological mechanisms that might render the bone mineral more negative and thereby increase its ability to passively concentrate sodium and potassium. The finding that bicarbonate increased the concentrative factors for sodium and potassium in synthetic hydroxyapatite (21,37) and preliminary data (not reported) demonstrating that citrate acted in a similar fashion give further support to the involvement of these anions in the bone mineral concentrative phenomenon.

Although polyanions such as hyaluronic acid and chondroitin sulfate can conceivably concentrate sodium and potassium and might be responsible for the excess accumulation of sodium observed in cartilage (15), these substances make up only a small fraction of the organic matrix of bone (10). By far the most likely candidate responsible for passively concentrating sodium and potassium in the skeleton is the zeta potential resulting from the charge imbalances at the surfaces of hydroxyapatite crystals.

As discussed previously, the hydroxyapatite zeta potential concentrates phosphate as well as calcium and other cations. There is evidence suggesting that chloride and bicarbonate are also concentrated above blood levels in the bone extracellular fluid. Skeletal chloride is completely exchangeable (22,38) and exists in bone at a greater concentration than can be accounted for by assuming that bone water is an ultrafiltrate of plasma (13). Since cells contain lower concentrations of chloride than

extracellular fluid, correcting for cell volume only raises the bone extracellular fluid concentration of chloride. When the exchangeable bicarbonate of bone is expressed in terms of bone water, concentrative factors of greater than 10 have been obtained (33,38). Thus, bicarbonate alters the hydroxyapatite zeta potential by binding to the crystal surface and also concentrates in the hydration water in response to the zeta potential (21). The presence of variable quantities of both polarizable cations and anions in the skeleton insures that there is no net charge associated with the bone mineral.

BIBLIOGRAPHY

1. Alfrey, A.C. and N.L. Miller: Bone magnesium pools in uremia. J. Clin. Invest. 52, 3019-3027 (1973).

2. Alfrey, A.C., N.L. Miller and D. Butkus: Evaluation of body magnesium stores. J. Lab. Clin. Med. 84, 153-162 (1974).

3. Barr, L. and R.L. Malvin: Estimation of extracellular spaces of smooth muscle using different-sized molecules. Am. J. Physiol. 208, 1042-1045 (1965).

4. Clark, I. and Bélanger, L.: The effects of alterations in dietary magnesium on calcium, phosphate and skeletal metabolism. Calc. Tiss. Res. 1, 204-218 (1967).

5. Cremaschi, D. and S. Hénin: Extracellular space determination in gall bladder mucosa. Biochim. Biophys. Acta 411, 291-294 (1975).

6. Forbes, G.B., G.L. Mizuer and A. Lewis: Effect of age on radio-sodium exchange in bone (rat). Am. J. Physiol. 190, 152-156 (1957).

7. Forbes, G.B., R.B. Tobin and A. Lewis: Response of bone sodium to acute changes in extracellular fluid composition (cat). Am. J. Physiol. 196, 69-73 (1959).

8. Grim, E., A. Simonds, C. Oeljen and J. Cheng: Inulin and mannitol distribution volumes in canine small intestinal tissues in vivo. Am. J. Physiol. 224, 186-190 (1973).

9. Kimmich, G.A.: Personal communication (1978).

10. Herring, G.M.: Methods for the study of the glycoproteins and proteoglycans of bone using bacterial collagenase: Determination of bone sialoprotein and chondroitin sulfate. Calc. Tiss. Res. 24, 29-36 (1977).

11. Holmes, J.M. and R.A. Beebe: Surface areas by gas adsorption on amorphous calcium phosphate and crystalline hydroxyapatite. Calc. Tiss. Res. 7, 163-174 (1971).

12. Leach, S.A.: Electrophoresis of synthetic hydroxyapatite. Arch. Oral Biol. 3, 48-56 (1960).

13. Lobeck, C.C.: Studies on chloride of bone in cat and rat. Proc. Soc. Exptl. Biol. Med. 98, 856-860 (1958).

14. MacManus, J. and F.W. Heaton: The influence of magnesium on calcium release from bone in vitro. Biochim. Biophys. Acta 215, 360-367 (1970).

15. Manery, J.F.: Connective tissue electrolytes. Fed. Proc. 25, 1799-1803 (1966).

16. Martindale, L. and F.W. Heaton: The relation between skeletal and extracellular fluid magnesium in vitro. Biochem. J. 97, 440-443 (1965).

17. Neuman, M.W.: Personal communication (1978).

18. Neuman, W.F. and M.W. Neuman: The Chemical Dynamics of Bone Mineral. Univ. of Chicago Press, Chicago, 1958.

19. Neuman, W.F., T.Y. Toribara and B.J. Mulryan: Synthetic hydroxyapatite crystals. I. Sodium and potassium fixation. Arch. Biochem. Biophys. 98, 384-390 (1962).

20. Neuman, W.F., R. Bjornerstedt and B.J. Mulryan: Synthetic hydroxyapatite crystals. II. Aging and strontium incorporation. Arch. Biochem. Biophys. 101, 215-224 (1963).

21. Neuman, W.F. and B.J. Mulryan: Synthetic hydroxyapatite crystals. III. The carbonate system. Calc. Tiss. Res. 1, 94-104 (1967).

22. Neuman, W.F., A.R. Terepka, F. Canas and J.T. Triffitt: The cycling concept of exchange in bone. Calc. Tiss. Res. 2, 262-270 (1968).

23. Neuman, W.F.: The milieu interieur of bone: Claude Bernard revisited. Fed. Proc. 28, 1846-1850 (1969).

24. Neuman, W.F. and B.J. Mulryan: On the nature of exchangeable sodium in bone. Calc. Tiss. Res. 3, 261-265 (1969).

25. Neuman, W.F. and B.J. Mulryan: Synthetic hydroxyapatite crystals. IV. Magnesium incorporation. Calc. Tiss. Res. 7, 133-138 (1971).

26. Neuman, W.F. and W.K. Ramp: The concept of a bone membrane: Some implications. In, Cellular Mechanisms for Calcium Transfer and Homeostasis, ed. by G. Nichols, Jr. and R.H. Wasserman. Academic Press, New York, pp. 197-206, 1971.

27. Neuman, W.F., B.J. Mulryan, M.W. Neuman and K. Lane: Calcium transport systems in the chick calvaria. Am. J. Physiol. 224, 600-606 (1973).

28. Neuman, W.F. and B.J. Bareham: Further studies on the nature of fluid compartmentalization in chick calvaria. Calc. Tiss. Res. 17, 249-255 (1975).

29. Neuman, W.F.: Personal communication (1978).

30. Nielson, S.P.: Effects of magnesium on the calcification of young bone in tissue culture. Calc. Tiss. Res. 11, 78-94 (1973).

31. Pak, C.Y.C. and F.C. Bartter: Ionic interaction with bone mineral. II. The control of Ca^{2+} and PO_4^{3-} exchange by univalent cation-Ca^{2+} substitution at the hydroxyapatite crystal surface. Biochim. Biophys. Acta 141, 410-420 (1967).

32. Palmer, L.G. and J. Gulati: Potassium accumulation in muscle: A test of the binding hypothesis. Science 194, 521-523 (1976).

33. Poyart, C.F., E. Bursaux and A. Fréminet: The bone CO_2 compartment: Evidence for a bicarbonate pool. Respiration Physiol. 25, 89-99 (1975).

34. Ramp, W.K., J.R. Thomas and P.D. Nifong: Magnesium and mineral metabolism of chick embryo tibiae in organ culture. Calc. Tiss. Res. 24, 93-98 (1977).

35. Reddy, C.R., J.W. Coburn, D.L. Hartenbower, R.M. Friedler, A.S. Brinkman, S.G. Massry and J. Jowsey: Studies on mechanisms of hypocalcemia of magnesium depletion. J. Clin. Invest. 52, 3000-3010 (1973).

36. Scarpace, P.J. and W.F. Neuman: The blood:bone disequilibrium. I. The active accumulation of K^+ into the bone extracellular fluid. Calc. Tiss. Res. 20, 137-149 (1976).

37. Stroll, W.R. and W.F. Neuman: The surface chemistry of bone mineral. X. The lack of interaction between sodium and carbonate ions. J. Physical Chem. 62, 377-379 (1958).

38. Triffitt, J.T., A.R. Terepka and W.F. Neuman: A comparative study of the exchange in vivo of major constituents of bone mineral. Calc. Tiss. Res. 2, 165-176 (1968).

39. Triffitt, J.T. and W.F. Neuman: The uptake of sodium-22 by bone and certain soft tissues. Calc. Tiss. Res. 6, 70-76 (1970).

CHAPTER THREE

STUDIES ON THE DISTRIBUTION OF WATER AND POTASSIUM IN NEONATAL RAT CALVARIA

INTRODUCTION

This study was undertaken in an attempt to explore the possible existence of an endocranial versus ectocranial asymmetry in the potassium concentration in neonatal rat calvaria. A potassium asymmetry in these rapidly growing bones was expected for the following reasons:

1) Bone potassium is decreased in hypophysectomized rats (1), and during vitamin D deficiency in chicks (1) and has been claimed to fall with age (1,16). Estimates of the cellular content of neonatal rat (3) and chick (8,15) calvaria suggested that the majority of the potassium in these bones was extracellular. These findings, and others (7), indicated that the concentration of potassium in the bone ECF was elevated above blood levels and the highest potassium concentrations were found in growing bone.

2) Immature calvaria appeared to be an ideal system for biochemical studies of skeletal apposition and resorption. Calvarial growth was thought to consist of ectocranial apposition and simultaneous endocranial resorption. The Ussing chamber studies of Scarpace and Neuman (14), involving the release of ^{45}Ca from prelabeled chick calvaria, supported this viewpoint. Therefore, if bone potassium were concentrated at areas of bone growth, the ectocranial side of the neonatal rat calvarium should have contained a higher concentration of potassium than the endocranial side.

During the course of this study, the premise of ectocranial skull apposition and endocranial resorption was found to be no longer valid. Histological examinations revealed little, if any, osteoclastic activity on the endocranial surface of fetal rat calvaria (13). Improved techniques for measuring the unidirectional fluxes of calcium to and from bone demonstrated that mineral apposition occurred on both sides of neonatal rat and young chick calvaria (9). Utilizing staining with vital dyes, Moss and coworkers (5) have shown beyond doubt that apposition occurs on both endocranial and ectocranial sides of a growing calvarium. The data from this study suggest that the potassium concentration, expressed in terms of water content, is equal on both sides of the neonatal rat calvarium.

Despite the fact that the major premise of this study was later found to be invalid, the experiments undertaken to examine the possible potassium asymmetry revealed several interesting properties of the Ussing chamber calvarial system. In particular, the following conclusions appear to be of interest to the chamber studies:

1) The exchange of water, and probably that of other diffusible species, between the incubation buffer and bone tissue is not restricted to the exposed cross-sectional area defined by the chamber. Although substances must flow through this defined area to enter the calvaria, once inside they can diffuse outward, towards the periphery of the calvaria. Similarly, a substance produced in peripheral areas, for example lactate, can diffuse inward and then efflux from the bone into the buffer.

2) As determined by $^{3}H_2O$ and ^{42}K contents, the endocranial side of the neonatal rat calvarium contains a greater proportion of soft tissue mass than the ecotcranial side. If this soft tissue asymmetry is assumed to result from a greater quantity of cells on the endocranial side, then the reported asymmetry in neonatal rat calvaria lactate fluxes (10), may be attributed in part to a difference in cellular volume with the endocranial side having the greater cell mass and lactate production.

3) Although the flow of water from one side of the Ussing chamber to the other side is relatively slow, fluxes can be determined. These fluxes must be related in some fashion to the overall resistance to diffusional flow in the calvaria and the physiological interpretations of these fluxes await further exploration.

MATERIALS AND METHODS

In all studies 3- to 4-day-old rats of the Sprague-Dawley (Holtzman) strain were used. These rats weighed approximately 10 grams and were always returned to their mothers following injection of isotope. ^{42}K (0.14 mCi/mg) and $^{3}H_2O$ (100 mCi/gm) were purchased from New England Nuclear Corporation. Potassium was determined on an Instrumentation Laboratory Model 143 flame photometer. For tritium scintillation counting, a cocktail consisting of 4% BBS-3 (solubilizer) plus 4 grams/liter of Omnifluor dissolved in toluene was used. Quenching was monitored by the channels-ratio approach and was found not to be a problem.

Exchange Study

Rat pups were injected with 10 microliters of ^{42}K subcutaneously in the back at 29, 6 and 1 hours before sacrifice. Upon sacrifice, blood was collected in heparinized capillary tubes and centrifuged within the following 15 minutes. For each rat, the plasma from several tubes was pooled and then 30 microliters were taken for radioisotope counting. The calvarium, brain and liver were then quickly removed and an 8 millimeter diameter punch was used to obtain a reproducible calvarial sample. Parts of the brain and liver were weighed on a Cahn Model 7500-7 Millibalance. The three tissues were then counted with the corresponding plasma sample for two minutes and a background subtracted from each sample. Each tissue was then dissolved in 2 N HNO_3 and aliquots taken for analysis of total potassium by flame photometry.

Data for bone, liver and brain have been expressed as tissue ^{42}K CPM per μmole K divided by CPM in 30 μliters of plasma. In addition, the total potassium per punched-out section (calvaria) or per mg tissue wet weight (liver and brain) was calculated.

Asymmetry Study

Rat pups were injected subcutaneously with 10 μliters of $^{3}H_2O$ or ^{42}K eight to 16 hours before the start of the experiment. Calvaria were dissected out and clamped in the Ussing chambers of Scarpace and Neuman (14). Each side of the chamber was filled with 2.5 to 3.5 ml of medium consisting of 125 mM NaCl, 5.3 mM KCl, 1.75 mM Na_2HPO_4, 2.0 mM $CaCl_2$, 2.0 mM $MgSO_4$, 30 mM HEPES, 500 mg.% glucose, 100 units penicillin/ml, 100 μg streptomycin/ml, 2 mg/ml BSA and 5% heat-inactivated newborn calf serum. The medium was adjusted to a pH of 7.4 at 37° C with NaOH.

At the indicated times, aliquots of medium were taken from both sides of the chamber and counted for either ^{42}K or $^{3}H_2O$. The total amount of isotope that had effluxed from the calvarium was calculated from these sample aliquots without correcting for the effect of the changing medium volume with sampling. An endocranial/ectocranial ratio of counts was calculated for each time point and the mean $\pm$ SEM determined for each bone sample. After taking the last sample, the calvaria were removed from the chambers, counted and the per cent of isotope that effluxed from the calvarium was calculated. In the $^{3}H_2O$ experiment 5 μl samples of plasma were also taken and used to determine the total volume of H_2O that was initially present in each calvarium.

RESULTS

Previous experience involving Ussing chamber transport studies (14) has demonstrated that the large calvarial mineral pool contains an excess of exchangeable calcium and phosphate sufficient to effectively prohibit the flow of calcium and phosphate isotopes between sides of the Ussing chamber. A preliminary experiment with 20-day embryonic chick calvaria was undertaken to quantitate the flow of $^{3}H_2O$ from one side of the Ussing chamber to the other side. The results are shown in Figure 1. Although $^{3}H_2O$ did cross the calvaria, the time required for full equilibration was many hours. After one hour, approximately 2% of the 3.2 ml of medium water on the two sides of chamber had exchanged. This exchange was slow enough so that any asymmetry present in the release of $^{3}H_2O$ from prelabelled calvaria could be adequately detected in analyses of medium from each side of the chamber. ^{42}K, being charged and hydrated, can be expected to have a slower trans-calvarial flux than $^{3}H_2O$. As indicated by the data presented in Tables 2 and 3, the small trans-calvarial fluxes of $^{3}H_2O$ and ^{42}K did not interfere with the asymmetry studies.

The potassium contents of neonatal rat brain and liver taken during the ^{42}K exchange study are given in Table 1. For comparative purposes, data from Ginsburg and Wilde (4) on 125-175 gram rats are included. Values of calvaria potassium contents taken during separate experiments are also presented. The older rats appear to have slightly higher potassium contents in liver and brain. Bone contains a lower

FIGURE 1. The Transfer of Medium Between Sides of the Ussing Chamber.

Twenty-day embryonic chick calvaria were dissected out and placed in the Ussing chambers. At time zero 3.2 ml of medium containing $^{3}H_2O$ at a concentration of 29,472 $\pm$221 CPM/50 µl (mean $\pm$ SEM, n = 12) were placed on the endocranial side of the calvaria and simultaneously 3.2 ml of unlabeled medium were placed on the ectocranial side of the calvaria. At various periods of time 50 µl aliquots of medium were taken from the ectocranial side of the calvaria and counted for $^{3}H_2O$ by liquid scintillation spectroscopy. The data are expressed both as CPM/50 µl appearing on the ectocranial side and as the per cent of the total medium water that transversed the calvaria. Background CPM were not subtracted from the data.

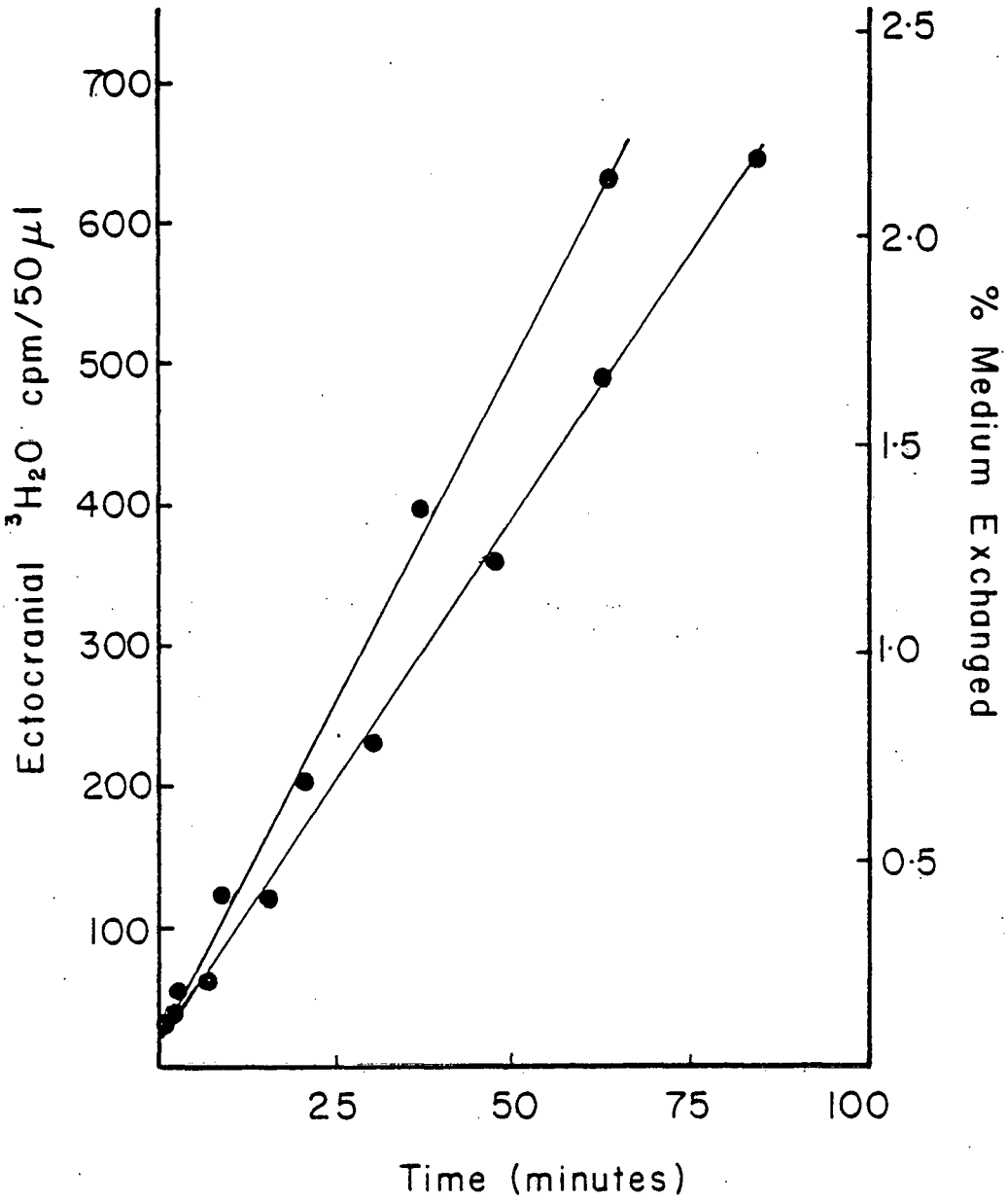

Ectocranial $^{3}H_2O$ cpm/50 µl
700
600
500
400
300
200
100
2·5
2·0
1·5
1·0
0·5
% Medium Exchanged
25
50
75
100
Time (minutes)

TABLE 1

Tissue Potassium Contents

Tissue	Potassium Content (μmoles/gm wet weight) 10 gm Rats	125-175 gm rats
Brain	74.4 ±0.6 (12)	98.6 ±2.2 (11)[a]
Liver	88.1 ±0.8 (12)	95.0 ±0.9 (61)[a]
Calvaria[b]	48.3 ±0.6 (9)	19.0 ±0.8 (9)

The data are given as the mean ± SEM for the no. of samples indicated in parentheses.

[a]Data taken from Ginsburg and Wilde (4).

[b]When expressed in terms of total bone water, the potassium concentrations of 10 and 125-175 gm rats are 68.2 ±0.8 and 62.9 ±1.8 mM, respectively (mean ± SEM, n = 9).

content of potassium than soft tissues due to a greater proportion of extracellular space and the presence of calcium-phosphate salts.

The relative exchange of potassium with time in liver, brain and calvaria is given in Figure 2. In agreement with previous studies on older rats (4,11,12,17), neonatal liver and bone exchange potassium rapidly while the exchange of potassium in brain is much slower. The existence of the capillary endothelial blood-brain barrier presumably accounts for the slow exchange of brain potassium.

The asymmetry data for $^{3}H_2O$ and ^{42}K are given in Tables 2 and 3, respectively. In both cases the flux of these isotopes out of the calvaria was very rapid as the samples taken during the first time point contained greater than one-half of the total counts that finally did efflux. Approximately two-thirds of the isotopes came out of the calvaria during the course of the experiment and the isotope remaining appears to be located in the fraction of the calvarium located far outside the 0.6 mm diameter opening and therefore not accessible to the medium for exchange. There was a slight asymmetry in the neonatal rat calvarial contents of both water (1.22 $\pm$.04) and potassium (1.13 $\pm$.08) with the endocranial side having the greater contents.

FIGURE 2. The Accumulation of ^{42}K in Liver, Calvaria and Brain as a Function of Time.

Rat pups were injected subcutaneously with ^{42}K at time zero. The data are expressed as the mean $\pm$ SEM for animals sacrificed at one hour (n = 3), six hours (n = 4) and 29 hours (n = 5).

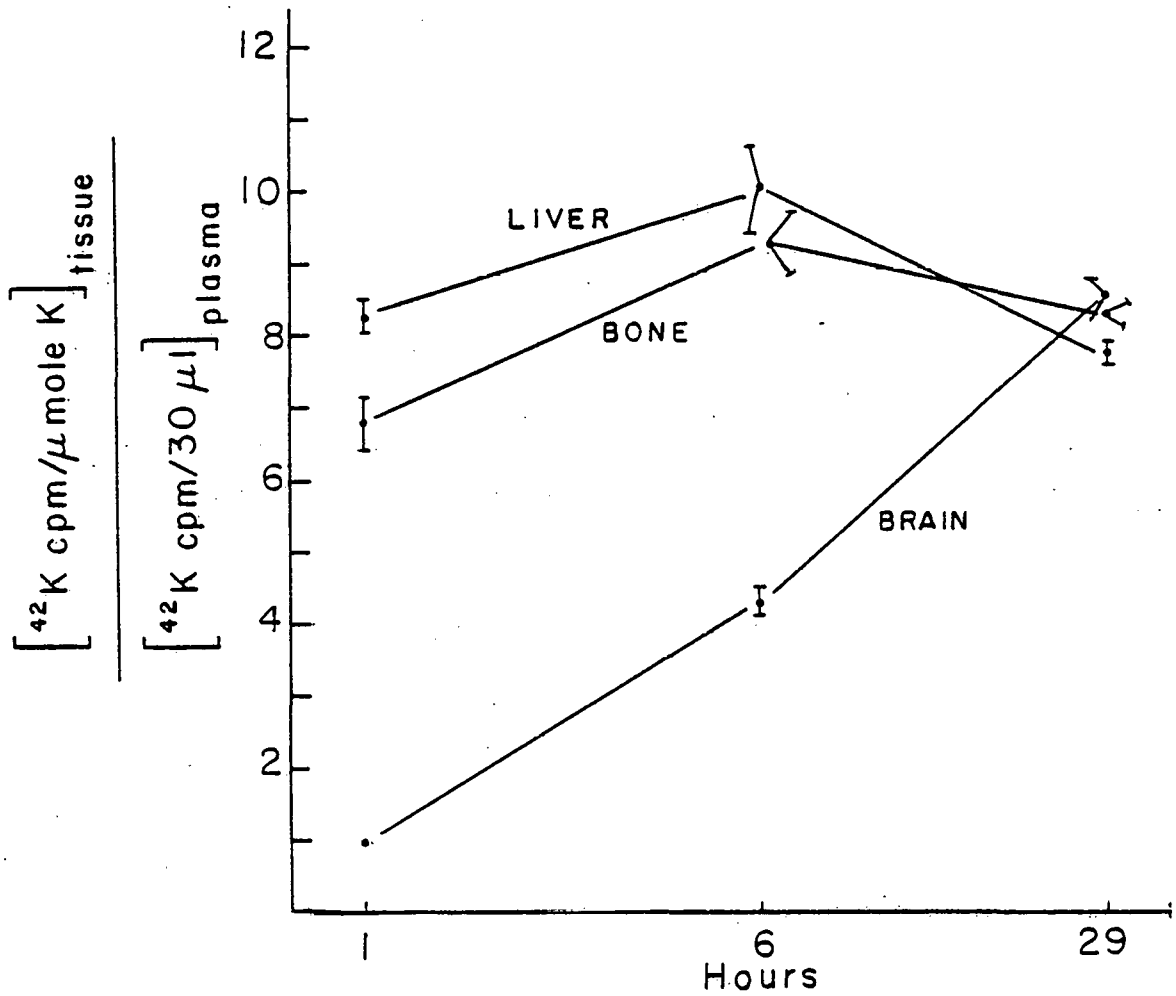

$\frac{[^{42}K\ cpm/\mu mole\ K]_{tissue}}{[^{42}K\ cpm/30\ \mu l]_{plasma}}$
12
10
8
6
4
2
LIVER
BONE
BRAIN
1
6
29
Hours

TABLE 2

Asymmetry of Calvaria Water

Sample	Times (min.)	Total H_2O (µl)	% H_2O Effluxed	Asymmetry
1	10,15	-	-	1.14 ±0.03
2	5,10,15,20	11.7	80	1.21 ±0.03
3	1,2,3,5,7,10,20	16.9	69	1.10 ±0.06
4	10,20	15.7	72	1.37 ±0.02
5	10,20	12.1	76	1.24 ±0.02
6	2,4,6,8,17	15.3	66	1.27 ±0.02
		14.3 ±1.0	73 ±2	1.22 ±0.04[a]

Asymmetry of calvaria water is defined as the mean ratio of the endocranial to ectocranial CPM of ^3H_2O in the medium for the times indicated.

[a]This value is the mean ± SEM of the individual asymmetries and is statistically different from 1.0 as evaluated by the Student t-test at $p < .01$.

TABLE 3

Asymmetry of Calvaria Potassium

Sample	Times (min.)	% ^{42}K Effluxed	Asymmetry
1	5,10,15	63	1.05 ±0.03
2	5,15	56	0.93 ±0.03
3	10,20,30	64	1.26 ±0.01
4	1,3,5	47	1.50 ±0.07
5	1,3,5	47	0.86 ±0.02
6	10,20,30	59	1.13 ±0.02
7	1,3,5	47	1.68 ±0.22
8	10,20,30	73	1.07 ±0.03
9[a]	5,10,15	78	1.04 ±0.01
10[a]	5,10,15	72	1.14 ±0.01
11[a]	5,10,15	61	0.80 ±0.03
			1.13 ±0.08[b]

Asymmetry of calvaria potassium is defined as the mean ratio (given with SEM) of the endocranial to ectocranial CPM of ^{42}K in the medium for the times indicated.

[a]These calvaria were briefly wiped with tissue paper before being placed in the Ussing chambers in order to determine if any excess fluid was an artifact of the dissection procedure. Wiping did not appear to have any effect.

[b]This value is the mean ± SEM of the individual asymmetries and is statistically different from 1.0 as evaluated by the Student t-test at $p < .10$.

DISCUSSION

The rate of exchange of any isotope in bone depends upon blood flow and the concentration of the material in both blood and bone. A simple model describing exchange in bone predicts an exchange time of 160 minutes for potassium in a 200 gram rat (6). This rather rapid exchange has been verified (6,11,12,15,16) and is not surprising considering the fact that potassium exchanges rapidly in most tissues (17), due in part to almost complete extraction from the capillaries with only one passage of blood.

The data presented in Figure 2 show that, with respect to potassium exchange, the neonatal rat is similar to older animals. Bone and liver have a rapid exchange of potassium while exchange is much slower in brain. Assuming a plasma potassium concentration of approximately 4 mM, the exchange of potassium in liver and calvaria was complete in approximately one hour. Full equilibration of bone potassium insures that the effluxing potassium in the Ussing chamber asymmetry experiments represents the entire potassium pool in bone. Edelman et al. (2) have shown full exchange of bone water in dogs at two to four hours so that $^{3}H_2O$ is a good marker for total bone water.

Since the effluxing isotopes came from a volume of approximately 10 µl and entered a volume of approximately 6 ml, backflux must have been negligible. Isotope effluxing into one side of the chamber could not flow through the entire bone into the other side of the chamber to any appreciable extent (Figure 1). Consequently, any asymmetry in

water or potassium contents that existed in the calvarium would be measured in this system.

Since the efflux of $^{3}H_2O$ and ^{42}K occur so rapidly, actual flux values cannot be obtained. Rather, the data indicate the total amounts of isotope present in the endocranial and ectocranial sides of the calvaria. These measurements do not distinguish between the relative proportion of water or potassium present in intracellular or extracellular spaces. Preliminary estimates of the extracellular fluid space with inulin in neonatal rat calvaria (3) and mannitol in four-day-old chick calvaria (8) have given values of 85 and 80% of the total water, respectively. The proportion, and hence concentration, of potassium in the bone extracellular fluid is still undetermined.

The contents of water and potassium in the neonatal rat calvarium appear to be greater on the endocranial side than the ectocranial side. The simplest interpretation of this finding is that there is slightly more soft tissue endocranially. Such an increased tissue volume probably corresponds to a greater cell mass and would explain, in part, the greater production of lactate by the endosteal surface (10). The ratio of the asymmetry of the potassium content to that of water content gives a reasonable indication of the asymmetry of the potassium concentration in the total bone water. Since the asymmetry in the potassium content (1.13 ± 0.08) is close to that of the water content (1.22 ± 0.04), the total potassium concentration appears to be nearly equal on endocranial and ectocranial sides of the calvarium.

As described in the Introduction, recent studies involving histology, vital staining and calcium flux determinations have conclusively demonstrated net appositional growth on both sides of the developing calvarium. Therefore, the lack of a demonstrable asymmetry in potassium concentration (1.13 $\pm$0.08/1.22 $\pm$0.04) reported in this study is of no help in discussions of the dependency of bone potassium levels upon skeletal growth. However, as seen in Table 1 and discussed in Chapter 1, more recent evidence does not indicate that bone potassium concentration falls with age.

From the data presented in Table 2, an average of 73% of the 14.3 µl of ^3H_2O initially present in the calvaria - or 10.4 µl - had effluxed through the 0.283 cm^2 opening of the Ussing chamber. Since these calvaria are known to contain 15.6 µl of water per cm^2 (10), only 4.4 µl of the 10.4 µl of water that effluxed could have been initially present within the 0.283 cm^2 area. Clearly, water can diffuse inside these calvaria beyond the area defined by the opening in the chamber. No doubt other diffusible substances can also move in a similar fshion inside the calvaria. This phenomenon may contribute to the large rate of lactate production by the neonatal rat pup calvaria (10).

From the data in Figure 1, the calculated unidirectional flux of ^3H_2O through embryonic chick calvaria is approximately 12 mmole/cm^2/hr. In terms of volume, an equivalent of 60 µl of water flow through the 0.283 cm^2 chamber opening per hour from each side of the chamber. Since these values are far greater than the actual water contents of the calvaria, the diffusion of water appears to be relatively uninhibited

by the presence of the calcified matrix. Further knowledge on the diffusibility of other substances through the calvaria would be of great interest in analyzing the availability of substrates and removal of metabolites to and from individual bone cells.

BIBLIOGRAPHY

1. Canas, F., A.R. Terepka and W.F. Neuman: Potassium and the milieu intérieur of bone. Am. J. Physiol. 217, 117-120 (1969).

2. Edelman, I.S., A.H. James, H. Baden and F.D. Moore: Electrolyte composition of bone and the penetration of radiosodium and deterium oxide into dog and human bone. J. Clin. Invest. 33, 122-131 (1954).

3. Geisler, J.Z. and W.F. Neuman: The membrane control of bone potassium. Proc. Soc. Exptl. Biol. Med. 130, 608-612 (1969).

4. Ginsburg, J.M. and W.S. Wilde: Distribution kinetics of intravenous radiopotassium. Am. J. Physiol. 179, 63-75 (1954).

5. Moss, M.L., M-A. Meehan and L. Salentijn: Transformative and translative growth processes in neurocranial development of the rat. Acta Anatomica 81, 161-182 (1972).

6. Neuman, W.F., A.R. Terepka, F. Canas and J.T. Triffitt: The cycling concept of exchange in bone. Calc. Tiss. Res. 2, 262-270 (1968).

7. Neuman, W.F.: The milieu intérieur of bone. Claude Bernard revisited. Fed. Proc. 28, 1846-1856 (1969).

8. Neuman, W.F. and B.J. Bareham: Further studies on the nature of fluid compartmentalization in chick calvaria. Calc. Tiss. Res. 17, 249-255 (1975).

9. Neuman, W.F., R. Brommage and C.R. Myers: The measurement of Ca^{2+} effluxes from bone. Calc. Tiss. Res. 24, 113-117 (1977).

10. Neuman, W.F., M.W. Neuman and R. Brommage: Aerobic glycolysis in bone: Lactate production and gradients in calvaria. Am. J. Physiol. 234, C41-C50 (1978).

11. Norman, N.: The participation of bone in the sodium and potassium metabolism in the rat. I. Simultaneous determination of the exchangeable body sodium and potassium, and the exchangeable and inexchangeable fractions of these ions in bone of the normal rat. Acta Physiol. Scand. 57, 363-372 (1963).

12. Olsson, K.A., R. Söremark and K.R. Wing: Uptake and distribution of rubidium-86 and potassium-43 in mice and rats--an autoradiographic study. Acta Physiol. Scand. 77, 322-332 (1969).

13. Rifkin, B.R.: Personal communication (1977).

14. Scarpace, P.J. and W.F. Neuman: Quantitation of calcium fluxes in chick calvaria. Biochim. Biophys. Acta 323, 267-275 (1973).

15. Scarpace, P.J. and W.F. Neuman: The blood:bone disequilibrium. I. The active accumulation of K^+ into the bone extracellular fluid. Calc. Tiss. Res. 20, 137-149 (1976).

16. Triffitt, J.T., A.R. Terepka and W.F. Neuman: A comparative study of the exchange in vivo of major constituents of bone mineral. Calc. Tiss. Res. 2, 165-176 (1968).

17. Wilde, W.S.: Potassium. In, Mineral Metabolism, vol. II, Part B pp. 73-107, 1962.

CHAPTER FOUR

MECHANISM OF THE MOBILIZATION OF BONE MINERAL BY 1,25-DIHYDROXYVITAMIN D_3

INTRODUCTION

Vitamin D plays an important role in the proper functioning of the skeleton. Unmineralized osteoid, a sign of rickets and osteomalacia, results from vitamin D-deficiency and the ability of vitamin D to mobilize bone calcium was demonstrated by Carlsson over 20 years ago (3). Despite these widely accepted interactions between vitamin D and bone, very little is known about the mechanism by which vitamin D acts on the skeleton.

Following the synthesis of high specific activity radiolabeled vitamin D_3, the hydroxylation of this compound to 25-hydroxycholecalciferol [25-$(OH)D_3$] and subsequent conversion to either 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$] or 24,25-dihydroxycholecalciferol [24,25-$(OH)_2D_3$] was demonstrated (10,18). The kidneys synthesize 1,25-$(OH)_2D_3$ from 25-$(OH)D_3$ in a regulated fashion and this 1,25-$(OH)_2D_3$ is believed to be the physiologically active metabolite of vitamin D_3 (10,18). When given to vitamin D-deficient animals, 1,25-$(OH)_2D_3$ localizes in bone and intestine and is the major metabolite found in these target tissues (12,66). Studies on the intestine suggest 1,25-$(OH)_2D_3$ has a mechanism of action similar to that of other steroid hormones; i.e., cytosolic receptor binding and activation followed by the translocation of the hormone-receptor complex to the nucleus where the promotion of the synthesis of specific proteins occurs (16).

1,25-$(OH_2)D_3$ mobilizes skeletal mineral in rats (4,19,59) and induces bone mineral resorption in tissue culture (45,53).

The presently available evidence suggests that 1,25-$(OH)_2D_3$ mobilizes skeletal calcium and phosphate to maintain the blood levels of these ions and that the skeletal mineralization observed after vitamin D repletion in deficient animals results from the increased intestinal absorption of calcium and phosphate (52). In other words, bone mineralization proceeds as long as normal blood levels of calcium and phosphate are present (8,64). 1,25-$(OH)_2D_3$, in concert with parathyroid hormone [PTH], maintains serum calcium and phosphate during dietary shortages of these ions by promoting their release from the skeleton.

Recently, a high affinity receptor for 1,25-$(OH)_2D_3$ has been identified in chick and fetal rat calvaria (22). Presumably, the 1,25-$(OH)_2D_3$-receptor complex induces the synthesis of a protein(s) which sets in motion a series of events resulting in the net flow of calcium and phosphate from bone to blood (8,9). There exist at least three mechanisms by which this mineral mobilization might occur (2). Bone cells have been proposed to [1] actively pump calcium and phosphate from the bone extracellular fluid [ECF] to blood (48,54,57); [2] maintain a variable but regulated pH gradient between the bone ECF and blood (33,35); and [3] secrete a mineral "solubilizer" (2). This investigation was undertaken to help decide among these theories by determining whether 1,25-$(OH)_2D_3$ alters bone lactate production and/or the mineral solubility of non-vital bone.

MATERIALS AND METHODS

The conditions of incubation used in these expeiments are modifications from the fetal rat long bone system of Raisz (44) and the neonatal mouse calvaria system of Reynolds (51). The electrolyte content of the medium was altered to reflect more closely the neonatal mouse levels shown in Table 1. Calvaria were "submerged" in the medium rather than placed at an air-fluid interface on mesh grids in petri dishes. Since ^{45}Ca release did not adequately reflect calcium movement (see Figure 2), measurements of total medium and buffer calcium and phosphate concentrations were used to determine mineral mobilization and solubility, respectively. Messer et al. (30) have described a similar problem with using ^{45}Ca in their studies. The 20 ng dose of 1,25-$(OH)_2D_3$ injected into the mice was taken from the midpoint of the dose response curve reported by Reynolds et al. (53). Since the mice weighed about three grams, the peak concentration of 1,25-$(OH)_2D_3$ was approximately 25 nanomolar.

Four- to five-day-old mice were sacrificed by decapitation and their calvaria (frontal and parietal bones) removed by careful dissection. All dissecting instruments were cleaned with 70% alcohol between dissections. The calvaria were placed in individual 13 x 100 mm borosilicate glass test tubes containing either 1 ml of medium or 0.75 ml of buffer (to be described below) and incubated for various periods of time at 37° C under an atmosphere saturated with water and containing 5% CO_2. At the conclusion of the incubation the medium (buffer) was collected and stored for analysis.

The medium used for these experiments consisted of a modified BGJ_b mixture (44). A 10x concentrate of BGJ_b amino acids and vitamins (except ascorbic acid) was purchased from Grand Island Biological Company and stored at 4° C until used. This concentrate was diluted 10x such that the resulting mixture contained the BJG_b concentrations of ascorbic acid, sodium acetate, penicillin and streptomycin and, in addition, 1 mM $MgSO_4$, 5 mM KCl, 25 mM $NaHCO_3$, 600 mg.% glucose, 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES], 50 mM NaCl and varying concentrations of $CaCl_2$ and Na_2HPO_4. Then, this mixture was further diluted by 5% with heat-inactivated newborn calf serum (Grand Island Biological Company). This medium was warmed to 37° C and the pH adjusted to 7.4 with 4 N NaOH. Filtering the medium through a 0.45 micron Millipore filter removed any bacteria present. One ml of this medium was dispensed into previously autoclaved test tubes and rewarmed to 37° C before addition of the calvaria.

The buffer used in these experiments consisted of 90 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 25 mM HEPES, 0.5 mM $MgSO_4$, 100 units penicillin/ml and 100 µg streptomycin/ml. The buffer pH was adjusted to 7.4 at 37° C with 4 N NaOH. Three-quarters of a ml of buffer per test tube was used for each calvarium.

Medium and buffer calcium concentrations were measured either by atomic absorption spectroscopy or ethyleneglycol-bis-(β-aminoethyl ether)N,N'tetraacetic acid [EGTA] titration on a Precision Systems Model 4008 Calcette titrator. Atomic absorption spectroscopy was

carried out on an Instrumentation Laboratories Model 153 spectrometer using strontium as an internal standard with 0.1% lanthanum, 0.5% HCl, 32.5 μg/ml of $SrCl_2 \cdot H_2O$ and 0.2% nonionic detergent as the diluent. Glucose was analyzed by the glucose oxidase method of Raabo and Teikeldsen (42) as modified in Sigma Technical Bulletin #510. For analyses of phosphate and lactate, protein was first precipitated with 8% perchloric acid. Phosphate was determined by the method of Chen et al. (6) while lactate determinations involved measuring the amount of β-nicotinamide adenine dinucleotide [NADH] produced from NAD^+ in the presence of lactate dehydrogenase as described in Sigma Technical Bulletin 826-UV. Plasma sodium and potassium were measured with an Instrumentation Laboratories Model 143 Flame Photometer while plasma calcium and phosphate were assayed as described above.

In one experiment 2 μCi of ^{45}Ca was injected subcutaneously into one-day-old mice and these mice were sacrificed four days later. Following the incubation each calvarium was wiped clean of medium and then extracted in 0.5 ml of 2 N HNO_3. Both medium and calvaria extracts were analyzed for ^{45}Ca content by liquid scintillation spectroscopy using 4% Beckman Biosolve BBS-3 in an Omnifluor-toluene cocktail. The bone extract was diluted 11-fold and analyzed for total calcium and phosphate as described previously. Bone "resorption" was calculated as per cent of the total calcium, phosphate and ^{45}Ca originally present in the calvaria which was released into the medium during the incubation. A negative value for resorption indicates the calvaria took up the

substance from the medium. The cells in some bone samples were "killed" by placing the calvaria in the usual medium following the dissection and then freezing and thawing the entire test tube contents 3x by repeated immersion into dry ice-acetone mixture.

For analyses of mouse pup plasma, blood was collected at decapitation in heparinized capillary tubes, centrifuged and the plasma removed. Plasma obtained in this fashion from 21 pups was pooled and then analyzed. The $1,25\text{-}(OH)_2D_3$ was a generous gift from Dr. Milan R. Uskokovic' of Hoffman-LaRoche, Inc. In initial experiments this compound was weighed and dissolved in 95% ethanol before injection into the mouse pups, but in later experiments (Table 2, Experiment 2 and Figure 4) the actual concentration of $1,25\text{-}(OH)_2D_3$ was determined from the ultraviolet absorption spectrum by using a molar extinction coefficient of 18,200 at 265 mμ.

RESULTS

The concentrations of calcium, phosphate, sodium and potassium in the plasma of neonatal mice are listed in Table 1. As has been reported for the rat (13), blood calcium tends to be high (11 mg.%) during the first week of life. Likewise, blood potassium is high at birth and gradually falls with age (56). Sodium and phosphate appear to be close to adult levels. The hematocrit value of 34 is in agreement with the low values observed in newborn animals.

The effects of omitting either glucose or HEPES from the medium are shown in Figure 1. The calvaria did not produce any lactate when glucose was not present and consequently medium glucose appears to be the sole substrate available for lactate production. The small amount of lactate present in the medium results from the inclusion of the 5% heat-inactivated newborn calf serum. In the absence of added glucose the calvaria must soon exhaust their energy reserves resulting in a decrease in medium levels of calcium and phosphate.

The use of HEPES to buffer the medium pH resulted in a more linear rate of lactate production. Since acid pH is known to inhibit bone lactate production (37), lactate already produced lowers the medium pH and inhibits the further production of lactate. By increasing the buffering capacity of the medium with the inclusion of HEPES, the fall in pH was decreased and lactate production proceeded at nearly the initial rate. As observed earlier (35), maintaining the medium pH also

TABLE 1

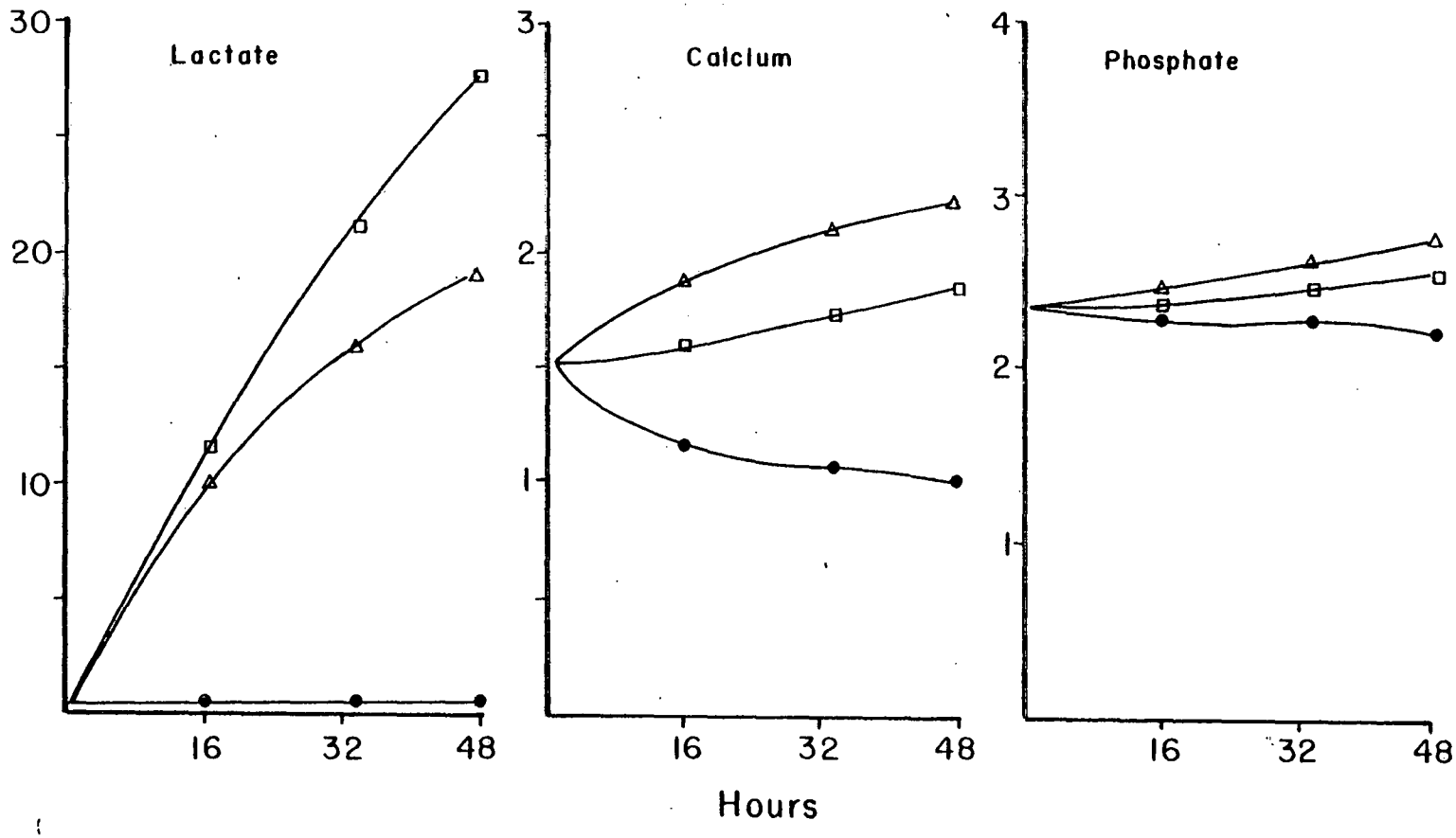
MOUSE PUP PLASMA ELECTROLYTE LEVELS

Calcium	10.94 mg.%
Phosphate	1.52 mM
Sodium	139.6 mM
Potassium	6.19 mM

Each value is the mean of a triplicate determination from 21 pooled plasma samples. The blood hematocrit was 34 $\pm$3 (standard deviation).

FIGURE 1. The Medium Concentrations of Lactate, Calcium and Phosphate are Given in mM.

Each point represents the mean from analyses of four calvaria. The medium was identical to that described in the text except for the following modifications: □ - 30 mM HEPES, 700 mg.% glucose; ● - 30 mM HEPES, no glucose; △ - 700 mg.% glucose, no HEPES.


Lactate
Calcium
Phosphate
30
20
10
3
2
1
4
3
2
1
16
32
48
16
32
48
16
32
48
Hours

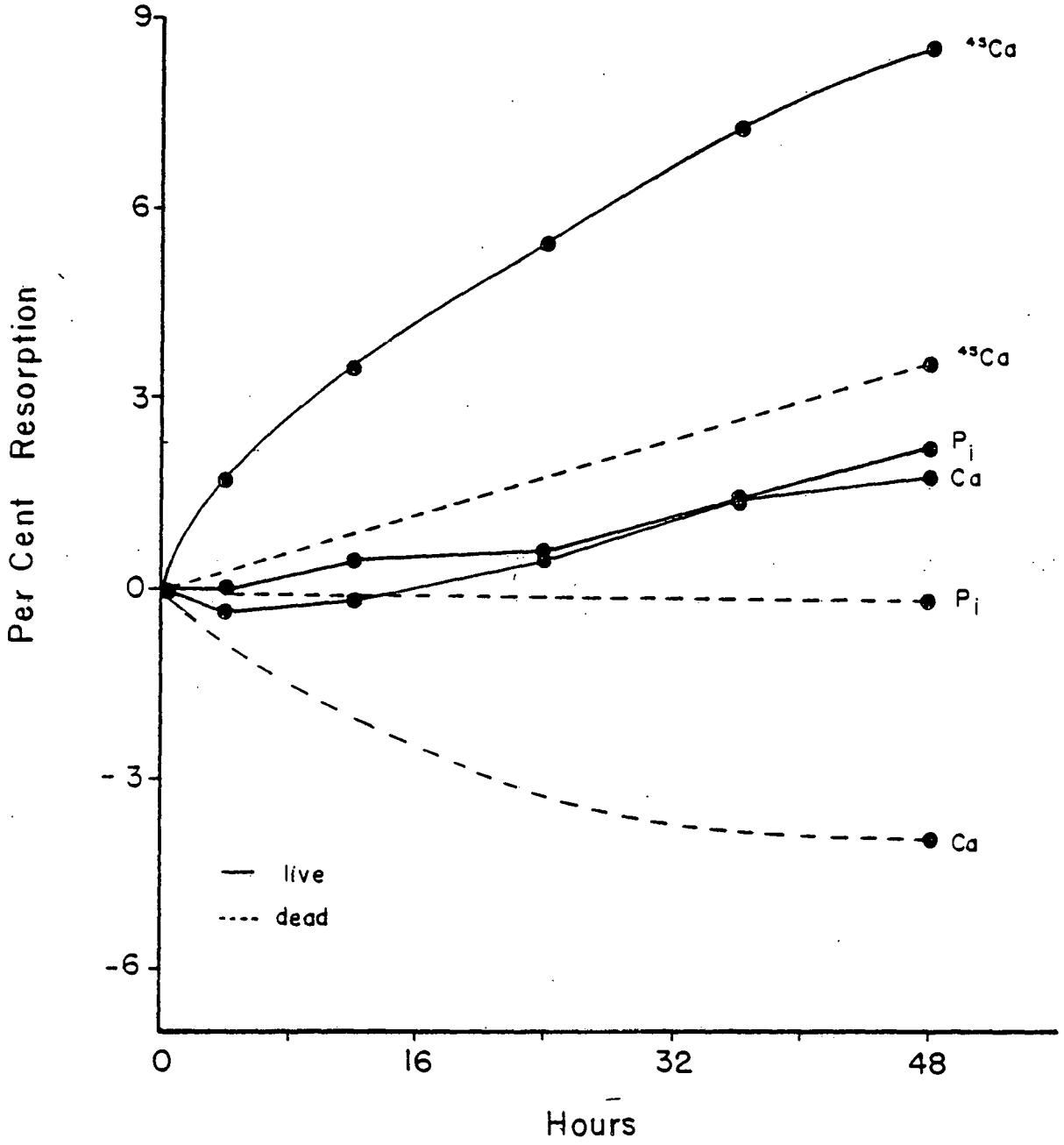
decreased the acid-induced solubilization of bone mineral and thereby minimized the rise in medium calcium and phosphate. No doubt the increases in medium calcium and phosphate seen in the presence of 30 mM HEPES result from a slight fall in medium pH. The concentration of HEPES in the medium was increased to 50 mM in the remaining experiments.

The results of an experiment in which the release of ^{45}Ca from prelabeled calvaria was measured are shown in Figure 2. Under normal incubation conditions ("live" bone) ^{45}Ca was released in a greater proportion than the release of total calcium. Thus, even four days after an exposure to a pulse injection, significant quantities of bone ^{45}Ca are readily exchangeable and released into the medium without the concurrent release of bone calcium. When the calvaria were "killed" by freezing and thawing three times ^{45}Ca was released into the medium despite the fact that calcium was actually taken up by the calvaria. Nevertheless, compared to "live" calvaria, "dead" calvaria released less ^{45}Ca to the medium.

Figure 2 also shows that calcium and phosphate were released into the medium in almost identical proportions during the course of the incubation and therefore either ion may be used as an index of bone mineral mobilization. In "dead" calvaria, as the calcium concentration fell, there was no change in the medium phosphate concentration. Apparently, the dead bone mineral underwent a spontaneous conversion to a higher Ca/P ratio (27,35,48). This phenomenon will be discussed in greater detail later.

FIGURE 2. The Time Course of the "Resorption" of Live and Dead Bone Mineral as Indicated by ^{45}Ca, Total Calcium and Total Phosphate.

Each point is the mean of 4 to 6 determinations. Solid lines indicate "live" bone, dotted lines indicate "dead" (3x frozen and thawed) bone. Per cent resorption is defined in the text. Initial medium calcium and phosphate levels were 1.53 and 1.62 mM, respectively. The calvaria had a Ca/P ratio of 1.52 $\pm$0.005 and a specific activity of 14,999 $\pm$163 CPM/μmole calcium (mean $\pm$ SEM, n = 30 in each case). The final calvaria Ca/P ratio of the "dead" calvaria was 1.549 $\pm$0.014 (n = 6).


9
6
3
0
-3
-6
Per Cent Resorption
0
16
32
48
Hours
45Ca
45Ca
Pi
Ca
Pi
Ca
live
dead

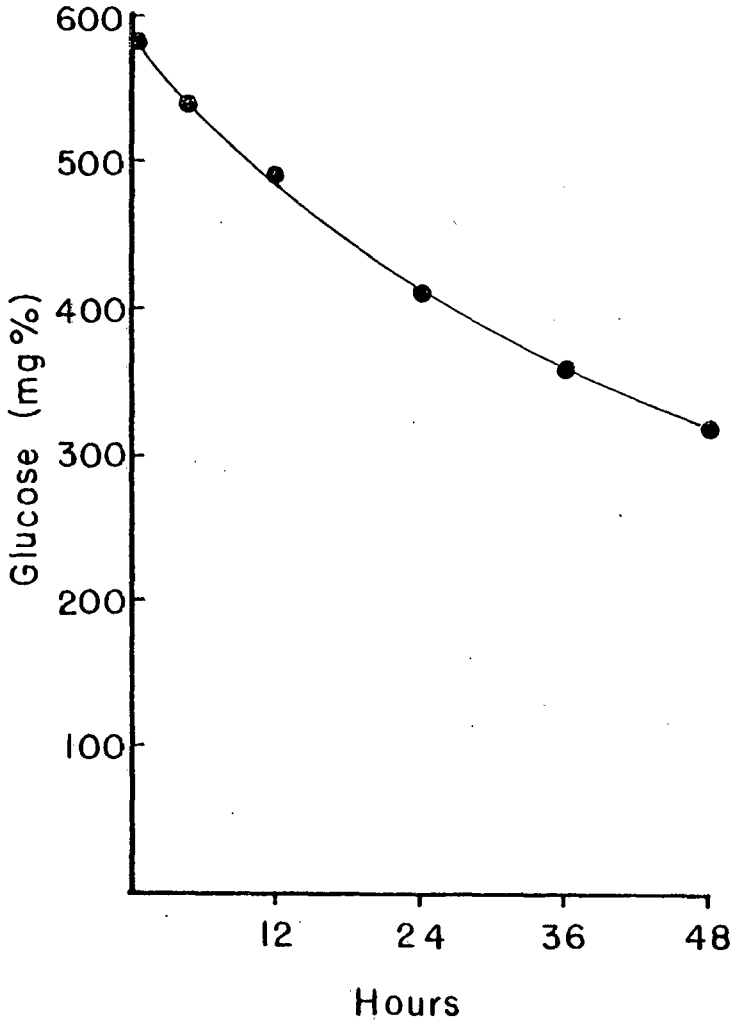
The utilization of medium glucose and the production of lactate are shown in Figure 3. These data were from the same experiment described in Figure 2. Although the rates of glucose consumption and lactate production were not perfectly linear, an excellent maintenance of cellular viability was indicated. The concentration of glucose was not a limiting factor after two days of incubation. Even with 50 mM HEPES present the release of over 25 μmoles of lactate depressed the medium pH and inhibited lactate production to a slight extent. Additional experiments demonstrated that the medium pH decreased to about 7.2 after 24 hours of incubation and to about 7.0 by the end of 48 hours.

The effects of injecting 20 ng (48 pmoles) of $1,25\text{-}(OH)_2D_3$ into mice pups 24 hours before the start of the in vitro incubation are given in Table 2. $1,25\text{-}(OH)_2D_3$ treatment resulted in a greater release of calcium and phosphate into the medium at all times investigated. This mobilization of bone mineral was not accompanied by an increase in bone lactate production at 8 or 24 hours. There was a tendency for $1,25\text{-}(OH)_2D_3$ to increase lactate production at 48 hours; however, since the dissolution of bone mineral consumes H^+, part of this tendency can be explained by a reduced fall in medium pH as a result of the mineral mobilization with $1,25\text{-}(OH)_2D_3$ treatment.

As demonstrated in Table 3, injecting $1,25\text{-}(OH)_2D_3$ at 8, 17 or 26 hours before the start of the incubation did not influence glucose consumption or lactate production over the 24-hour incubation period. The effect of $1,25\text{-}(OH)_2D_3$ upon calcium and phosphate release from the

FIGURE 3. The Utilization of Medium Glucose and the Release of Lactate Into the Medium.

Each point is the mean of 4 or 5 determinations. Medium without calvaria contained 0.28 $\pm$0.01 mM lactate (n = 6) and this value was subtracted from all lactate data. Medium from "dead" calvaria (48 hours of incubation) had 0.14 mM lactate and 568 mg.% glucose (n = 6 in both cases).


600
500
400
300
200
100
Glucose (mg%)
12
24
36
48
Hours

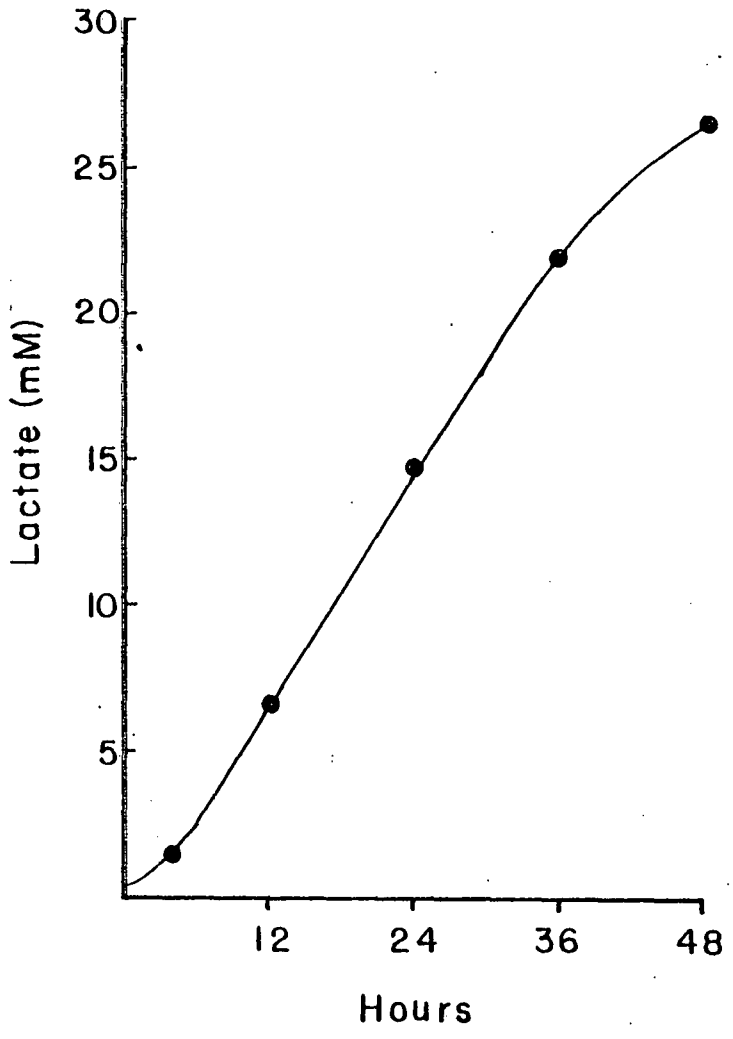

30
25
20
15
10
5
Lactate (mM)
12
24
36
48
Hours

TABLE 2

The Effects of 1,25-$(OH)_2D_3$ Upon the Medium Concentrations of Calcium, Phosphate and Lactate as a Function of Incubation Time

	Time (hrs)	Experiment 1		Experiment 2	
		Control	1,25-$(OH)_2D_3$	Control	1,25-$(OH_2D_3$
Calcium	0	1.39 ±0.01		1.44 ±0.01	
	8	1.30 ±0.02	1.48 ±0.05*	1.38 ±0.02	1.52 ±0.02*
	24	1.49 ±0.04	1.75 ±0.02*	1.42 ±0.03	1.63 ±0.03*
	48	1.87 ±0.09	2.20 ±0.06*	1.52 ±0.03	1.74 ±0.06*
Lactate	8	6.96 ±0.31	7.55 ±0.46	5.34 ±0.31	5.50 ±0.15
	24	18.26 ±0.78	18.56 ±0.79	13.94 ±0.47	14.15 ±0.62
	48	35.44 ±1.09	37.99 ±0.86	21.98 ±0.55	23.89 ±1.13
Phosphate	0			2.24 ±0.01	
	8			2.17 ±0.01	2.29 ±0.01*
	24			2.23 ±0.02	2.35 ±0.03*
	48			2.30 ±0.01	2.44 ±0.02*

Each value is the mean ± SEM of 4 or 5 calvaria. The mice were injected with 20 ng of 1,25-$(OH)_2D_3$ or the 95% ethanol vehicle 24 hours before the start of the incubation.

*Significantly greater than the control value at $p < .01$ as evaluated by the Student t-test.

TABLE 3

Effects of the Time Interval Between 1,25-$(OH)_2D_3$ Injection and the Start of Injection

	No Injection	8-hr. Interval	17-hr. Interval	26-hr. Interval
Media Ca (mM)	1.66 ±0.03	1.73 ±0.02	1.74 ±0.02	1.95 ±0.04
Media P_i (mM)	2.03 ±0.01	2.03 ±0.02	2.10 ±0.02	2.19 ±0.04
Glucose consumption (μmoles/24 hrs)	13.1 ±0.5	13.5 ±0.4	11.6 ±0.2	12.7 ±0.5
Lactate production (μmoles/24 hrs)	21.8 ±1.1	23.3 ±1.4	20.1 ±0.4	21.9 ±0.9

Each value is the mean ± SEM of 5 or 6 determinations. The in vitro incubation lasted for 24 hours; 20 ng of 1,25-$(OH)_2D_3$ was injected per pup. The initial medium concentrations of calcium and phosphate were 1.52 and 1.84 mM respectively.

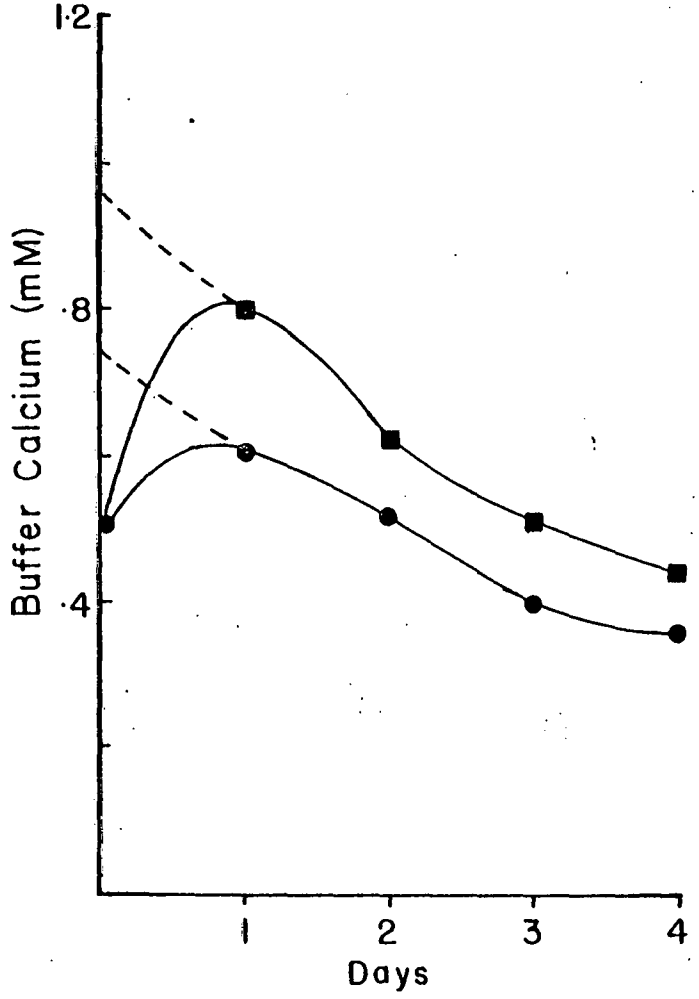
calvaria was greatest at 26 hours and diminished as the time interval between injection and incubation was shortened. Clearly, the mineral mobilization observed in Table 2 did not occur as the result of an increase in bone lactate production before the initiation of the incubation. This time course of 1,25-$(OH)_2D_3$ action is consistent with data in vivo in the vitamin D-deficient rat (4,19,59).

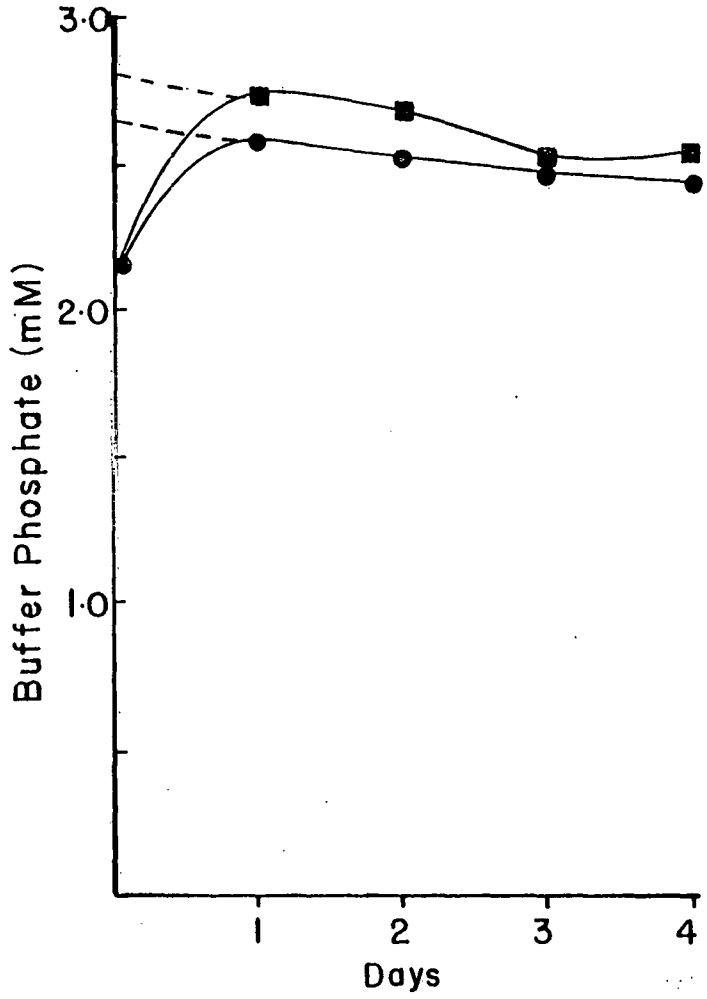
In order to study the effects of 1,25-$(OH)_2D_3$ upon the solubility of bone mineral in the absence of living cells, a simple buffer solution was devised. Preliminary experiments demonstrated that removal of glucose from the medium was equivalent to freezing and thawing three times with respect to levels of calcium and phosphate in the medium. Therefore, this buffer did not contain any glucose, vitamins, amino acids or serum.

The effect of 1,25-$(OH)_2D_3$ on the solubility of bone mineral as measured by the buffer concentrations of calcium and phosphate is shown in Figure 4. The solubility of both control and 1,25-$(OH)_2D_3$-treated bones decreased from the first to the fourth day of incubation. However, prior injection of 1,25-$(OH)_2D_3$ 24 hours before the start of the incubation into the mice pups increased the mineral solubility relative to vehicle injected pups throughout the incubation period. This effect was greatest during the early phases of the incubation and gradually diminished with time. The rise in buffer calcium concentration was greater than the rise in buffer phosphate concentration, presumably a result of the bone Ca/P ratio of 1.5 and the lower initial concentration of calcium in the buffer.

FIGURE 4. Buffer Concentrations of Calcium and Phosphate with Time.

Squares represent values from mice given a subcutaneous injection of 20 ng of $1,25\text{-}(OH)_2D_3$ 24 hours prior to the start of the incubation while circles represent vehicle injected mice. Each point represents the mean value from four calvaria. The dotted lines indicate extrapolations of the buffer concentrations back to zero time in order to estimate the bone mineral solubility at the time of sacrifice.


1·2
·8
·4
Buffer Calcium (mM)
1
2
3
4
Days


3·0
2·0
1·0
Buffer Phosphate (mM)
1
2
3
4
Days

DISCUSSION

The purpose of this investigation was to explore the mechanism(s) by which 1,25-$(OH)_2D_3$ promotes the mobilization of bone mineral. In bone 1,25-$(OH)_2D_3$ has been shown to bind to a cytoplasmic receptor (22) and localize in the nucleus (62). Actinomycin D obliterates the mineral mobilizing action of 1,25-$(OH)_2D_3$ in the rat (59). Since 1,25-$(OH)_2D_3$ is thought to act upon bone without further modification (66), actinomycin D presumably interferes with the synthesis of the unknown 1,25-$(OH)_2D_3$-dependent bone protein(s) claimed to be responsible for mineral mobilization (8,9). Recently, a 1,25-$(OH)_2D_3$-dependent protein with immunological cross-reactivity to the 1,25-$(OH)_2D_3$-induced intestinal calcium binding protein has been discovered in chick tibia (7). The physiological roles of this protein(s) in bone and intestine are unknown. Wergedal (63) studied various enzymatic activities in vitamin D-deficient bone and saw only minor differences compared to pair-fed controls. 1,25-$(OH)_2D_3$ decreases the oxidation of citrate in an "osteoblast-like" population of isolated bone cells (65) and also decreases the synthesis of collagen in bone tissue culture (47).

By inducing the synthesis of a specific protein(s), an agent such as 1,25-$(OH)_2D_3$ might stimulate bone mineral mobilization by one of three non-mutually exclusive mechanisms (2). While all three mechanisms involve the direct action of bone cells to produce a net efflux of calcium and phosphate from the skeleton, each mechanism uniquely couples

cellular activity with the efflux of these ions. The existence of a "bone membrane" to regulate the flow of substances to and from the skeleton has been advanced (32,34,57). This membrane has been proposed to maintain a regulated blood/bone pH gradient to dissolve bone mineral (33,35) and to actively pump calcium and phosphate from bone to blood (48,54,57). Alternatively, bone cells might secrete variable amounts of a mineral "solubilizer" which binds to the surface of bone mineral and increases both the bone fluid and blood levels of calcium and phosphate without the need for a bone membrane (2).

The large lactic acid production by bone cells has been proposed to be the source of protons for the blood/bone pH gradient (33,35). The stimulation of bone lactic acid production by PTH is thought to decrease the pH in the bone ECF and by dissolving bone mineral, to increase the flux of calcium and phosphate from bone to blood. The effect of vitamin D upon bone lactate production has previously been studied by giving vitamin D to deficient animals several days before the incubation of bone in vitro. In this type of study vitamin D has been reported both to increase (38,39) and decrease (1) bone lactate production. An extract of the leaves of Solanum glaucophyllum, which contains a glycosylated form of 1,25-$(OH)_2D_3$ (15), increased lactate production in chick embryo calvaria (42).

The results of these studies on the alteration of bone lactate production by vitamin D are complicated by several factors. Solanum glaucophyllum extracts contain relatively high levels of calcium,

magnesium and phosphate (61) and use of the crude extract has caused problems in a mouse calvaria culture system (24). In addition, it is uncertain whether a 13-day-old chick embryo is normally exposed to 1,25-$(OH)_2D_3$ (32). When vitamin D is given to deficient animals numerous changes in serum electrolyte and hormone levels occur for several days. Gradually, both the types and number of bone cells change. In this study, by giving low doses of the physiologically active metabolite of vitamin D to normal mice and determing bone lactate production as a function of both time of incubation and time between injection and incubation, these objections have been minimized.

The data presented in Tables 2 and 3 clearly show that bone lactate production was not affected by 1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ did not increase lactate production either preceding or during the mobilization of bone mineral. Such a lack of effect is in marked contrast to the well known action of PTH to increase lactate production by bone (33). Despite the fact that PTH and 1,25-$(OH)_2D_3$ both mobilize bone mineral, their initial actions on bone cells appear to be quite different. PTH is thought to act upon a plasma membrane receptor to influence adenylate cyclase activity (5) and the plasma membrane permeability to calcium (49), while 1,25-$(OH)_2D_3$ binds to a cytoplasmic receptor (22) and does not alter the bone cell concentration of 3',5'-adenosine monophosphate [cAMP] (28,29,40,65) or uptake of calcium (11). Hekkelman et al. (17) have proposed that the PTH-induced increase in bone lactate production results from a prior increase in bone cell cAMP levels.

Since 1,25-$(OH)_2D_3$ can be shown to induce the mobilization of bone mineral without a concurrent increase in bone lactate production, 1,25-$(OH)_2D_3$ apparently does not act by increasing the proposed lactate-mediated blood/bone pH gradient. The possibility that 1,25-$(OH)_2D_3$ might promote bone mineral mobilization through the action of a solubilizing agent(s) has received some support (2). For instance, Nichols et al. (38) have shown that vitamin D-treated bone maintains higher buffer levels of calcium and phosphate than vitamin D-deficient bone even when cellular metabolism is abolished. Similar observations have been made from bones taken from rats treated with PTH (20,43,47), while calcitonin treatment lowers buffer calcium and phosphate concentrations (21). These findings indicate that the solubility of the mineral phase can be altered by an unidentified agent(s) secreted by bone cells under the influence of PTH and 1,25-$(OH)_2D_3$.

This solubilizing agent(s), by interacting with the bone mineral surface, presumably increases the bone mineral solubility and thereby raises the concentrations of calcium and phosphate in the bone ECF. Eventually, the calcium and phosphate released from the bone mineral distribute throughout the entire body ECF. Once bone cells have secreted the solubilizing agent(s) they are no longer necessary for the increase in bone mineral solubility. Therefore, the solubility of non-vital bone reflects the physiological state of the animal at the time of sacrifice. Bone from animals in PTH- and vitamin D-deficient hypocalcemic states can be expected to contain little of this

solubilizing agent(s). If this agent(s) were removed entirely the solubility of the bone mineral would approach that of synthetic apatite. Naturally, the solubilizing agent(s) must eventually be removed from the mineral surface and inactivated. Since calcitonin lowers bone mineral solubility (21), bone cells may be involved in some fashion with the removal and/or inactivation of the solubilizer.

The initial precipitation of calcium phosphate near neutral pH involves a transitory phase [amorphous calcium phosphate or ACP] of lower Ca/P ratio and higher solubility than the final hydroxyapatite phase (36,58). Upon poisoning bone cells, bone mineral spontaneously converts to a phase with a higher Ca/P ratio; presumably an ACP to hydroxyapatite transformation occurs. Although this conversion was first recognized by Neuman and Bareham (35), decreases in buffer calcium with simultaneous increases in buffer phosphate during incubation of non-vital bone had been previously observed (27,48). Apparently, active bone cells stabilize the presence of ACP in bone. Preliminary work in this investigation fully supported the earlier work and led to the choice of a high buffer phosphate to calcium ratio in the solubility studies. During 48 hours of incubation (Figure 2), the bone Ca/P ratio increased from 1.512 $\pm$0.005 to 1.549 $\pm$0.014. The data in Figure 4 demonstrate that this increase was not complete in 48 hours and it is difficult to predict whether the hydroxyapatite stoichiometric ratio of 1.667 can be attained in a melieu containing magnesium and carbonate.

Figure 4 shows the mineral solubility of the mouse pup calvaria decreased with time over the 4-day incubation period. Consistent with the expected ACP to hydroxyapatite phase transition, the buffer calcium concentration decreased to a much greater extent than the buffer phosphate concentration. 1,25-$(OH)_2D_3$ increased the mineral solubility of the calvaria throughout the four days of incubation but did not appear to influence the gradual decline in buffer concentrations of calcium and phosphate. Further work is required to determine the interaction of the postulated solubilizing agent with the ACP-hydroxyapatite phase transition.

The evidence obtained in this investigation clearly demonstrates that 1,25-$(OH)_2D_3$ exerts an effect upon the solubility of bone mineral in a manner entirely consistent with its physiological action of transferring calcium and phosphate from bone to blood (4,19,59). Thus, 1,25-$(OH)_2D_3$ appears to have been the active metabolite in an earlier study of the effects of vitamin D on bone mineral solubility (38). Since PTH (20,43,47) and calcitonin (21) also alter the solubility of bone mineral, the action of the proposed solubilizing agent(s) may be a general mechanism by which bone mineral mobilizing agents act. This does not imply that the pump and pH gradient theories are incorrect, only that they cannot fully explain the actions of these hormones. The key arguments in favor of the "solubilizer" theory are (a) the effects of these hormones persist after bone cell metabolism is abolished, a fact difficult to explain by the pump theory and (b) 1,25-$(OH)_2D_3$

increases the mobilization of bone mineral without a concurrent increase in lactate production, a fact difficult to explain by the lactate-mediated pH gradient theory.

$1,25\text{-}(OH)_2D_3$ might act to induce either the synthesis of the solubilizer directly or the enzymatic machinery necessary for the full development of the action of the solubilizer(s). Since colchicine has been shown to inhibit the action of $1,25\text{-}(OH)_2D_3$ (46), microtubules may be involved in the secretion of the solubilizer(s). The solubilizer(s) may or may not be a protein. It is interesting to note that PTH increases the synthesis of hyaluronic acid by bone in vitro (25,26) and that $1,25\text{-}(OH)_2D_3$ alters the lipid composition of the intestinal brush border membrane (50). Although the exact identity of the proposed solubilizer is presently unknown, the solubilizer(s) might be related to: i) one of the recently discovered proteins found in bone: calcium-binding protein (7), bone sialoprotein (18), α_2-HS glycoprotein (60) and the γ-carboxyglutamic acid-containing osteocalcin (14) or, ii) the peptides and other low molecular weight components described by Learer and Shuttelworth (23).

BIBLIOGRAPHY

1. Au, W.Y.W. and F.C. Bartter: Effect of vitamin D on in vitro bone calcium metabolism. Endocrinol. 78, 1100-1104 (1966).

2. Brommage, R., M.W. Neuman and W.F. Neuman: Aerobic glycolysis, ion fluxes and the bone membrane. In, Endocrinology of Calcium Metabolism, ed. by D.H. Copp and R.V. Talmage. Excerpta Medica, Amsterdam, pp. 79-84, 1978.

3. Carlsson, A.: Tracer experiments on the effect of vitamin D on the skeletal metabolism of calcium and phosphorous. Acta Physiol. Scand. 26, 212-220 (1952).

4. Castillo, L., Y. Tanaka and H.F. DeLuca: The mobilization of bone mineral by 1,25-dihydroxyvitamin D_3 in hypophosphatemic rats. Endocrinol. 97, 995-999 (1975).

5. Chase, L.R., S.A. Fedak and G.D. Aurbach: Activation of skeletal adenyl cyclase by parathyroid hormone in vitro. Endocrinol. 84, 761-768 (1969).

6. Chen, P.S., T.Y. Toribara and H. Warner: Microdetermination of phosphorous. Anal. Chem. 28, 1756-1758 (1956).

7. Christakos, S. and A.W. Norman: Vitamin D-dependent calcium binding protein in bone. Fed. Proc. 37, 408 (1978).

8. DeLuca, H.F.: Mechanism of action and metabolic fate of vitamin D. Vitamins and Hormones 25, 315-367 (1967).

9. DeLuca, H.F., H. Morii and M.J. Melancon, Jr.: The interaction of vitamin D, parathyroid hormone and thyrocalcitonin. In, Parathyroid Hormone and Thyrocalcitonin (Calcitonin), ed. by R.V. Talmage, L.F. Belanger and I. Clark. Excerpta Medica, Amsterdam pp. 448-454, 1968.

10. DeLuca, H.F. and H.K. Schnoes: Metabolism and mechanism of action of vitamin D. Ann. Rev. Biochem. 45, 631-666 (1976).

11. Dziak, R.M.: The effects of D_3 metabolites on bone cell calcium. In, Endocrinology of Calcium Metabolism, ed. by D.H. Copp and R.V. Talmage. Excerpta Medica, Amsterdam, pg. 372, 1978.

12. Frolik, C.A. and H.F. DeLuca: Metabolism of 1,25-dihydroxycholecalciferol in the rat. J. Clin. Invest. 51, 2900-2906 (1972).

13. Gerel, J.-M. and J.-P. Barlet: Calcium metabolism in newborn animals: The interrelationship of calcium, magnesium and inorganic phosphorous in newborn rats, foals, lambs and calves. Pediatric Res. 10, 749-754 (1976).

14. Hauschka, P.V., J.B. Lian and P.M. Gallop: Vitamin K and mineralization. Trends in Biochem. Sci. 3, 75-78 (1978).

15. Haussler, M.R., R.H. Wasserman, T.A. McCain, M. Peterlik, K.M. Bursac and M.R. Hughes: 1,25-dihydroxyvitamin D_3-glycoside: Identification of a calcinogenic principle of solanum malacoxylon. Life Sciences 18, 1049-1056 (1976).

16. Haussler, M.R. and T.A. McCain: Basic and clinical comments related to vitamin D metabolism and action. New England J. Med. 297, 974-983 (1977).

17. Hekkelman, J.W., M.P.M. Hermann-Erlee, J.N.M. Heersche and P.J. Gaillard: Studies on the mechanism of parathyroid hormone action on embryonic bone in vitro. In, Calcium-Regulating Hormones, ed. by R.V. Talmage, M. Owen and J.A. Parsons. Excerpta Medica, N.Y., pp. 185-194, 1975.

18. Herring, G.M.: The organic matrix of bone. Chapter 5 in, The Biochemistry and Physiology of Bone, ed. by G.H. Bourne, 2nd. edition. Academic Press, N.Y., pp. 127-189, 1972.

19. Holick, M.F., M. Garabedian and H.F. DeLuca: 1,25-dihydroxcholecalciferol: Metabolite of vitamin D_3 active on bone in anephric rats. Science 176, 1146-1147 (1972).

20. Johnston, C.C., Jr., E.B. Miner, D.M. Smith and W.P. Deiss: Influence of parathyroid activity on the chemical equilibrium of bone calcium in vitro. J. Lab. Clin. Med. 60, 689-694 (1962).

21. Khan, B.T., F.C. Ewald and M.O. Tachdjian: In vivo effect of thyrocalcitonin on bone solubility. Endocrinol. 81, 398-399 (1967).

22. Kream, B.E., M. Jose, S. Yamada and H.F. DeLuca: A specific high-affinity binding macromolecule for 1,25-dihdyroxyvitamin D_3 in fetal bone. Science 197, 1086-1088 (1977).

23. Learer, A.G. and C.A. Shuttleworth: Studies on the peptides, free amino acids and certain related compounds isolated from ox bone. Arch. Oral Biol. 13, 509-525 (1968).

24. Lloyd, W., H. Wells, M.W. Walling and D.V. Kimberg: Stimulation of bone resorption in organ culture by salt-free extracts of Solanum glaucophyllum. Endocrine Res. Commun. 2, 159-166 (1975).

25. Luben, R.A., J.F. Goggins and L.G. Raisz: Stimulation by parathyroid hormone of bone hyaluronate synthesis in organ culture. Endocrinol. 94, 737-745 (1974).

26. Luben, R.A. and D.V. Cohn: Effects of parathormone and calcitonin on citrate and hyaluronate metabolism in cultured bone. Endocrinol. 98, 413-419 (1976).

27. MacManus, J. and F.W. Heaton: The influence of magnesium on calcium release from bone in vitro. Biochim. Biophys. Acta 215, 360-367 (1970).

28. Mahgoub, A. and H. Sheppard: Early effect of 25-hydroxycholecalciferol (25-OH-D_3) and 1,25-dihydroxycholecalciferol (1,25-$(OH)_2$-D_3) on the ability of parathyroid hormone (PTH) to elevate cyclic AMP of intact bone cells. Biochem. Biophys. Res. Commun. 62, 901-907 (1975).

29. Marcus, R., F. Orner, G. Arvensen, K. Collias and A. Brinkman: Potentiation of PTH-dependent cAMP formation in bone: A unique effect of 24,25$(OH)_2$ vitamin D_3. Clin. Res. 26, 421A (1978).

30. Messer, H.H., W.D. Armstrong and L. Singer: Fluoride, parathyroid hormone and calcitonin: Inter-relationships in bone calcium metabolism. Calc. Tiss. Res. 13, 217-225 (1973).

31. Moriuchi, S. and H.F. DeLuca: Metabolism of vitamin D_3 in the chick embryo. Arch. Biochem. Biophys. 164, 165-171 (1974).

32. Neuman, W.F.: The Milieu Intérieur of bone: Claude Bernard revisited. Fed. Proc. 28, 1846-1850 (1969).

33. Neuman, W.F.: Aerobic glycolysis in bone in the context of membrane compartmentalization. Calc. Tiss. Res. 22 (Suppl.) 169-178 (1977).

34. Neuman, W.F. and W.K. Ramp: The concept of a bone membrane: Some implications. In, Cellular Mechanisms for Calcium Transfer and Homeostasis, ed. by G. Nichols, Jr. and R.H. Wasserman. Academic Press, N.Y., pp. 197-206, 1971.

35. Neuman, W.F. and B.J. Bareham: Evidence for the presence of secondary calcium phosphate in bone and its stabilization by acid production. Calc. Tiss. Res. 18, 161-172 (1975).

36. Neuman, W.F., R. Bjornerstedt and B.J. Mulryan: Synthetic hydroxyapatite crystals. II. Aging and strontium incorporation. Arch. Biochem. Biophys. 101, 215-224 (1963).

37. Neuman, W.F., M.W. Neuman and R. Brommage: Aerobic glycolysis in bone: Lactate production and gradients in calvaria. Am. J. Physiol. 234, C41-C50 (1978).

38. Nichols, G., Jr., S. Schartum and G.M. Vaes: Some effects of vitamin D and parathyroid hormone on the calcium and phosphorous metabolism of bone in vitro. Acta Physiol. Scand. 57, 51-60 (1963).

39. Paterson, C.R. and P. Fourman: Collagen synthesis and carbohydrate metabolism of rachitic bone. Biochem. J. 109, 101-106 (1968).

40. Peck, W.A. and I. Dowling: Failure of 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$] to modify cyclic AMP levels in parathyroid hormone-treated and untreated bone cells. Endocrine Res. Commun. 3, 157-166 (1976).

41. Puche, R.C. and M.E. Loeatto: Effects of Solanum malacoxylon on embryonic bone in vitro and on isolated mitochondria. Calc. Tiss. Res. 16, 219-226 (1974).

42. Raabo, E. and T.C. Terkildsen: On the enzymatic determination of blood glucose. Scand. J. Clin. Lab. Invest. 12, 402-407 (1960).

43. Raisz, L.G., W.Y.W. Au and J. Tepperman: Effect of changes in parathyroid activity on bone metabolism in vitro. Endocrinol. 68, 783-794 (1961).

44. Raisz, L.G. and I. Nieman: Effect of phosphate, calcium and magnesium on bone resorption and hormonal responses in tissue culture. Endocrinol. 85, 446-452 (1969).

45. Raisz, L.G., C.L. Trummel, M.F. Holick and H.F. DeLuca: 1,25-dihydroxycholecalciferol: A potent stimulator of bone resorption in tissue culture. Science 175, 768-769 (1972).

46. Raisz, L.G., M.E. Holtrop and H.A. Simmons: Inhibition of bone resorption by colchicine in organ culture. Endocrinol. 92, 556-562 (1973).

47. Raisz, L.G., D.M. Maina, S.C. Gworek, J.W. Dietrich and E.M. Canalis: Hormonal control of bone collagen synthesis in vitro: Inhibitory effect of 1-hydroxylated vitamin D metabolites. Endocrinol. 102, 731-735 (1978).

48. Ramp. W.K.: Cellular control of calcium movements in bone: Inter-relationships of the bone membrane, parathyroid hormone and alkaline phosphatase. Clin. Orthoped. Related Res. 106, 311-322 (1975).

49. Rasmussen, H. and P. Bordier: The Physiological and Cellular Basis of Metabolic Bone Disease. The Williams & Wilkins Co., Baltimore, 1974.

50. Rasmussen, H., O. Fontaine, E. Max and D.B.P. Goodman: Changes in brush border membrane lipid structure in 1,25-$(OH)_2D_3$ action. Clin. Res. 26, 562A (1978).

51. Reynolds, J.J.: Skeletal tissue in culture. In, The Biochemistry and Physiology of Bone, 2nd edition, vol. 1, pp. 69-126 (1972).

52. Reynolds, J.J.: The role of 1,25-dihydroxycholecalciferol in bone metabolism. Biochem. Soc. Spec. Publ. 3, 91-102 (1974).

53. Reynolds, J.J., M.F. Holick and H.F. DeLuca: The role of vitamin D metabolites in bone resorption. Calc. Tiss. Res. 12, 295-301 (1973).

54. Robertson, W.G.: Cellular calcium and calcium transport. In, Calcium, Phosphate and Magnesium, ed. by B.E.C. Nordin. Churchill-Livingstone, New York, pp. 230-256, 1976.

55. Schartum, S. and G. Nichols, Jr.: Calcium metabolism of bone in vitro. Influence of bone cellular metabolism and parathyroid hormone. J. Clin. Invest. 40, 2083-2091 (1961).

56. Solomon, S., H.H. Bengele and W.D. Smith: Postnatal plasma concentration and urinary excretion of Na and K in the rat. Proc. Soc. Exptl. Biol. Med. 157, 12-17 (1978).

57. Talmage, R.V.: Calcium homeostasis--calcium transport-- parathyroid action. The effects of parathyroid hormone on the movement of calcium between bone and fluid. Clin. Orthoped. Related Res. 67, 210-224 (1969).

58. Termine, J.D.: Mineral chemistry and skeletal biology. Clin. Orthoped. Related Res. 85, 207-241 (1972).

59. Tanaka, Y. and H.F. DeLuca: Bone mineral mobilizing activity of 1,25-dihydroxycholecalciferol, a metabolite of vitamin D. Arch. Biochem. Biophys. 146, 574-578 (1971).

60. Triffitt, J.T.: Plasma proteins present in human cortical bone: Enrichment of the α_2HS-glycoprotein. Calc. Tiss. Res. 22, 27-33 (1976).

61. Walling, M.W. and D.V. Kimberg: Calcium absorption by intestine: Stimulation in vitamin D-deficient nephrectomized rats by Solanum glaucophyllum. Gastroenterol. 69, 200-205 (1975).

62. Weber, J.C., V. Pons and E. Kodicek: The localization of 1,25-dihydroxycholecalciferol in bone cell nuclei of rachitic chicks. Biochem. J. 125, 147-153 (1971).

63. Wergedal, J.E.: Enzymes of protein and phosphate catabolism in rat bone. II. Changes in vitamin D deficiency. Calc. Tiss. Res. 3, 67-73 (1969).

64. Wergedal, J., M. Stauffer, D. Baylink and C. Rich: Inhibition of bone matrix formation, mineralization and resorption in thyro-parathyroidectomized rats. J. Clin. Invest. 52, 1052-1058 (1973).

65. Wong, G.L., R.A. Luben and D.V. Cohn: 1,25-dihydroxycholecalciferol and parathormone: Effects on isolated osteoclast-like and osteoblast-like cells. Science 197, 663-665 (1977).

66. Wong, R.G., J.F. Myrtle, H.C. Tsai and A.W. Norman: Studies on calciferol metabolism. V. The occurrence and biological activity of 1,25-dihydroxyvitamin D_3 in bone. J. Biol. Chem. 247, 5728-5735 (1972).

CHAPTER FIVE

AN INVESTIGATION OF THE REQUIREMENTS FOR VITAMIN D IN THE MATERNAL AND NEONATAL RAT

INTRODUCTION

Vitamin D_3 (cholecalciferol) is a natural fat-soluble steroid associated with normal body growth and bone calcification. Although all species do not react identically to a deficiency in vitamin D, typical symptoms include lack of weight gain, muscle weakness, loss of appetite, enlarged parathyroid glands, uncalcified (rachitic) cartilage, hypocalcemia and hypophosphatemia. In severe cases death from hypocalcemic tetany can occur. Bone and intestine are the most widely studied target organs for the action of vitamin D. Vitamin D increases the mobilization of bone mineral and the intestinal absorption of calcium and phosphate; both of these actions result in increased blood levels of calcium and phosphate. The parathyroid glands (28), salivary glands (20) and kidneys (5) also appear to be target organs in vitamin D action.

The synthesis of high specific activity radiolabeled vitamin D_3 led to the identification of 25-(OH)D_3, a metabolite produced by the liver. Although 25-(OH)D_3 is the major circulating form of vitamin D_3, further metabolism to 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ occurs in the kidneys. Low concentrations of 25,26-$(OH)_2D_3$ (63) and 1,24,25-$(OH)_3D_3$ (69) have also been found under certain circumstances. In terms of potency, 1,25-$(OH)_2D_3$ is the most active natural derivative of vitamin D_3 in promoting bone resorption in vitro (61,62), binding to the intestinal receptor protein (14,47) and in increasing tibia ash weight (7). This high activity, coupled with a rapid time course of action and the

observations that the synthesis of 1,25-$(OH)_2D_3$ is under metabolic control, has led to the belief that 1,25-$(OH)_2D_3$ is the physiologically active metabolite of vitamin D_3 (40,46).

In contrast to these findings however, three recent reports have suggested that 1,25-$(OH)_2D_3$ may not be responsible for all of the actions of vitamin D_3. Bordier et al. (6) could not fully duplicate the actions of 25-$(OH)D_3$ with 1,25-$(OH)_2D_3$ in man. Norman et al. (41) have shown that 1,25-$(OH)_2D_3$ does not fully support growth in chickens over a 30-week period while Sunde et al. (64) found that embryonic chicks did not develop properly when hens were given 1,25-$(OH)_2D_3$ instead of vitamin D_3. In the first part of this investigation another aspect of the long-term actions of 1,25-$(OH)_2D_3$ has been studied; namely, the efficacy of 1,25-$(OH)_2D_3$ treatment in terms of body weight gain, normalization of plasma calcium, and reproductive ability in young adult vitamin D-deficient female rats.

Since 1,25-$(OH)_2D_3$ has a rapid biological turnover (21,32,42), animals previously deficient in vitamin D and given 1,25-$(OH)_2D_3$ as their sole source of vitamin D should quickly become vitamin D-deficient upon cessation of the 1,25-$(OH)_2D_3$ treatment. Such a procedure was employed in order to obtain vitamin D-deficient rat pups. By terminating injections of 1,25-$(OH)_2D_3$ at delivery into female rats being maintained with this metabolite, rat pups and dams soon showed signs of vitamin D deficiency. An additional experiment was undertaken to determine whether normal impregnated female rats could be rendered vitamin D-deficient by the time of delivery.

MATERIALS AND METHODS

All rats were of the Sprague-Dawley strain and purchased from Holtzman Company. These rats were housed in a room free from ultraviolet light and kept on a 12 hour/day light cycle. The three vitamin D-deficient diets were purchased from Teklad Test Diets and contained: (1) no calcium, 0.4% phosphorous (TD 76524); (2) 0.2% calcium, 0.4% phosphorous (TD 76428); and (3) 1.4% calcium, 1.0% phosphorous (TD 77192). The 1,25-$(OH)_2D_3$ was a generous gift from Dr. Milan R. Uskokovic' of Hoffman LaRoche, Inc. and the vitamin D_3 was purchased from ICN Pharmaceuticals, Inc. Stock solutions of 1,25-$(OH)_2D_3$ were checked for actual 1,25-$(OH)_2D_3$ concentration by taking ultraviolet absorption spectra and using a molar extinction coefficient of 18,200 at 265 mμ. One unit of vitamin D_3 or 1,25-$(OH)_2D_3$ is defined as 65 pmoles.

Rats were bled by snipping off the tip of their tails (usually under ether anesthesia) and collecting blood in heparinized capillary tubes. Blood from rat pups was obtained at decapitation. Plasma calcium was determined, usually in triplicate, on 20 microliter samples with a Calcette titrator. Plasma phosphate was determined after deproteinization with 8% perchloric acid by the method of Chen et al. (8). The medium employed for the in vitro incubation (Table 4) consisted of 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.65 mM Na_2HPO_4, 1 mM $MgSO_4$, 30 mM HEPES, 500 mg.% glucose, 2 mg/ml bovine serum albumin, 100 units/ml penicillin and 100 μg/ml streptomycin diluted by 5% with heat-inactivated newborn

calf serum and set to pH 7.4. Medium calcium and phosphate were analyzed in the same fashion as the plasma determinations. Sodium and potassium concentration in serum and bone extracts were measured on an Instrumentation Laboratories Model 143 Flame Photometer. Bone wet and dry weights were taken on a Cahn Millibalance. The glucose oxidase method of Raabo and Terkildsen (48) was used to determine medium and serum glucose while medium lactate was analyzed according to the procedure described in Sigma Technical Bulletin #826-UV. Calvarium thickness was measured with a Mitutoyo micrometer.

RESULTS

Experiment 1

Figure 1 contains a flow chart outlining the procedures followed in this experiment.

Twenty-three 40-day-old female rats weighing approximately 140 grams were placed on the 0.2% calcium, 0.4% phosphorous, vitamin D-deficient diet upon arrival from Holtzman Company. Body weights were determined every several days. After 36 days five rats were given 500 units of vitamin D_3 by intraperitoneal injection. Four of these vitamin D_3-injected and six of the non-injected rats were bled 21 days later. Plasma calcium values were 10.9 $\pm$0.1 and 5.9 $\pm$0.1 mg.% (mean $\pm$ SEM) for the vitamin D_3-injected and non-injected rats, respectively. The body weights of these rats during this period are shown in Figure 2. The non-injected, vitamin D-deficient rats gained less than 40 grams while the rats given vitamin D_3 gained over 90 grams of body weight.

The 18 non-injected rats were divided into four groups: one group of six was not injected while three groups of four rats were injected with 0.25, 1.0 and 10.0 units of 1,25-$(OH)_2D_3$ per day for one week. The 1,25-$(OH)_2D_3$ was initially dissolved in 95% ethanol and then diluted to the proper dosage level with saline. Plasma calcium values obtained after this week of injections are given in Table 1. There was a clear dose-dependent increase in plasma calcium but normalization to values near 10 mg.% did not occur. All three injected groups of rats gained

FIGURE 1. Flow Chart Describing the Procedures Employed for Generating the 1,25-$(OH)_2D_3$-Replete Female Rats Used for Mating.

A full description of the experimental details is given in the RESULTS section under "Experiment 1".

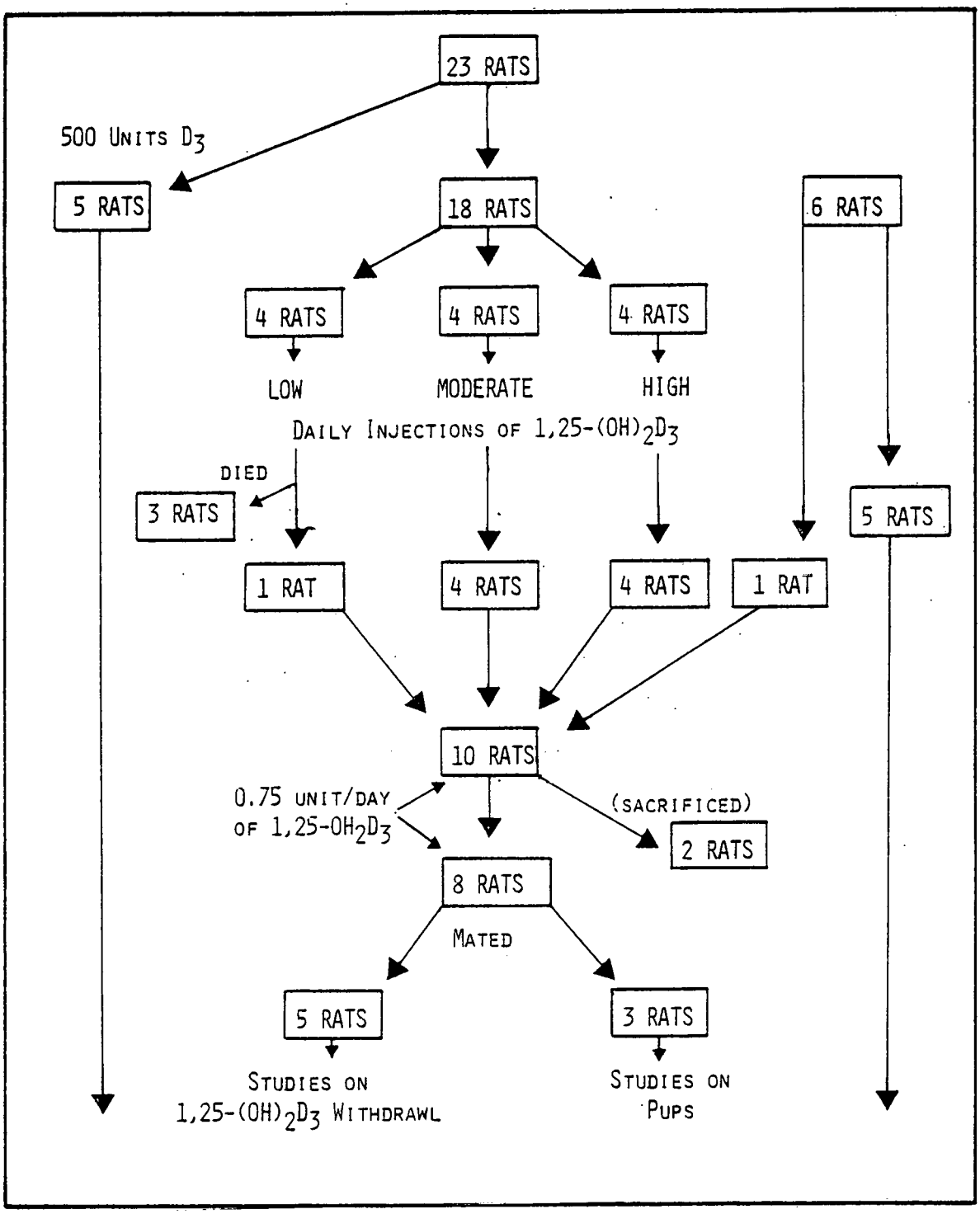

23 RATS
500 UNITS D_3
5 RATS
18 RATS
6 RATS
4 RATS
4 RATS
4 RATS
LOW
MODERATE
HIGH
DAILY INJECTIONS OF 1,25-$(OH)_2D_3$
DIED
3 RATS
5 RATS
1 RAT
4 RATS
4 RATS
1 RAT
10 RATS
0.75 UNIT/DAY OF 1,25-OH_2D_3
(SACRIFICED)
2 RATS
8 RATS
MATED
5 RATS
3 RATS
STUDIES ON 1,25-$(OH)_2D_3$ WITHDRAWL
STUDIES ON PUPS

FIGURE 2. Time Course of the Body Weights (mean ± SEM) of Female Rats Placed on the 0.2% Calcium 0.4% Phosphorous, Vitamin D-Deficient Diet After Purchase from Holtzman Company.

The age indicated is the actual age of the rats. At the times indicated by the arrows, 500 units of vitamin D_3 were injected intraperitoneally into 5 of the rats. The remaining 18 rats did not receive any vitamin D_3. Plasma calcium values (mean ± SEM) obtained on the number of rats given in parentheses, at 97 days of age, are also indicated.

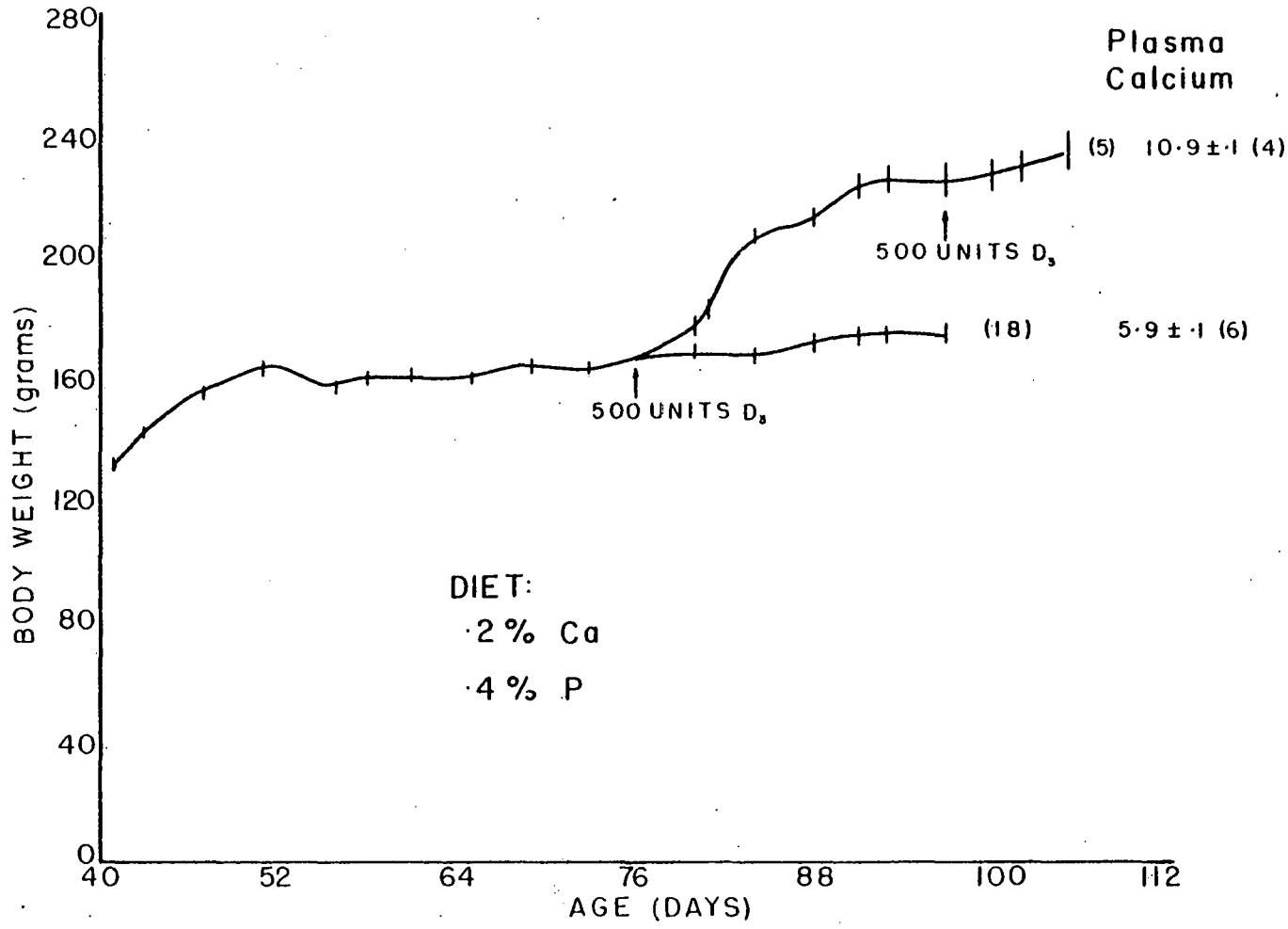

Plasma
Calcium
(5) 10·9 ± ·1 (4)
500 UNITS D3
(18) 5·9 ± ·1 (6)
500 UNITS D3
DIET:
·2 % Ca
·4 % P
BODY WEIGHT (grams)
0
40
80
120
160
200
240
280
40
52
64
76
88
100
112
AGE (DAYS)

TABLE 1

The Effect of 1,25-$(OH)_2D_3$ Upon Plasma Calcium After 7 Days of Injection

Daily Dose of 1,25-$(OH)_2D_3$ (units)	N	Plasma Calcium (mg.%)
0	6	5.9 $\pm$0.1
0.25	4	6.3 $\pm$0.2
1.0	4	7.0 $\pm$0.2
10.0	4	8.1 $\pm$0.5

Seven daily intraperitoneal injections with saline as a vehicle were given and the rats were bled one day after the last injection. Plasma calcium values are given as the mean $\pm$ SEM; N refers to the number of rats in each group.

about 15 grams in body weight while the non-injected group gained 5 grams.

Due to the possibility of oxidation of the 1,25-$(OH)_2D_3$ occurring in the saline diluent, attempts were made to continue these injections with propylene glycol in place of the saline. This procedure did not prove successful since the 200 microliter propylene glycol injections caused severe pain in the rats. All subsequent injections were made with 95% ethanol as the vehicle and the injected volume was reduced to either 25 or 50 microliters. Problems were also encountered in changing the diet from powder to pellet form. As a result of these two delays, a three-month period passed between the initial saline vehicle injections (Table 1) and the injections with ethanol as the vehicle described below. During this time the rats continued to appear healthy but did not gain body weight.

The three previously injected groups of rats were injected with 0.05, 0.2 and 1.0 units of 1,25-$(OH)_2D_3$ per day. The non-injected and 0.05 unit/day injected rats did not gain any body weight. The rats on the high dose of 1,25-$(OH)_2D_3$ had an approximately linear rate of body weight gain over the next four weeks. Two of the four rats receiving the intermediate dose of 1,25-$(OH)_2D_3$ gained weight at the same rate as the rats receiving the high dose while the other two rats on the intermediate dose lost weight. This weight loss appeared to result from the injections as the loss stopped after terminating the injections. Plasma calcium values obtained 38 days after the start of the injections

are given in Table 2. The 1.0 unit/day dose of 1,25-$(OH)_2D_3$ was effective in returning plasma calcium to normal. The 0.05 unit/day dose was not effective while the results of the 0.2 unit/day dose of 1,25-$(OH)_2D_3$ were inconclusive in the normalization of plasma calcium.

Since the effective daily dose of 1,25-$(OH)_2D_3$ as judged by body weight gain and normalization of plasma calcium appeared to be between 0.2 and 1.0 unit per day, a dose of 0.75 unit/day was chosen for the remainder of the injections. Ten rats (one previously non-injected, four given 1.0 unit/day, four given 0.2 unit/day and one rat given 0.05 unit/day of 1,25-$(OH)_2D_3$) were injected daily with 0.75 unit of 1,25-$(OH)_2D_3$ for five to seven weeks. During this time the two rats that did not adjust to the earlier intermediate dosage again had problems resulting from the injections and were sacrificed. The mean body weights throughout this period of the non-injected and four rats who had previously received the high dose of 1,25-$(OH)_2D_3$ are given in Figure 3.

The eight female rats receiving 0.75 unit/day of 1,25-$(OH)_2D_3$ were mated with normal young males for a period of eight days. Two males were placed with two females. After the males were removed the females were put into individual cages for the remainder of the experiment. Three of these females produced litters of 7, 9 and 10 pups. The females did not receive any further injections of 1,25-$(OH)_2D_3$ once they had given birth.

The 1,25-$(OH)_2D_3$ injections were terminated on four of the five rats that did not give birth. These rats weighed an average of 326 grams

TABLE 2

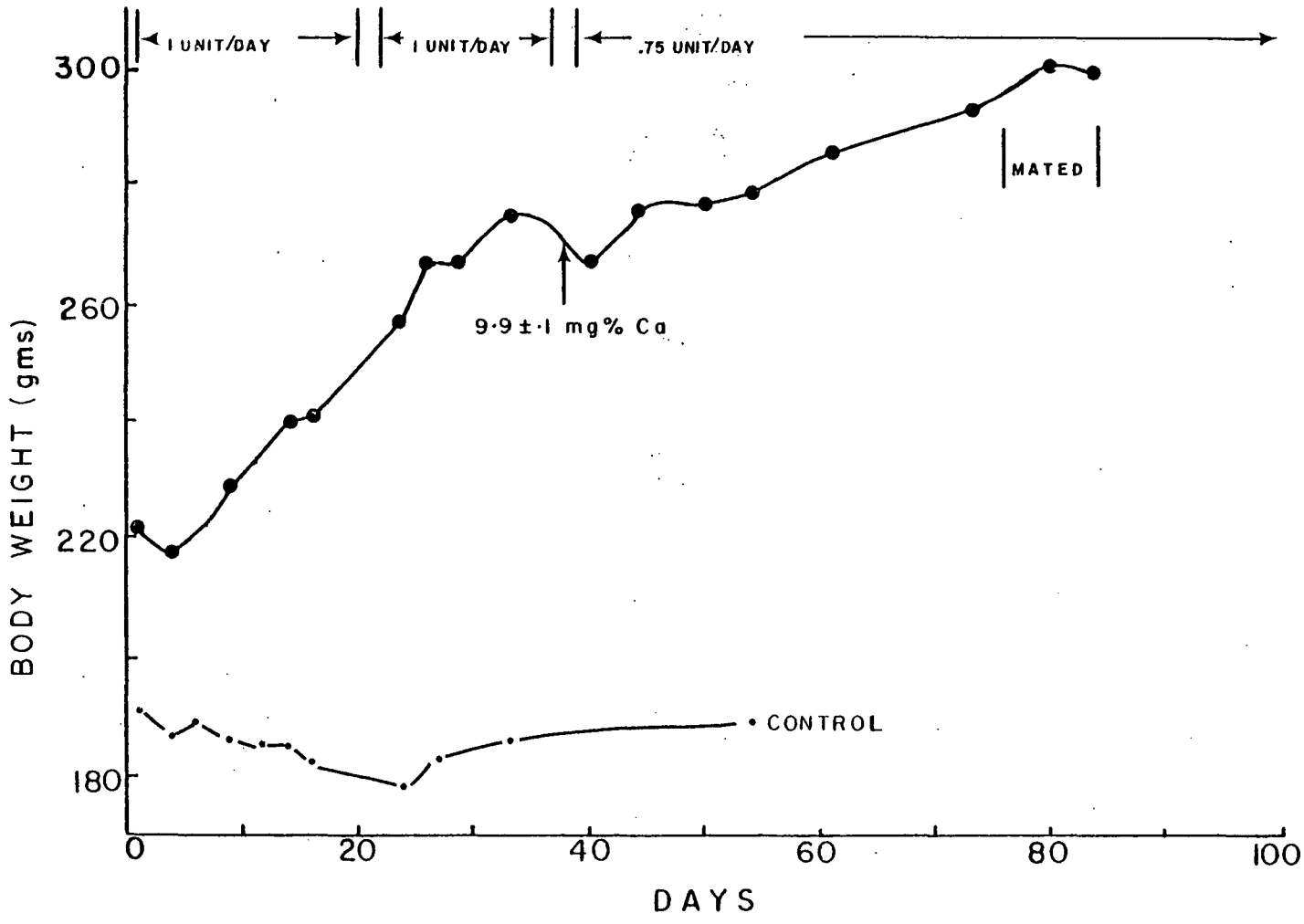
The Effect of 1,25-$(OH)_2D_3$ Upon Plasma Calcium After 38 Days of Injection

Daily Dose of 1,25-$(OH)_2D_3$ (units)	Plasma Calcium (mg.%)
0.05	7.0
0.2	8.7
0.2	10.0
1.0	9.8
1.0	9.8
1.0	9.9
1.0	9.9

The 1,25-$(OH)_2D_3$ was given daily by intraperitoneal injection with 95% ethanol as the vehicle. Body weights for non-injected and 1.0 unit/day 1,25-$(OH)_2D_3$-injected rats during this period are shown in Figure 2.

FIGURE 3. Mean Body Weights of 1,25-$(OH)_2D_3$-Injected Rats (n = 4) Following the Start of the Injections; Non-Injected Controls are Included for Comparison.

The daily doses of 1,25-$(OH)_2D_3$ injected controls are given at the top of the figure. Plasma calcium averaged 9.9 $\pm$0.1 mg.% (mean $\pm$ SEM) after 38 days of injection. The injected female rats were mated with normal males during the indicated eight-day interval.


1 UNIT/DAY
1 UNIT/DAY
.75 UNIT/DAY
MATED
9·9 ± 1 mg% Ca
CONTROL
BODY WEIGHT (gms)
300
260
220
180
0
20
40
60
80
100
DAYS

and were considered to be fully grown. The requirement for vitamin D in these animals was studied by taking plasma samples and analyzing for calcium. The data presented in Table 3 show that plasma calcium did not fall over a period of three and one-half weeks. As judged by their ability to maintain normal levels of plasma calcium, these adult rats did not have any requirement for vitamin D. Possibly, analyses of additional parameters would have demonstrated a deficiency in vitamin D.

Seven pups were born to the first dam who delivered. One of these pups died on the second day and another died on the tenth day after birth. All the pups appeared normal upon visual inspection and suckled uneventfully. The body weights of these pups are shown in Figure 4; although growth was slow the first few days, the pups gained an average of 12% of their body weight per day after the fourth day. Throughout this period the dam was kept on a vitamin D-deficient diet containing 0.2% calcium and 0.4% phosphorous. At 11 days of age the five pups were sacrificed and found to have a plasma calcium of 10.3 $\pm$0.1 mg.% (mean $\pm$ SEM). The corresponding plasma calcium value in the dam was 5.7 mg.%. After her pups were sacrificed, the dam was placed on the calcium-free, 0.4% phosphorous, vitamin D-deficient diet overnight. The next day she went into tetany, had a plasma calcium of 5.4 mg.% and was sacrificed. Whereas adult vitamin D-deficient rats not subject to a calcium stress maintain a normal plasma calcium (Table 3), 11 days of lactation reduce plasma calcium by nearly one-half.

As was the case with the first dam, the daily injections of $1,25\text{-}(OH)_2D_3$ were terminated at delivery for the second and third dams

TABLE 3

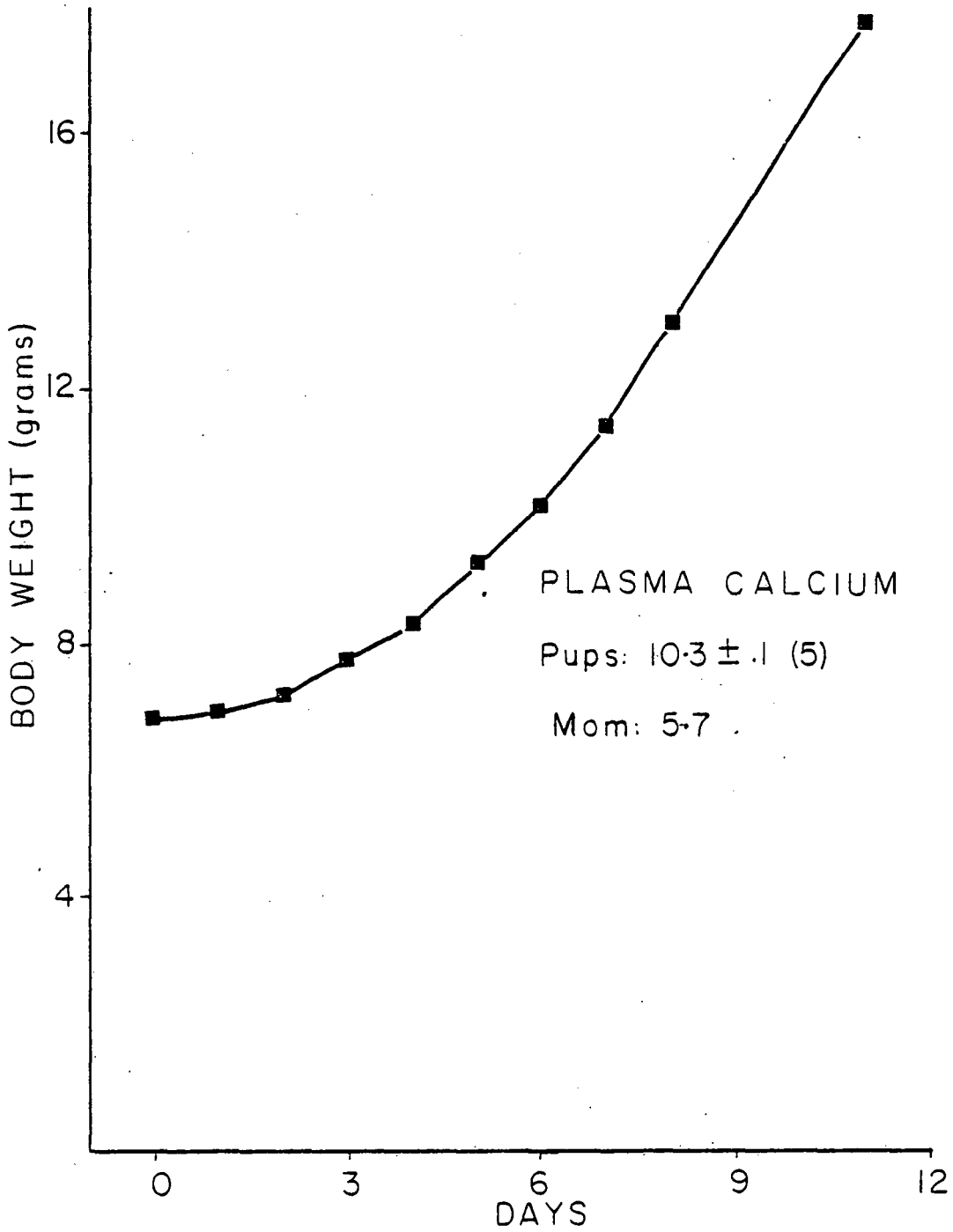
Plasma Calcium Following Withdrawal of $1,25\text{-}(OH)_2D_3$

Days Since Last $1,25\text{-}(OH)_2D_3$ Injection	Plasma Calcium (mg.%)	
	$1,25\text{-}(OH)_2D_3$-Injected Rats	D_3 Injected Rats
1	10.6 ± 0.3	10.5
4	10.5 ± 0.2	10.5
8	10.4 ± 0.2	10.8
15	10.4 ± 0.3	-
25	10.7 ± 0.1	10.6

Plasma calcium values for the $1,25\text{-}(OH)_2D_3$-injected rats are the mean ± SEM for four rats while values for the vitamin D_3-injected rats are the mean of two rats.

FIGURE 4. Mean Body Weights of the First Litter of Pups Born to the Rats Maintained on 1,25-$(OH)_2D_3$.

The dam was kept on the 0.2% calcium, 0.4% phosphorous, vitamin D-deficient diet after delivery but the 1,25-$(OH)_2D_3$ injections were terminated. The pups were sacrificed at 11 days of age and their plasma analyzed for calcium. The dam was bled at the same time for determination of plasma calcium.


BODY WEIGHT (grams)
16
12
8
4
0
3
6
9
12
DAYS
PLASMA CALCIUM
Pups: 10·3 ± .1 (5)
Mom: 5·7

that gave birth. However, these two dams were placed on the calcium-free, 0.4% phosphorous, vitamin D-deficient diet at delivery. The body weights of the third litter (8 pups plus 2 born dead) are given in Figure 5. The pups grew for the first nine days but then lost weight and died on days 13 through 16. On the 12th day after delivery the dam had a plasma calcium of 4.1 mg.%. The dam was placed on the 1.4% calcium, 1.0% phosphorous, vitamin D-deficient diet and in 12 days she gained over 50 grams in body weight and had an increase in plasma calcium to 8.0 mg.%.

The gains in body weight for the pups in the second litter (8 pups plus 1 born dead) are given in Figure 6. Normal growth occurred for eight days but then plateaued over the next four days. On the 12th day the dam was put on the 1.4% calcium, 1.0% phosphorous, vitamin D-deficient diet and by the next day the pups had begun to gain weight. By the 17th day the pups were beginning to eat solid food and in order to evaluate their growth on a more restrictive calcium intake, the 0.2% calcium, 0.4% phosphorous, vitamin D-deficient diet was given to the pups and dam. The pups were not weaned but continued to both suckle and eat solid pellets. Except for their small size, roughly one-half the normal body weight, these pups appeared normal. They had a full coat of fur and actively scampered around the cage. Eye opening occurred on day 16, within the normal range (15).

Ten units of vitamin D_3 were given by intraperitoneal injection to three pups at 26 days of age; three other pups were injected with

FIGURE 5. Mean Body Weights of the Third Litter of Pups Born to the Rats Maintained on 1,25-$(OH)_2D_3$.

At delivery the dam was placed on the calcium-free, 0.4% phosphorous, vitamin D-deficient diet and the daily injections of 1,25-$(OH)_2D_3$ were terminated. The dam was bled and a plasma calcium determination carried out on day 12. The pups died on days 14 through 16.

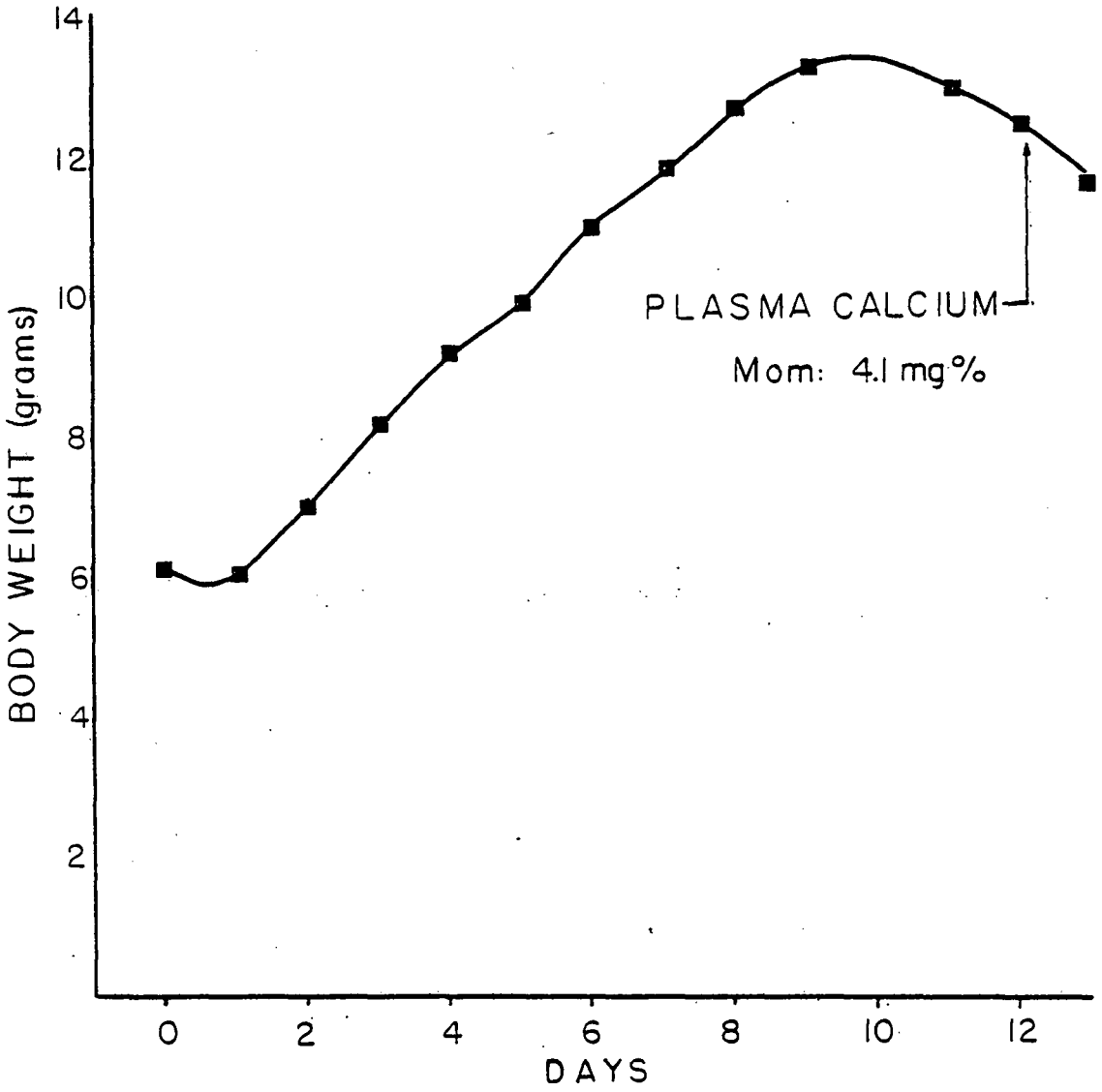
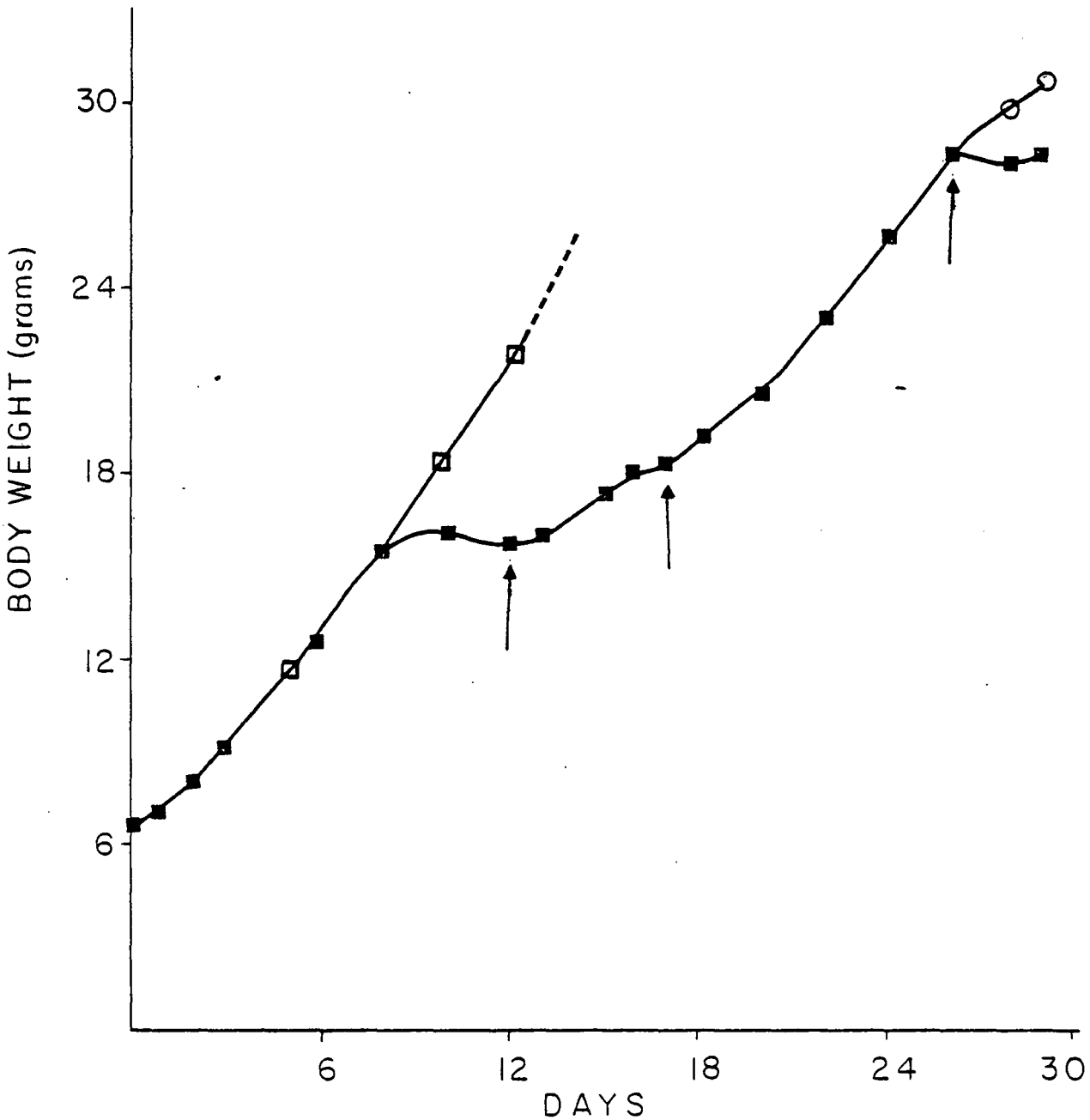

14
12
10
8
6
4
2
BODY WEIGHT (grams)
PLASMA CALCIUM
Mom: 4.1 mg%
0
2
4
6
8
10
12
DAYS

FIGURE 6. Mean Body Weights of the Second Litter of Pups Born to the Rats Maintained on 1,25-$(OH)_2D_3$ (solid squares).

At delivery the dam was placed on the calcium-free, 0.4% phosphorous, vitamin D-deficient diet. The open squares represent the mean body weights of the four litters of pups in "Experiment 2" whose dams had been given 500 units of vitamin D_3 on the fifth day after delivery. On the 12th day (first arrow), the dam was placed on the 1.4% calcium, 1.0% phosphorous, vitamin D-deficient diet and on the 17th day (second arrow) the dam was switched to the 0.2% calcium, 0.4% phosphorous, vitamin D-deficient diet. Ten units of vitamin D_3 were given by subcutaneous injection to one-half of the pups on the 26th day (third arrow). The mean body weights of these pups are given by the solid circles. The remaining pups received an injection of the 95% ethanol vehicle and all pups were sacrificed at 28 days of age.


BODY WEIGHT (grams)
30
24
18
12
6
6
12
18
24
30
DAYS

the 95% ethanol vehicle. Three days later, following an overnight fast the pups were sacrificed, plasma collected and the calvaria dissected out, rapidly weighed and then incubated in three ml of medium for seven hours. Initial and final medium concentrations of calcium, phosphate, glucose and lactate were determined. Following the incubation, the calvaria were dried overnight at 110° C and then extracted overnight with 2 ml of 2 N HNO_3. This extract was analyzed for calcium after a 20-fold dilution.

The data in Table 4 demonstrate that the vitamin D_3 injection dramatically increased plasma calcium and slightly increased plasma phosphate in these young rats. According to the water and calcium contents of the calvaria, there was no effect of vitamin D_3 upon bone mineralization. This fact is not surprising considering the short time interval between injection of vitamin D_3 and sacrifice. Incubated calvaria from vitamin D_3-injected rats maintained significantly higher levels of medium calcium than controls. Since medium phosphate was also higher with vitamin D_3 treatment, the vitamin D_3-treated calvaria supported a significantly higher calcium X phosphate product than vitamin D-deficient control calvaria. Vitamin D_3 increased calvarial lactate production without a corresponding increase in glucose consumption.

Experiment 2

Twelve sperm-positive females were purchased from Holtzman Company and placed on the 0.2% calcium, 0.4% phosphorous, vitamin D-deficient

TABLE 4

Effects of Vitamin D_3 Injection on Four-Week-Old Rats

	Control	10 Units D_3
Plasma calcium (mg.%)	5.24 ±0.03	8.62 ±0.31[1]
Plasma phosphate (mM)	2.74 ±0.29	3.23 ±0.29[2]
Calvaria water (% wet wt.)	56.6 ±0.9	55.9 ±2.3
Calvaria calcium (% dry wt.)	18.2 ±0.5	17.9 ±0.2
Medium calcium (mM)	1.67 ±0.04	1.90 ±0.02[3]
Medium phosphate (mM)	1.47 ±0.05	1.54 ±0.05[2]
Medium Ca x P_i (mM^2)	2.45 ±0.03	2.93 ±0.08[3]
Glucose consumption (μmoles/hr/gm wet wt.)	9.60 ±0.44	10.23 ±0.93
Lactate production (μmoles/hr/gm wet wt.)	13.62 ±0.33	17.13 ±0.81[4]

Rats were injected with vitamin D_3 or the ethanol vehicle at 26 days of age and upon sacrifice three days later plasma was collected and calvaria dissected out. Full details are given in the text. There were three rats in each group; each value is the mean ± SEM. p values were determined with the Student t-test.

[1] $p < .0005$ compared to control value

[2] $p < .20$ compared to control value

[3] $p < .005$ compared to control value

[4] $p < .01$ compared to control value

diet and housed individually. These rats were received two days after they were certified to be sperm-positive. Ten of these rats delivered pups and were put on the calcium-free, 0.4% phosphorous, vitamin D-deficient diet after delivery. Litter sizes were evened out such that each dam had eight pups. Four of the 80 pups died within two days. On the fifth day after delivery 500 units of vitamin D_3 were given by intraperitoneal injection to four of the dams. The pups were weighed at least every alternate day and the normalized body weight gains (defined in the figure) are plotted in Figure 7. After one week the pups from the dams not injected with vitamin D_3 stopped growing while the pups from dams given vitamin D_3 grew normally. This phenomenon of cessation of growth at about one week of age was also observed in the first experiment of this investigation (Figures 5 and 6).

To better understand the cause of this rat pup growth failure, one-half of the pups in each litter were injected subcutaneously with 10 microliters of 95% ethanol containing 50 units of vitamin D_3 at 10 days of age. The remaining pups were injected with 10 microliters of the ethanol vehicle. On days 12 through 15 one pup from each group in each litter was sacrificed and plasma collected. For the pups sacrificed on days 12, 13 and 14, plasma calcium was determined. The data presented in Table 5 clearly show that none of the pups were hypocalcemic and vitamin D_3 had little effect, if any, upon plasma calcium. Plasma calcium values were slightly lower the last two days as a result of additional care in preventing calcium-rich milk from contaminating the

FIGURE 7. The Growth of the Pups in "Experiment 2" as Evaluated by their Normalized Body Weight Gain (mean $\pm$ SEM).

The normalized weight gain of an individual litter is defined as the mean body weight of the litter divided by the litter's mean body weight at one day of age. At delivery the dams were switched from the 0.4% phosphorous, vitamin D-deficient diet containing 0.2% calcium to one that was calcium-free. Four dams were given intraperitoneal injections of 500 units of vitamin D_3 five days after delivery and the normalized weight gains of their pups are represented by solid squares. The remaining six dams did not receive any vitamin D_3 and the normalized weight gains of their pups are represented by the solid circles.

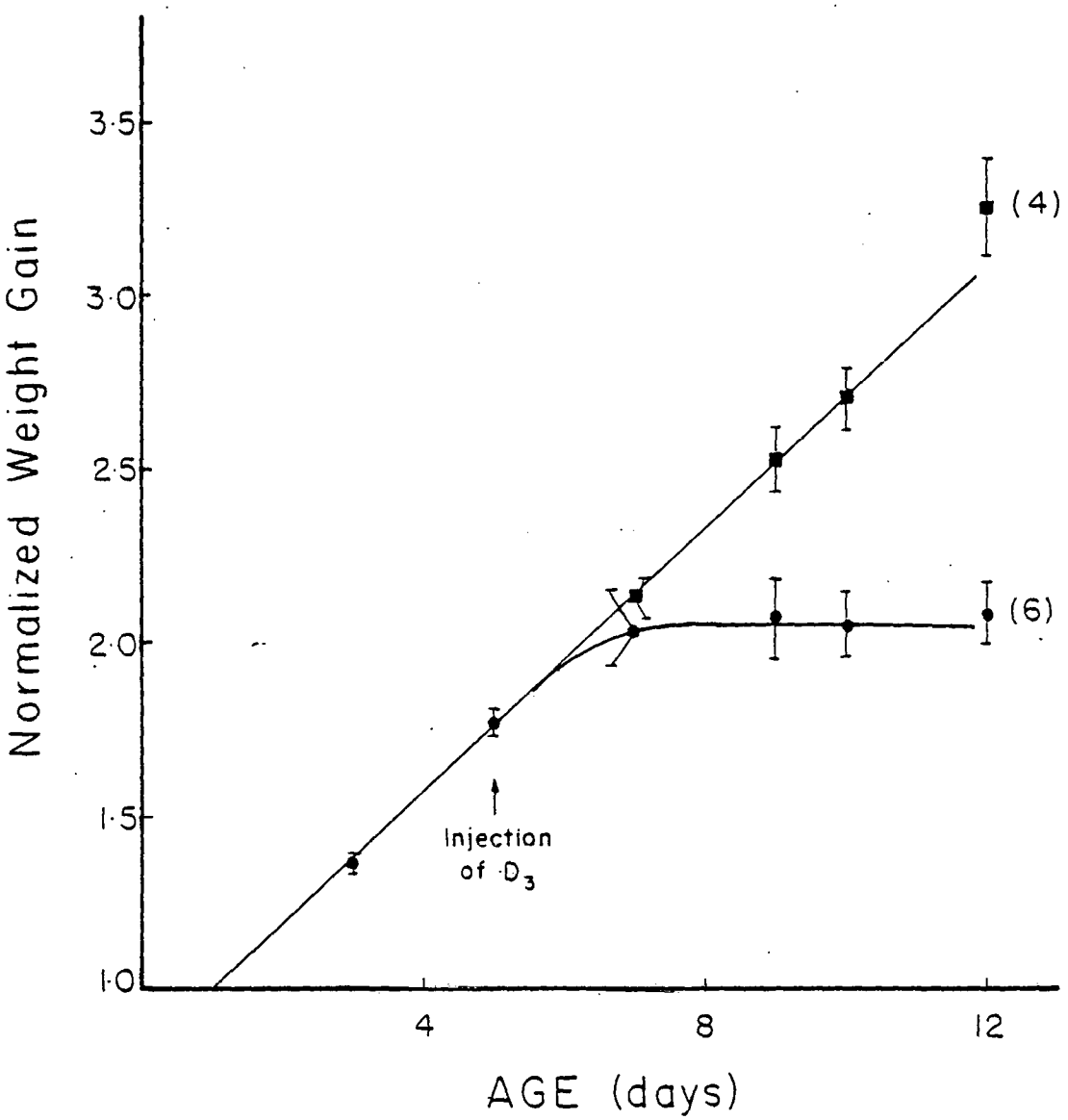

Normalized Weight Gain
3·5
3·0
2·5
2·0
1·5
1·0
4
8
12
AGE (days)
(4)
(6)
Injection
of D3

TABLE 5

Pup Plasma Calcium Values Following Vitamin D_3 Injection

Age (Days)	Dam Given D_3	Pups Given D_3	N	Plasma Calcium (mg.%)
12	Yes	Yes	4	11.6 ±0.1
	Yes	No	4	11.7 ±0.1
12	No	Yes	6	12.0 ±0.3
	No	No	5	11.7 ±0.2
13	No	Yes	4	10.5 ±0.3
	No	No	4	10.2 ±0.3
14	No	Yes	4	10.8 ±0.3
	No	No	4	10.3 ±0.3

Vitamin D_3 injected dams were given 500 units 5 days after delivery. At 10 days of age, one-half of the pups in each litter were given 50 units of vitamin D_3 as described in the text. Plasma calcium values are given as the mean ±SEM; N refers to the number of pups in each group.

plasma samples. On the 15th day plasma phosphate was determined as were the water, calcium, sodium and potassium contents of the calvaria; the results are given in Table 6. Plasma phosphate was significantly increased in the vitamin D_3-injected pups from the vitamin D-deficient dams. Changes in the calvarial contents of water, calcium and sodium were all in the direction of increased mineralization in these vitamin D_3-treated pups, although the change in each individual parameter was barely statistically significant. Vitamin D_3 did not appear to influence bone potassium, a reasonable index of cellularity.

In addition to determining plasma calcium levels on the pups sacrificed at 12 through 14 days of age (Table 5), the pups' calvaria were dissected out and incubated in the recently developed system for skeletal ion flux analyses (39).[1] The calvaria from the vitamin D_3-treated pups maintained a higher final level of buffer calcium after a four-hour incubation compared to the calvaria taken from the vehicle-injected littermates in 12 out of 12 cases. Since vitamin D_3 had a skeletal effect at a physiological dose of 50 units, the vehicle-injected pups appear to have been deficient in vitamin D. Further work is required in order to understand how this apparent increase in bone mineral solubility is promoted by vitamin D_3.

On the 15th day all the remaining pups were removed from the dams and sacrificed. The dams were starved overnight and then sacrificed

---

[1] These experiments were performed by C.R. Myers.

TABLE 6

Plasma Phosphate and Bone Data from 15-Day-Old Pups

	- Vitamin D_3	+ Vitamin D_3
Plasma phosphate (mM)	2.86±0.08	3.25±0.11[1]
Calvaria water (% wet wt.)	46.5 ±0.3	45.1 ±0.9[2]
Calvaria calcium (% dry wt.)	18.95±0.27	19.33±0.18[3]
Calvaria sodium (μmole/100 mg dry wt.)	18.27±0.19	18.68±0.24[3]
Calvaria potassium (mmoles/liter bone H_2O)	81.4 ±4.3	83.3 ±1.5

These pups were from dams that had not received any vitamin D_3. At ten days of age one-half of the pups from each litter were given 50 units of vitamin D_3 as described in the text. Values are given as the mean ±SEM for four pups (one from each litter). p values were determined with the Student t-test.

[1] $p < .025$ compared to vehicle-injected pups.

[2] $p < .10$ compared to vehicle-injected pups.

[3] $p < .20$ compared to vehicle-injected pups.

the following day. Sera were collected and the calvaria dissected out. The results of several assays performed on the sera and calvaria are given in Table 7. Vitamin D-deficient dams had lower serum calcium but higher serum phosphate values. No changes were observed in serum glucose, sodium or potassium with vitamin D status. Vitamin D_3-treated dams mobilized a significant fraction of their skeletal mineral as indicated by an increase in water content and a corresponding decrease in the calcium content of their calvaria. The skeleton was the only source of calcium available to the pups through milk since these dams were on a calcium-free diet since delivery. Vitamin D status did not appear to affect the thickness of the calvaria. One observation made but not quantitated was the fact that the vitamin D_3-treated dams ate considerably more food than the vitamin D-deficient dams.

TABLE 7

Comparison of Vitamin D_3-Injected Dams with Non-Injected Dams

	+ Vitamin D_3	Non-Injected
Serum calcium (mg.%)	9.8 ±0.3 (4)	8.6 ±0.4 (3)[1]
Serum phosphate (mM)	2.1 ±0.1 (4)	3.1 ±0.1 (3)[2]
Serum glucose (mg.%)	99 ± 5 (4)	116 ± 11 (3)
Serum sodium (mM)	130 ± 1 (4)	130 ± 1 (3)
Serum potassium (mM)	6.7 ±0.4 (4)	6.8 ±0.2 (3)
Calvaria water (% wet wt.)	38.1 ±0.8	27.6 ±1.0 (4)[3]
Calvaria calcium (% dry wt.)	19.2 ±0.8 (4)	22.2 ±0.3 (4)[4]
Calvaria thickness (millimeters)	0.85 ±0.09 (4)	0.86 ±0.06 (4)

Vitamin D_3-injected dams were given 500 units of vitamin D_3 by intraperitoneal injection on the fifth day after delivery. The dams were sacrificed on day 16 after an overnight fast; the last of the pups had been removed on the previous day. Values are given as the mean ± SEM for the number of data points in parentheses. p values were determined with the Student t-test.

[1] $p < .05$ when compared to vitamin D_3-injected dams

[2] $p < .005$ when compared to vitamin D_3-injected dams

[3] $p < .0005$ when compared to vitamin D_3-injected dams

[4] $p < .01$ when compared to vitamin D_3-injected dams

DISCUSSION

The daily dose of 1,25-$(OH)_2D_3$ required to maintain rats in a normal calcium and phosphorous balance depends upon age, sex and the dietary levels of calcium and phosphorous. The increased requirements for calcium and phosphate during growth are reflected in higher plasma levels of 1,25-$(OH)_2D_3$ in children (16,23). Norman et al. (41) have demonstrated a sex difference in growth rates of chickens given daily doses of 1,25-$(OH)_2D_3$. As dietary calcium is decreased, the efficiency of the intestinal absorption of this cation is increased and 1,25-$(OH)_2D_3$ is thought to be the "endogenous factor" mediating this phenomenon. Hughes et al. (27) have shown that blood levels of 1,25-$(OH)_2D_3$ increase when dietary calcium or phosphorous is lowered.

Under normal circumstances, the variable requirement for 1,25-$(OH)_2D_3$ is achieved through a complex interplay of various physiological factors. The synthesis of 1,25-$(OH)_2D_3$ by the kidney cortex mitochondria is influenced by parathyroid hormone (19), serum phosphate (66), insulin (57), estrogen (2), glucocorticoids (60) and possibly prolactin (59) and growth hormone (35) but not calcitonin (34). The mechanism(s) by which these agents act is currently under investigation in several laboratories and, at least in the case of parathyroid hormone, cAMP[2] appears to be involved (25). There is little information available about the control

---

[2] 3',5'-adenosine monophosphate.

of blood 1,25-$(OH)_2D_3$ levels by degradative pathways although fecal excretion, side chain oxidation and conversion to more polar metabolites do occur. 1,25-$(OH)_2D_3$ speeds up its own metabolic breakdown (18). Recent experiments have shown that when radiolabeled 1,25-$(OH)_2D_3$ is given daily to rats for two weeks at a dose level similar to the one used in this experiment, several unidentified metabolites could be distinguished by chromatographic analysis (53). When vitamin D-deficient rats are maintained on daily injections of 1,25-$(OH)_2D_3$ the physiological control mechanisms for regulating blood levels of 1,25-$(OH)_2D_3$ by synthesis are bypassed. Care must be taken in order that hypercalcemia does not develop (67).

There are several additional complications in choosing an effective maintenance dose of 1,25-$(OH)_2D_3$. As the need for calcium and phosphate increase during pregnancy and lactation, the requirement for 1,25-$(OH)_2D_3$ can also be expected to increase. The elevated blood levels of 1,25-$(OH)_2D_3$ observed in lactating rats (3) and during human pregnancy (23) are consistent with this expectation. Thus, a dose of 1,25-$(OH)_2D_3$ sufficient for an adult rat might be too low if that same rat became pregnant. The route of administration of 1,25-$(OH)_2D_3$ may also be an important factor. Since 1,25-$(OH)_2D_3$ was ineffective when given by repeated oral doses in the study of Tanaka et al. (65), the 1,25-$(OH)_2D_3$ was given by intraperitoneal injections in this investigation. A single daily injection of 1,25-$(OH)_2D_3$ with the subsequent oscillations of blood and tissue concentrations can at best only approximate the normal continuous

synthesis and breakdown. As might be expected for a relatively rapidly metabolized hormone, on a daily basis, chicks require less 1,25-$(OH)_2D_3$ when they are given injections daily rather than on alternate days (10).

In this investigation, a daily injection of 0.75 unit of 1,25-$(OH)_2D_3$ proved to be an adequate maintenance dose for young female rats on a diet of 0.2% calcium and 0.4% phosphorous. Although direct comparisons to other studies are difficult, the dose of 1,25-$(OH)_2D_3$ employed in this study does appear reasonable. Tanaka and DeLuca (65,66) used similar doses of 1,25-$(OH)_2D_3$ but their rats were fed diets with extremely low contents of either calcium or phosphorous. Rizzoli et al. (54) used a dose of 0.4 unit of 1,25-$(OH)_2D_3$ divided into two daily injections for parathyroidectomized rats. However, their rats were given higher dietary levels of calcium and phosphate than used in this study and thyroparathyroidectomy does not fully stop the synthesis of 1,25-$(OH)_2D_3$ (27). If the 0.75 unit/day dose of 1,25-$(OH)_2D_3$ required by a 300 gram rat is extrapolated to the requirement for a 70 kg human, assuming the dose goes as body weight to the 0.75 power (56), then an estimated value of 1.2 μg/day is obtained. This value is in excellent agreement with the clinical doses currently employed (6,21).

Under normal dietary conditions vitamin D is clearly necessary for an organism's development to full potential. Vitamin D-deficient animals typically suffer from muscle weakness, swollen joints and loss of appetite. These symptoms, together with the failure of the intestine to properly absorb calcium and phosphate, result in greatly

reduced intakes of calcium and phosphate. Although hypersecretion of parathyroid hormone can temporarily mobilize calcium and phosphate from bone, the skeleton eventually becomes refractive to parathyroid hormone and both hypocalcemia and hypophosphatemia occur. A vicious cycle is set up since the failure to maintain normal serum electrolytes aggravates the muscle weakness and loss of appetite.

Upon repleting deficient animals with vitamin D, enhanced intestinal absorption and the combined bone mineral mobilizing effects of parathyroid hormone and 1,25-$(OH)_2D_3$ result in increased blood levels of calcium and phosphate. The subsequent mineralization of cartilage and rapid body growth give the impression that vitamin D is an anabolic hormone. However, these anabolic actions are probably secondary to the normalization of blood calcium and phosphate levels. With proper dietary manipulation, normal skeletal growth and maturation occur in rats deprived of vitamin D (26) or parathyroid hormone (77). Several reviews have emphasized that there has been no experiment reported demonstrating a direct effect of any vitamin D metabolite on bone mineralization (13,51,52). The anabolic effects on the skeleton observed with low doses of parathyroid hormone have also not been satisfactorily dissociated from increases in the blood levels of calcium and phosphate (44). Both of these hormones are obviously required during dietary shortages of calcium and/or phosphate.

If vitamin D is not required for full development from weaning with proper management of dietary calcium and phosphorous, then rats

might not need vitamin D to produce and rear young successfully. Maternal parathyroidectomy does not interfere with reproduction and lactation (4,58). Hypophysectomized rats will deliver young but not nurse properly without prolactin (12). In both cases, each fetus has its own full complement of hormones. Although severely hypocalcemic vitamin D-deficient rats cannot reproduce (49), there has been no reported attempt to breed vitamin D-deficient rats made normocalcemic and normophosphatemic by dietary manipulation. If such rats can successfully reproduce and rear their young to sexual maturity, then a convincing argument could be made that vitamin D is not necessarily a required vitamin.

The experiments reported in this investigation demonstrate that $1,25\text{-}(OH)_2D_3$ can successfully support reproduction in rats given a vitamin D-deficient, low (0.2%) calcium, normal (0.4%) phosphorous diet. $1,25\text{-}(OH)_2D_3$ appears to fully mimic the actions of vitamin D in maintaining the appropriate physiological environment necessary for reproduction. Whether all of the responses to vitamin D in all species can be achieved by proper $1,25\text{-}(OH)_2D_3$ treatment is still an area for future research. Certainly, the studies of Bordier et al. (6) on man and those of Norman et.al. (41) and Sunde et. al. (64) on chickens strongly suggest that a metabolite of vitamin D_3 other than $1,25\text{-}(OH)_2D_3$ may be required for some of the diverse effects of vitamin D_3. The earlier discussion concerning the possibility that vitamin D and its metabolites might not be required at all assumed that proper dietary manipulation of calcium

and phosphorous could be achieved. Neither in this investigation, nor in those of Bordier et al. (6), Norman et al. (41) and Sunde et al. (64) have any attempts been made to reduce, if not eliminate altogether, the requirement for vitamin D and its metabolites by dietary manipulation.

The status of vitamin D activity in milk has yet to be resolved. Since little vitamin D activity can be obtained from milk with procedures used for lipid extractions, claims have been made that vitamin D in milk may exist in a water-soluble sulfated form (24,31). Osborn and Norman (43) have reported that milk contains some substance which displaces 25-$(OH)D_3$ from the chick serum binding protein and this substance did not appear to be 25-$(OH)D_3$. A binding protein for vitamin D metabolites has been found in human milk (73). Since the possibility exists that maternal 1,25-$(OH)_2D_3$ might be secreted into milk, the 1,25-$(OH)_2D_3$ injections into the dams were terminated at delivery. This procedure insured that the pups were not exposed to any form of vitamin D from birth.

The biological turnover of 1,25-$(OH)_2D_3$ is an important parameter to consider in evaluating the time course of the development of vitamin D-deficiency following the termination of 1,25-$(OH)_2D_3$ treatment. Following injection, the blood levels of 1,25-$(OH)_2D_3$ rise rapidly and then fall as metabolism and target tissue extraction occur. In the target tissues, 1,25-$(OH)_2D_3$ binds to receptors and proceeds to elict specific responses. Even after 1,25-$(OH)_2D_3$ has left the target tissues these responses continue for an additional period of time; thus, vitamin D-

deficiency might be present several days before the appearance of any symptoms indicative of the deficiency.

The plasma turnover of 1,25-$(OH)_2D_3$ is relatively rapid; Lawson and Emtage (32) have data indicating a four- to five-hour "half-life" in vitamin D-deficient chicks while Omdahl et al. (42) estimated the entire plasma pool of 1,25-$(OH)_2D_3$ is renewed every 20 hours in normal chicks. The body stores of 1,25-$(OH)_2D_3$ in man have been estimated to turnover approximately twice a day (21). By 24 hours, 20 to 30% of an injected dose of 1,25-$(OH)_2D_3$ has undergone side chain oxidation in the rat (30) and additional amounts of 1,25-$(OH)_2D_3$ have presumably been excreted and/or metabolized differently. As discussed previously, when single daily injections of 1,25-$(OH)_2D_3$ are given to vitamin D-deficient animals the maintenance dose obtained is probably an overestimate of the natural production rate. Assuming the daily synthesis of 1,25-$(OH)_2D_3$ by a 300 gram rat (with a plasma volume of 12 ml) is 0.4 unit and the steady state blood level is 20 ng/100 ml (27), then, if its turnover follows first order kinetics, the plasma half-life of 1,25-$(OH)_2D_3$ is 3.7 hours.

Wong et al. (78) have observed that, following a single injection to rachitic chicks, 1,25-$(OH)_2D_3$ reached its peak concentration in bone within four hours and by 48 hours only 10% of the 1,25-$(OH)_2D_3$ in bone remained. The time course of 1,25-$(OH)_2D_3$ action on intestine and bone has been investigated by giving a single injection of 1,25-$(OH)_2D_3$ to vitamin D-deficient animals and observing the induction and decay of

the intestinal absorption of calcium and phosphate and the mobilization of calcium and phosphate from bone mineral (33,38,68,75). Since these studies involved rats and chicks of various ages and dietary histories and utilized several procedures for analyzing intestinal absorption and bone mineral mobilization, the data on the length of time $1,25\text{-}(OH)_2D_3$ was active varied. Generally, one to three days after the injection of $1,25\text{-}(OH)_2D_3$ the bone and intestinal responses decayed back to baseline levels. However, in one instance, the intestinal absorption of calcium was still enhanced five days after $1,25\text{-}(OH)_2D_3$ treatment (68).

In this study, after terminating the daily maintenance injections of $1,25\text{-}(OH)_2D_3$ into dams at delivery, the growth of the rat pups ceased at about one week of age (Figures 5 and 6). A similar cessation of growth was observed (Figure 7) when normal impregnated female rats were placed on a vitamin D-deficient diet soon after mating. In both cases the dams had been placed on a calcium-free, vitamin D-deficient diet at delivery and normal growth of the pups occurred during the first week. When a low (0.2%) calcium diet was used rather than the calcium-free diet, the pups grew normally at least to 11 days of age (Figure 4).

Although the exact mechanism behind the defect responsible for the pup's growth failure has not been determined, several interesting observations can be made about this phenomenon. Pups grow normally when dams are placed on a calcium-free diet if there is an adequate source of vitamin D (Figure 7 and Reference 17). Thus in the presence of vitamin D, the mobilization of calcium and phosphate from the skeleton

provides the environment necessary in the dam for supporting normal neonatal development. Toverud et al. (72) have estimated that in a lactating rat as much as 130 mg of calcium may be transferred to milk every day and consequently, the plasma pool of calcium must be renewed roughly every 12 minutes. Calvaria from vitamin D_3-treated dams underwent significant resorption compared to those of vitamin D-deficient dams as indicated by an increase in water content and a decrease in calcium content (Table 7). During the skeletal resorption accompanying lactation trabecular bone is resorbed to a greater extent than cortical bone and cortical bone resorption occurs primarily at the endosteal surface (11,74). Under normal circumstances, enhanced levels (55) and potency of calcitonin (72) appear to minimize the destructive effects of lactation on the skeleton (70).

The failure of the vitamin D-deficient dams to mobilize sufficient bone mineral resulted in severe hypocalcemia (Figures 4 and 5). A similar group of rats who did not deliver pups and were kept on the 0.2% calcium, 0.4% phosphorous, vitamin D-deficient diet did not become hypocalcemic in the three and one-half weeks following termination of the daily injections of 1,25-$(OH)_2D_3$ (Table 3). Apparently, parathyroid hormone was able to mobilize sufficient skeletal calcium to maintain normocalcemia in these rats but was ineffective when the severe calcium drain accompanying lactation was present. Although several studies have shown that the skeleton is refractive to parathyroid hormone in young vitamin D-deficient rats (22,29,37,50), parathyroid hormone mobilized bone mineral when

infused along with calcium (25) and when normocalcemia was maintained by dietary manipulation (1). Further knowledge of the mechanisms of action of both 1,25-$(OH)_2D_3$ and parathyroid hormone on the skeleton will no doubt help to clarify these discrepancies. Nevertheless, in the absence of vitamin D, parathyroid hormone cannot mobilize sufficient bone mineral to prevent hypocalcemia in lactating dams.

The pups from the vitamin D-deficient dams must have been vitamin D-deficient themselves. Surprisingly, despite the fact that they stopped growing, these pups were not hypocalcemic (Figure 4 and Table 5). Simply placing the dams on a high (1.4%) calcium, high (1.0%) phosphorous diet allowed the pups to start growing again and they continued to grow slowly (Figure 6) despite the fact that they eventually became hypocalcemic (Table 4).

Very little information is available on the absorption of calcium and phosphate from the gut in neonatal rats. These rats do absorb gamma globulin antibodies and other large molecules by pinocytosis and "closure" does not occur until 18 days of age (9). Most of the calcium in milk is in a colloidal rather than ionic form and consequently, active transport of calcium by the neonatal gut might not be necessary. No attempt has been reported to find calcium-binding protein in the intestines of suckling rats despite the fact that this protein is induced by 1,25-$(OH)_2D_3$ in the intestines of weaned rats. Although rat pups have been shown to metabolize their endogenous supply of 25-$(OH)D_3$ (36), Weisman et al. (76) found little evidence of 1,25-$(OH)_2D_3$ production in these animals.

Naturally, as long as a rat pup is suckled and can absorb the ingested calcium, it has little need for the mineral mobilizing actions of 1-25-$(OH)_2D_3$.

Toverud (71) has shown that the calcium content of rat milk is independent of the plasma calcium in the dam as long as the parathyroid glands are intact. The mechanism by which calcium is transported from blood to milk does not appear to depend upon the blood calcium concentration. Therefore, the growth failure in the vitamin D-deficient pups probably did not result from a deficiency in milk calcium. Since the dams were on a normal (0.4%) phosphorous diet and had elevated serum phosphate levels (Table 7), a deficiency in milk phosphate is also an unlikely explanation for the growth failure in the pups. The observations that these pups had normal blood levels of calcium (Table 5) and nearly normal blood levels of phosphate (Table 6) support these claims.

One possible reason for the failure of the neonatal rats to continue growing might be that they were deficient in vitamin D. However, as discussed previously, the anabolic actions of vitamin D are thought to result from primary effects on blood calcium and phosphate concentrations. These non-growing pups were normocalcemic and only slightly hypophosphatemic. In addition, when 50 units of vitamin D_3 were given to one-half of the pups in each vitamin D-deficient litter, there did not appear to be any growth surge over the next several days. The primary defect seems to have been a maternal one and correctable, at least in part, by increasing the maternal intake of calcium (Figures 4 and 6).

Since the vitamin D-deficient dams were severely chronically hypocalcemic, an alteration of some calcium-dependent metabolic process responsible for the production of a normal milk might have been affected. Under this hypothesis, the vitamin D-deficient dams could not mobilize sufficient skeletal calcium to maintain normocalcemia and an increase in blood calcium brought about by either an increased dietary intake of calcium or a $1,25\text{-}(OH)_2D_3$-induced mobilization of skeletal calcium would at least partially correct the hypocalcemia and permit production of milk containing all of the proper nutrients. A preliminary analysis of the dam's condition showed that serum levels of sodium, potassium and glucose were all normal (Table 7).

A relatively small change in milk composition can have a drastic effect on neonatal development. For example, there exists a strain of mice with a genetic abnormality resulting from a recessive mutation. Females with this "lethal milk" syndrome give birth to normal pups but neonatal growth does not occur solely as a result of a 34% decrease in the zinc content of the dam's milk (45). A similar deficiency of an essential nutrient found in milk would explain the cessation of growth observed in the pups of the vitamin D-deficient dams placed on a calcium-free diet. In this case however, the abnormal milk is hypothesized to result from hypocalcemia rather than a genetic defect.

Whatever the exact mechanism of the defect resulting in the failure of the vitamin D-deficient pups turns out to be, two conclusions can be drawn from this investigation. First, $1,25\text{-}(OH)_2D_3$ can support

reproduction in female rats otherwise devoid of vitamin D. Second, the successful production of rat pups deficient in vitamin D opens up new experimental possibilities for studies on the actions and metabolism of vitamin D_3. In particular, immature, vitamin D-deficient bone can be used for in vitro investigations on the mechanism of action of $1,25\text{-}(OH)_2D_3$ on the mobilization of bone mineral.

BIBLIOGRAPHY

1. Au, W.Y.W. and L.G. Raisz: Restoration of parathyroid responsiveness in vitamin D-deficient rats by parenteral calcium or dietary lactose. J. Clin. Invest. 46, 1572-1578 (1967).

2. Baksi, S.N. and A.D. Kenny: Does estradiol stimulate in vivo production of 1,25-dihydroxyvitamin D_3 in the rat?. Life Sciences 22, 787-792 (1978).

3. Boass, A., S.V. Toverud, T.A. McCain, J.W. Pike and M.R. Hausler: Elevated serum levels of 1α,25-dihydroxycholcalciferol in lactating rats. Nature 267, 630-632 (1977).

4. Bodansky, M. and V.B. Duff: Effects of parathyroid deficiency and calcium and phosphorous of the diet on pregnant rats. J. Nutrition 21, 179-192 (1941).

5. Bonjour, J.-P., C. Preston and H. Fleisch: Effect of 1,25-dihydroxyvitamin D_3 on the renal handling of P_i in thyroparathyroidectomized rats. J. Clin. Invest. 60, 1419-1428 (1977).

6. Bordier, P., H. Rasmussen, P. Marie, L. Miravet, J. Gueris and A. Ryckwaert: Vitamin D metabolites and bone mineralization in man. J. Clin. Endocrinol. & Metab. 46, 284-294 (1978).

7. Boris, A., J.F. Hurley and T. Trmal: Relative activities of some metabolites and analogs of cholecalciferol in stimulation of tibia ash weight in chicks otherwise deprived of vitamin D. J. Nutrition 107, 194-198 (1977).

8. Chen, P.S., T.Y. Toribara and H. Warner: Microdetermination of phosphorous. Anal. Chem. 28, 1756-1758 (1956).

9. Clark, S.L.: The ingestion of proteins and colloidal materials by columar absorptive cells of the small intestine in suckling rats and mice. J. Biophys. Biochem. Cytol. 5, 41-49 (1959).

10. Cork, D.J., M.R. Haussler, M.J. Pitt, E. Rizzardo, R.H. Hesse and M.M. Pechet. 1α-hydroxyvitamin D_3: A synthetic sterol which is highly active in preventing rickets in the chick. Endocrinol. 94, 1337-1345 (1974).

11. Currey, J.D. and S.M. Hughes: The effects of pregnancy and lactation on some mechanical properties of the femora of the rat. Calc. Tiss. Res. 11, 112-123 (1973).

12. Daugaday, W.H. and M. Kapadia: Maintenance of serum somatomedin activity in hypophysectomized pregnant rats. Endocrinol. 102, 1317-1320 (1978).

13. DeLuca, H.F.: Mechanism of action and metabolic fate of vitamin D. Vitamins and Hormones 25, 315-367 (1967).

14. DeLuca, H.F.: Vitamin D - 1976. In, Endocrinology, vol. 2, ed. by V.H.T. James, Excerpta Medica, Amsterdam-Oxford, pp. 262-267, 1976.

15. Donaldson, H.H. (ed.): The Rat Data and Reference Tables for the Albino Rat and the Norway Rat. Memoirs of Wister Institute of Anatomy and Biol., 2nd edition, No. 6, 1924.

16. Eisman, J.A., A.J. Hamstra, B.E. Kream and H.F. DeLuca: 1,25-dihydroxyvitamin D in biological fluids: A simple and sensitive assay. Science 193, 1021-1023 (1976).

17. Ellinger, G.M., J. Duckworth, A.C. Dalgarno and M.H. Quenoville: Skeletal changes during pregnancy and lactation in the rat: Effect of different levels of dietary calcium. Brit. J. Nutrition 6, 235-253 (1952).

18. Frolik, C.A. and H.F. DeLuca: The stimulation of 1,25-dihydroxy-cholecalciferol metabolism in vitamin D-deficient rats by 1,25-dihydroxcholcalciferol treatment. J. Clin. Invest. 52, 543-548 (1973).

19. Garabedian, M., M.F. Holick, H.F. DeLuca and I.T. Boyle: Control of 25-hydroxycholecalciferol metabolism by parathyroid glands. Proc. Natl. Acad. Sci. 69, 1673-1676 (1972).

20. Goodwin, D., D. Noff and S. Edelstein: The parotid gland: A new target organ for vitamin D action. Biochim. Biophys. Acta 539, 249-252 (1978).

21. Gray, R.W., A.E. Caldas, D.R. Wilz, J. Lemann, Jr., G.A. Smith and H.F. DeLuca: Metabolism and excretion of ^{3}H-1,25-$(OH)_2D_3$-vitamin D_3 in health adults. J. Clin. Endocrinol. Metab. 46, 756-765 (1978).

22. Harrison, H.C., H.E. Harrison and E.A. Park: Vitamin D and citrate metabolism: Effect of vitamin D in rats fed diets adequate in both calcium and phosphorous. Am. J. Physiol. 192, 432-436 (1958).

23. Haussler, M.R., M.R. Hughes, J.W. Pike and T.A. McCain: Radio-ligand receptor assay for 1,25-dihydroxyvitamin D: Biochemical, physiologic and clinical applications. In, Vitamin D: Biochemical Chemical and Clinical Aspects Related to Calcium Metabolism, ed. by A.W. Norman et al., Walter de Gruyter, N.Y., pp. 473-482, 1977.

24. Hollis, B.W. and H.H. Draper: Vitamin D metabolites in milk. Fed. Proc. 37, 409 (1978).

25. Horiuchi, N., T. Suda, H. Takahashi, E. Shimazawa and E. Ogata: In vivo evidence for the intermediary role of 3',5'-cyclic AMP in parathyroid hormone-induced stimulation of 1α,25-dihydroxy-vitamin D_3 synthesis in rats. Endocrinol. 101, 969-974 (1977).

26. Howard, G.A., R. Haller, J.E. Wergedal and D.J. Baylink: Normocalcemia corrects the defect in bone matrix formation and maturation induced by vitamin D deficiency. Clin. Res. 25, 392A (1977).

27. Hughes, M.R., P.F. Brumbaugh, M.R. Haussler, J.E. Wergedal and D.J. Baylink: Regulation of serum 1α,25-dihydroxyvitamin D_3 by calcium and phosphate in the rat. Science 190, 578-580 (1975).

28. Hughes, M.R. and M.R. Haussler: 1,25-Dihydroxyvitamin D_3 receptors in parathyroid glands. J. Biol. Chem. 253, 1065-1073 (1978).

29. Kakuta, S., T. Suda, S. Sasaki, N. Kimura and N. Nagata: Effects of parathyroid hormone on the accumulation of cyclic AMP in bone of vitamin D-deficient rats. Endocrinol. 97, 1288-1293 (1975).

30. Kumar, R., D. Harnden and H.F. DeLuca: Metabolism of 1,25-dihydroxy-vitamin D_3: Evidence for side chain oxidation. Biochemistry 15, 2420-2423 (1976).

31. Lakdawala, D.R. and E.M. Widdowson: Vitamin D in human milk. The Lancet 1, 167-168 (1977).

32. Lawson, D.E.M. and J.S. Emtage: Localization and function of 1,25-dihydroxycholecalciferol in chick intestine. Biochem. Soc. Spec. Pub. 3, 75-90 (1974).

33. Lawson, D.E.M., M. Charman, P.W. Wilson and R. Spencer: Intestinal calcium transport and the biogenesis of intestinal proteins inducing calcium binding protein. In, Endocrinology of Calcium Metabolism, ed. by D.H. Copp and R.V. Talmage, Excerpta Medica Amsterdam, Oxford, pp. 191-196, 1978.

34. Lorenc, R., Y. Tanaka, H.F. DeLuca and G. Jones: Lack of effect of calcitonin on the regulation of vitamin D metabolism in the rat. Endocrinol. 100, 468-472 (1977).

35. MacIntyre, I., K.W. Colston, M. Szelke and E. Spanos: A survey of the hormonal factors that control calcium metabolism. Ann. N.Y. Acad. Sci. 307, 345-354 (1978).

36. Mendelsohn, M. and J.G. Haddad, Jr. Postnatal fall and rise of circulating 25-hydroxyvitamin D in the rat. J. Lab. Clin. Med. 86, 32-37 (1975).

37. Morii, H. and H.F. DeLuca: Relationship between vitamin D deficiency, thyrocalcitonin and parathyroid hormone. Am. J. Physiol. 213, 358-362 (1967).

38. Morrissey, R.L., D.T. Zolock, D.D. Bikle, R.N. Empon, Jr. and T.J. Bucci: Intestinal response to 1α,25-dihydroxycholecalciferol. I. RNA polymerase, alkaline phosphatase, calcium and phosphorous uptake in vitro, and in vivo calcium transport and accumulation. Biochem. Biophys. Acta 538, 23-33 (1978).

39. Neuman, W.F., R. Brommage and C.R. Myers: The measurement of Ca^{2+} effluxes from bone. Calc. Tiss. Res. 24, 113-117 (1977).

40. Norman, A.W. and H. Henry: 1,25-dihydroxycholecalciferol - A hormonally active form of vitamin D_3. Recent Prog. in Hormone Res. 30, 431-480 (1974).

41. Norman, A.W., T. Okamoto and H. Henry: Differential response of male and female chickens to dihydroxylated vitamin D metabolites. Fed. Proc. 37, 409 (1978).

42. Omdahl, J.L., G. Jelinek and R.P. Eaton: Kinetic analysis of 25-hydroxyvitamin D_3 metabolism in strontium-induced rickets in the chick. J. Clin. Invest. 60, 1202-1210 (1977).

43. Osborn, T.W. and A.W. Norman: A "25-hydroxycholecalciferol-like compound" in milk as determined by specific competitive binding radioassays. In, Vitamin D: Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism, ed. by A.W. Norman et al. Walter de Gruyter, N.Y., pp. 523-525, 1977.

44. Parsons, J.A.: Parathyroid physiology and the skeleton. Chapter 5 in, The Biochemistry and Physiology of Bone, ed. by G.H. Bourne, second edition, vol. IV, Academic Press, pp. 159-225, 1976.

45. Piletz, J.E. and R.E. Ganschow: Zinc deficiency in murine milk underlies expression of the lethal milk (lm) mutation. Science 199, 181-183 (1978).

46. Pitt, M.J. and M.R. Haussler: Vitamin D: Biochemistry and clinical applications. Skeletal Radiol. 1, 191-208 (1977).

47. Procsal, D.A., W.H. Okamura and A.W. Norman: Structural requirements for the interaction of 1α,25-$(OH)_2D_3$-vitamin D_3 with its chick intestinal receptor system. J. Biol. Chem. 250, 8382-8388 (1975).

48. Raabo, E. and T.C. Terkildsen: On the enzymatic determination of blood glucose. Scand. J. Clin. Lab. Invest. 12, 402-407 (1960).

49. Raisz, L.G.: Discussion following - Physiologic and pharmacologic regulation of bone resorption. New England J. Med. 282, 909-916 (1970).

50. Rasmussen, H., H.F. DeLuca, C. Arnaud, C. Hawker and M. Von Stedingk: The relationship between vitamin D and parathyroid hormone. J. Clin. Invest. 42, 1940-1946 (1963).

51. Rasmussen, H. and P. Bordier: The Physiological and Cellular Basis of Metabolic Bone Disease. The Williams & Wilkins Company, Baltimore, Md., 1974.

52. Reynolds, J.J.: The role of 1,25-dihydroxycholecalciferol in bone metabolism. Biochem. Soc. Spec. Publ. 3, 91-102 (1974).

53. Ribovich, M.L. and H.F. DeLuca: 1,25-dihydroxyvitamin D_3 metabolism: The effect of dietary calcium and phosphorous. Arch. Biochem. Biophys. 188, 164-171 (1978).

54. Rizzoli, R., H. Fleisch, and J.-P. Bonjour: Effect of thyroparathyroidectomy on calcium metabolism in rats: Role of 1,25-dihydroxyvitamin D_3. Am. J. Physiol. 233, E160-E164 (1977).

55. Samaan, N.A., G.D. Anderson and M.E. Adam-Mayne: Immunoreactive calcitonin in the mother, neonate, child and adult. Am. J. Obs. Gynecol. 12, 622-625 (1975).

56. Schmidt-Nielsen, K.: Energy metabolism, body size and problems of scaling. Fed. Proc. 29, 1524-1532 (1970).

57. Schneider, L.E., H.P. Schedl, T. McCain and M.R. Haussler: Experimental diabetes reduces circulating 1,25-dihydroxyvitamin D in the rat. Science 196, 1452-1454 (1977).

58. Sinclair, J.G.: Fetal rat parathyroids as affected by changes in maternal serum calcium and phosphorous through parathyroidectomy and dietary control. J. Nutrition 23, 141-152 (1942).

59. Spanos, E., J.W. Pike, M.R. Haussler, K.W. Colston, I.M.A. Evans, A.M. Goldner, T.A. McCain and I. MacIntyre: Circulating 1 alpha, 25-dihydroxyvitamin D in the chicken: Enhancement by injection of prolactin and during egg laying. Life Sciences 19, 1751-1756 (1976).

60. Spanos, E., K.W. Colston and I. MacIntyre: Effect of glucocorticoids on vitamin D metabolism. FEBS Letters 75, 73-76 (1977).

61. Stern, P.H., C.L. Trummel, H.K. Schnors and H.F. DeLuca: Bone resorbing activity of vitamin D metabolites and congeners in vitro: Influence of hydroxyl substituents in the A ring. Endocrinol. 97, 1552-1558 (1975).

62. Stern, P.H., T. Mavreas, C.L. Trummel, H.K. Schnoes and H.F. DeLuca: Bone-resorbing activity of analogues of 25-hydroxycholecalciferol and 1,25-dihydroxycholecalciferol: Effects of side chain modification and stereoisomerization on responses of fetal rat bones in vitro. Mol. Pharmacol. 12, 879-886 (1976).

63. Suda, T., H.F. DeLuca, H.K. Schnoes, Y. Tanaka and M.F. Holick: 25,26-dihydroxycholecalciferol, a metabolite of vitamin D_3 with intestinal calcium transport activity. Biochemistry 9, 4776-4780 (1970).

64. Sunde, M.L., C.M. Turk and H.F. DeLuca: The essentiality of vitamin D metabolites for embryonic chick development. Science 200, 1067-1069 (1978).

65. Tanaka, Y., H. Frank and H.F. DeLuca: Role of 1,25-dihydroxy-cholecalciferol in calcification of bone and maintenance of serum calcium concentration in the rat. J. Nutrition 102, 1569-1578 (1972).

66. Tanaka, Y. and H.F. DeLuca: The control of 25-hydroxyvitamin D metabolism by inorganic phosphorous. Arch. Biochem. Biophys. 154, 566-574 (1973).

67. Tanaka, Y., H. Frank and H.F. DeLuca: Biological activity of 1,25-dihydroxyvitamin D_3 in the rat. Endocrinol. 92, 417-422 (1973).

68. Tanaka, Y. and H.F. DeLuca: Role of 1,25-dihydroxyvitamin D_3 in maintaining serum phosphorous and curing rickets. Proc. Natl. Acad. Sci. 71, 1040-1044 (1974).

69. Tanaka, Y., L. Castillo, H.F. DeLuca and N. Ikekawa: The 24-hydroxylation of 1,25-dihydroxyvitamin D_3. J. Biol. Chem. 252, 1421-1424 (1977).

70. Taylor, T.G., P.E. Lewis and O. Balderstone: Role of calcitonin in protecting the skeleton during pregnancy and lactation. J. Endocrinol. 66, 297-298 (1975).

71. Toverud, S.V.: Calcium-vitamin D-parathyroid interrelationships in lactating rats. In, The Transfer of Calcium and Strontium Across Biological Membranes, ed. by R.H. Wasserman, Academic Press, N.Y., pp. 341-358 (1963).

72. Toverud, S.V., C. Harper and P.L. Munson: Calcium metabolism during lactation: enhanced effects of thyrocalcitonin. Endocrinol. 99, 371-378 (1976).

73. Van Baelen, H., R. Bouillon and P. DeMoor: Binding of 25-hydroxycholecalciferol in tissues. J. Biol. Chem. 252, 2515-2518 (1977).

74. Warnock, G.M. and J. Duckworth: Changes in the skeleton during gestation and lactation in the rat. Biochem. J. 38, 220-224 (1944).

75. Wasserman, R.H., A.N. Taylor and C.S. Fullmer: Vitamin D-induced calcium-binding protein and the intestinal absorption of calcium. Biochem. Soc. Special Pub. 3, 55-74 (1974).

76. Weisman, Y., R. Sapir, A. Harell and S. Edelstein: Maternal-perinatal interrelationships of vitamin D metabolism in rats. Biochim. Biophys. Acta 428, 388-395 (1976).

77. Wergedal, J., M. Stauffer, D. Baylink and C. Rich: Inhibition of bone matrix formation, mineralization and resorption in thyroparathyroidectomized rats. J. Clin. Invest. 52, 1052-1058 (1973).

78. Wong, R.G., J.F. Myrtle, H.C. Tsai and A.W. Norman: Studies on calciferol metabolism. V. The occurrence and biological activity of 1,25-dihydroxyvitamin D_3 in bone. J. Biol. Chem. 247, 5728-5735 (1972).