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EFFECT OF
DIETARY MINERALS AND DIABETES
ON
RENAL VITAMIN D HYDROXYLATION
AND
CALCIUM HOMEOSTASIS

by

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submitted in partial fulfilment of the
requirements for the degree of
Doctorate of Philosophy
in Biochemistry

ABSTRACT

Mg deficiency is associated with profound changes in Ca homeostasis which may involve complex interactions between PTH and vitamin D. To examine the aetiology of the aberrant Ca homeostasis in Mg deficiency a series of studies were conducted in chicks to assess indices of vitamin D metabolism and PTH target tissue effects. Mg deficient chicks fed adequate Ca had normal circulating $1,25(OH)_2D_3$, 1αhydroxylase activity and retention of Ca in bone, despite hypocalcaemia. In contrast chicks consuming diets low in Ca concomittant with or subsequent to Mg depletion exhibited elevated circulating $1,25(OH)_2D_3$, decreased bone Ca and normocalcaemia. Mg deficient chicks which received $1,25(OH)_2D_3$ supplementation maintained normal extracellular Ca. Collectively these results suggest alterations in vitamin D metabolism are pivotal to the genesis of hypomagnesaemic hypocalcaemia.

The inappropriate 1αhydroxylase responsiveness to the prevailing hypocalcaemia in Mg deficiency was not associated with decreased circulating $25(OH)D_3$, elevated circulating $24,25(OH)_2D_3$, increased metabolic clearance of $1,25(OH)_2D_3$ or alterations in renal tubule viability; nor was renal resistance to PTH indicated. Since neither vitamin D resistance nor renal or skeletal PTH resistance were consistently observed in Mg deficiency, it is possible that alterations in cell Ca, induced by Mg deficiency, are involved in the aetiology of hypomagnesaemic hypocalcaemia.

Alterations in vitamin D metabolism and Ca homeostasis are consistently observed in insulin dependent diabetes. The aetiology of the aberrant vitamin D metabolism in diabetes was investigated in experimental spontaneous (BB) and chemically induced (STZ) diabetes. Circulating and net synthesis of $1,25(OH)_2D_3$ were reduced in both models of diabetes. These reductions were observed 72 hours after insulin deficiency in STZ diabetes. In vivo insulin therapy normalized circulating $1,25(OH)_2D_3$ and 1αhydroxylase activity in diabetes, however 1αhydroxylase activity was not increased in

diabetic renal tubules exposed to insulin in vitro. Thus insulin does not directly alter basal $1,25(OH)_2D_3$ synthesis or acutely influence vitamin D metabolism. Renal resistance to in vitro PTH was not observed in diabetes. $1,25(OH)_2D_3$ production was enhanced in renal tubules from diabetic rats on low dietary Ca. PKC activity was elevated in STZ diabetic rat renal tubule membranes 24 hours prior to the onset of decreased circulating and net synthesis of $1,25(OH)_2D_3$. PKC activity correlated with renal hypertrophy in diabetes, and PKC activation was associated with decreased 1αhydroxylase activity in isolated renal tubules. Therefore the alterations in vitamin D metabolism associated with insulin dependent diabetes, may be related to elevated renal PKC activity and renal hypertrophy.

DEDICATION

TO THE MEMORY OF MY FATHER
whose enthusiastic intellectual curiosity
and tenacity inspired me
AND DAVID
whose consistent encouragement and support
made this thesis possible

William Weaver died of brain cancer, December 5, 1986.
David Spurr was diagnosed with brain cancer,
December 1, 1990.

After experience had taught me that all things which frequently take place in ordinary life are vain and futile, and when I saw that all the things I feared, and which feared me, had nothing good or bad in them save in so far as the mind was affected by them; I determined at last to inquire whether there was anything which might be truly good, and able to communicate its goodness, and by which the mind might be affected to the exclusion of all other things; I determined, I say, to inquire whether I might discover and attain the faculty of enjoying throughout eternity continual supreme happiness...I could see the many advantages acquired from honor and riches, and that I should be debarred from acquiring these things if I wished seriously to investigate a new matter...But the more one possesses of either of them, the more the pleasure is increased, and the more one is in consequence encouraged to increase them; whereas if at any time our hope is frustrated, there arises in us the deepest pain. Fame has also this great drawback, that if we pursue it we must direct our lives in such a way as to please the fancy of men, avoiding what they dislike and seeking what pleases them...But the love towards a thing eternal and infinite alone feeds the mind with a pleasure secure from all pain...The greatest good is the knowledge of the union which the mind has with the whole of nature...The more the mind knows, the better it understands its forces and the order of nature; the more it understands its forces or strength, the better it will be able to direct itself and lay down the rules for itself; and the more it understands the order of nature, the more easily it will be able to liberate itself from useless things; this is the whole method.

SPINOZA - ON THE IMPROVEMENT OF THE INTELLECT

ONLY KNOWLEDGE, THEN, IS POWER AND FREEDOM; AND THE ONLY PERMANENT HAPPINESS IS THE PURSUIT OF KNOWLEDGE AND THE JOY OF UNDERSTANDING.

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Nor may the weary in mind withstand his fate
Nor is high heart his helping
For the doom-eager oft bindeth fast his thought
In blood - bedabbled breast
THE SEAFARER
Ezra Pound

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
α	alpha
AIN	American Institute of Nutrition
β	beta
$25(OH)D_3$	calciferol
Ca	calcium
$1,25(OH)_2D_3$	calcitriol
cm	centimeter
D_3	cholecalciferol
cpm	counts per minute
cAMP	cyclic adenosine 3',5'-monophosphate
P450	cytochrome P450
DNA	deoxyribonucleic acid
diabetes	Diabetes Mellitus
DAG	diacylglycerol
$24,25(OH)_2D_3$	24,25-dihydroxycholecalciferol
D_2	ergocalciferol
EDTA	ethylenediaminetetra-acetate
fmoles	femtomoles
g	gram
G	guanine
HPLC	high pressure liquid chromatography
$25(OH)D_3$ hydroxylase	25 hydroxy vitamin D hydroxylase
$25(OH)D_3$ 1αhydroxylase, 1αhydroxylase	25 hydroxy vitamin D 1αhydroxylase
24 hydroxylase	25 hydroxy vitamin D 24 hydroxylase
$25(OH)D_3$ hydroxylation	25 hydroxy vitamin D hydroxylation
$25(OH)D_3$ 1αhydroxylation	25 hydroxy vitamin D 1αhydroxylation
IP_3	inositol trisphosphate
IGF-1	insulin like growth factor I
IMCAL	integral membrane binding protein
IU	international units
$[Ca]_i$	intracellular calcium
$[Ca^{2+}]_i$	intracellular free ionized calcium
$[Mg^{2+}]_i$	intracellular free ionized magnesium
$[Mg]_i$	intracellular magnesium
k	kilo
Mg	magnesium
mRNA	messenger RNA
IBMX	1-methyl-3-isobutyl-xanthine
μM	micromolar
mg	milligrams

mM	millimolar
mU	milliunits
M	molar
nm	nanometer
nmoles	nanomoles
NADPH	nicotinamide-adenine dinucleotide phosphate reduced
NADH	nicotinamide-adenine dinucleotide, reduced
PTG	parathyroid gland
PTE	parathyroid gland extract
PTH	parathyroid hormone
%	percentage
PBS	phosphate buffered saline
P	phosphorus
PI	phosphoinositide signalling pathway
4αPDD	4αphorbol 12,13 didecanoate
pg	picograms
PNF	post nuclear fraction
PKC	protein kinase C
RNA	ribonucleic acid
BB	spontaneously diabetic
SDS	sodium dodecylsulfate
SHIP	steroid hydroxylase inducing proteins
STZ	streptozotocin
TPA	12-O-tetra-decanoyl phorbol 13-acetate
UV	ultra violet
VDR	vitamin D receptor
H_2O	water

DEFINITIONS

circulating	concentration of a metabolite, peptide or mineral (measured) in serum or plasma
$1,25(OH)_2D$	refers to active metabolite of vitamin D - calcitriol including D_2 and D_3
$1,25(OH)_2D_3$	refers to active metabolite of vitamin D - calcitriol the D_3 form
n1-34 PTH	N terminal portion (first 34 amino acids) active peptide fragment of PTH
transcalciferin	vitamin D binding protein
vitamin D	refers to all vitamin D metabolites in general

GENERAL INTRODUCTION

Magnesium (Mg) is one of the most abundant ions present in living organisms (Altura et al., 1987). It catalyzes or activates more than 300 enzymatic reactions and is pivitol in the transfer, storage and utilization of cellular energy (Wester, 1987). As such, Mg is essential to a great many metabolic processes and functions such as glucose utilization, synthesis of fat, protein and nucleic acids, muscle contraction, and some membrane transport systems (Gunther, 1981). Consistent with these actions, negative Mg balance (denoted Mg deficiency) may contribute to the pathogenesis of various disease states, including atherosclerosis (Altura et al., 1990) cardiac arhythmias and myocardial damage (Prasad et al., 1980) arterial hypertension (Wester and Dyckner, 1987) and kidney stone disease (Bunce et al., 1974).

Primary Mg deficiency, (defined as inadequate intake) is rare. In contrast, secondary Mg deficiency (that due to increased requirements, for example; during growth, pregnancy or lactation), excessive gastrointestinal losses (for example; malabsorption syndromes), inadequate renal reabsorption (for example; excessive use of diuretics, kidney disease) and endocrine and metabolic derangements (diabetes mellitus, alcoholism) has a high prevalence (Flink, 1981).

Mg deficiency affects intracellular and extracellular calcium (Ca) homeostasis and is characterized by secondary hypocalcaemia (Shils, 1988) with soft tissue calcification and preferential mitochondrial accumulation of Ca (George and Heaton, 1975). Indeed many of the clinical manifestations of Mg deficiency (including neuromuscular hyperactivity, psychiatric disturbances and cardiac arhythmias) may be related to the prevailing hypocalcaemia (Flink, 1981).

The aetiology of disturbed Ca homeostasis during Mg deficiency is currently unknown, although alterations in the parathyroid hormone (PTH)/vitamin D axis have been implicated (Shils, 1988). PTH directly modulates extracellular Ca levels via its actions on bone Ca resorption and

renal Ca reabsorption and also enhances renal production of calcitriol ($1,25(OH)_2D_3$), the active metabolite of vitamin D (Slatopolsky et al., 1990). Increased circulating $1,25(OH)_2D_3$ in turn increases active intestinal Ca absorption, modulates bone Ca resorption and enhances renal Ca reabsorption (DeLuca, 1984). Thus the perturbed Ca homeostasis associated with Mg deficiency may be related to impaired PTH secretion (Suh et al, 1973, Breitenbach et al, 1973, Rayssiguier et al, 1977) and/or target organ refractoriness to PTH (Forbes and Parker, 1980). While chronic Mg depletion results in failure of parathyroid gland (PTG) function (Chase and Slatopolsky, 1974) circulating PTH is often increased in acute Mg deficiency (Rayssiguier et al., 1982, Anast and Forte, 1983, Welsh and Weaver, 1988) and PTG hypertrophy has been described in hypocalcaemic Mg deficient chicks (Welsh et al., 1981). Reports that elevated PTH in Mg deficiency failed to alleviate the hypocalcaemia imply target organ resistance to PTH (Levi et al., 1974). One of the most important effects of PTH, in the correction of hypocalcaemia, is stimulation of $1,25(OH)_2D_3$ production; exemplified by significant hypocalcaemia during vitamin D deficiency despite hyperparathyroidism (DeLuca, 1984). Mg is a cofactor for the renal $25(OH)D_3$ 1αhydroxylase (1αhydroxylase) (Gray et al., 1972) the enzyme which synthesizes $1,25(OH)_2D_3$, and is also a cofactor for PTH enhancement of $1,25(OH)_2D_3$ synthesis, through the adenylate cyclase signal transduction pathway (Rude and Oldham, 1985). Vitamin D metabolism has not been extensively studied in uncomplicated Mg deficiency although patients with hypomagnesaemia due to malabsorption or alcoholism exhibited sub-normal circulating levels of $1,25(OH)_2D_3$ despite hypocalcaemia (Fuss et al., 1985, Rude et al., 1985). Reddy and Sivakumer (1976) reported two hypomagnesemic children with overt rickets who were unresponsive to vitamin D administration until plasma Mg was normalized. Additional evidence of alterations in vitamin D metabolism in both humans (Rosler and Rabinowitz, 1973, Medalle et al., 1976) and animals (Miravet et al., 1980, Traba et al., 1985) with hypomagnesaemia suggests that alterations in

Ca metabolism in Mg deficiency may involve complex interactions between PTH and vitamin D.

In this thesis vitamin D metabolism and Ca homeostasis were examined during the development of acute uncomplicated Mg deficiency in chicks. Target tissue responsiveness to PTH in Mg deficiency, was examined by in vitro and in vivo PTH stimulation studies. The role of vitamin D in the pathogenesis of the hypomagnesaemic hypocalcaemia was evaluated with $1,25(OH)_2D_3$ supplementation trials during Mg depletion.

Disturbances in Ca homeostasis are consistently observed in diabetes mellitus (Hough, 1987). Clinical (Nyomba et al., 1986, Frazer et al., 1986, Gertner et al., 1979) and experimental (Schneider et al., 1977, Wongsurawat et al., 1983) insulin dependent diabetes is associated with hypocalcaemia and alterations in bone growth and turnover. Young insulin dependent diabetics have reduced bone mass due to a reduced rate of accretion of bone during the growth period (resulting in growth retardation) whereas older insulin dependent diabetics have reduced bone mineral content due to increased bone loss (resulting in increased bone fracture rates, osteoporosis and arthritic lesions) (Auwerx et al., 1988). The aetiology of these alterations is unknown although abberations in vitamin D and/or PTH metabolism have been implicated (Colette et al., 1989, Hough, 1987). Decreased plasma $1,25(OH)_2D_3$ is consistently observed in human diabetes (Nyomba et al., 1986) and decreased $1,25(OH)_2D_3$ and 1αhydroxylase activity have been reported in experimental diabetes (Schneider et al., 1977, Wongsurawat et al., 1983). These alterations may be a direct or indirect consequence of insulin deficiency. Insulin deficiency might decrease $1,25(OH)_2D_3$ levels by influencing its synthesis and/or degradation. Thus, insulin deficiency could decrease $1,25(OH)_2D_3$ synthesis by compromising renal metabolic integrity (Bachelet et al., 1986) affecting steady state levels of one or more protein components of the 1αhydroxylase (Wang and Taub, 1991, Meisler and Howard, 1989) or by altering substrate (Nyomba et al., 1989) or cofactor concentrations (Mooradian

and Morley, 1987) or by 1αhydroxylase stimulation or inhibition (Ikeda et al., 1985). Alternately, insulin deficiency could decrease circulating $1,25(OH)_2D_3$ by increasing $1,25(OH)_2D_3$ catabolism (Wongsurawat et al., 1983).

Diabetes is the most frequent chronic disease associated with hypomagnesaemia (Jackson and Meier, 1968). Human and animal studies have indicated that diabetes is associated with lower circulating, tissue and bone Mg concentrations (Ewald, Gebre-Medhin and Tuvemo, 1983, Bhimji et al., 1986) secondary to enhanced renal loss of Mg (hypermagnesuria) (Paolisso et al., 1990). The hypermagnesuria during diabetes is inversely related to the degree of hyperglycaemia and the prevailing insulin levels (Paolisso et al., 1990). Insulin causes redistribution of Mg from the extracellular to the intracellular space; conversely insulin deficiency reduces intracellular Mg $[Mg]_i$. Mg deficiency in diabetes has been linked to insulin resistance (Paolisso et al., 1990) retinopathy (McNair, et al., 1978) ischemic heart disease (Sellig and Heggtveit, 1974) and alterations in Ca homeostasis (Mooradian and Morley, 1987). Saggese et al., (1991) reported that Mg administration to hypomagnesaemic insulin dependent diabetic children elevated circulating Mg, corrected the hypocalcaemia and normalized circulating $1,25(OH)_2D_3$. Thus the perturbations in the PTH/vitamin D/Ca axis prevalent in diabetes may be, in part, related to secondary Mg deficiency (Mooradian and Morley, 1987).

In the second series of studies described in this thesis vitamin D metabolism and Ca homeostasis were systematically examined in two experimental models of acute diabetes. The role of adenylate cyclase and PKC signal transduction pathways in the altered vitamin D metabolism and Ca homeostasis associated with diabetes was assessed both in vitro and in vivo. To elucidate the pathophysiology of the aberrant vitamin D metabolism and Ca homeostasis, in diabetes, temporal studies of diabetes induction and insulin withdrawal were conducted.

Overall these studies were intended to contribute to an understanding of the physiological and biochemical factors involved in the regulation of steady

state $1,25(OH)_2D_3$ levels and net synthesis of $1,25(OH)_2D_3$. It was of interest to determine whether observations on vitamin D metabolism and Ca homeostasis in primary Mg deficiency were relevant to diabetes, a form of secondary Mg deficiency.

OBJECTIVES

The primary objectives of these studies were:

1. To determine if alterations in vitamin D metabolism were relevant to the aberrant Ca homeostasis characteristic of primary Mg deficiency.

This objective was achieved by examining vitamin D metabolism and Ca homeostasis in Mg deficient chicks.

2. To ascertain the aetiology of the alterations in vitamin D metabolism and Ca homeostasis characteristic of insulin dependent diabetes.

This objective was achieved by examining vitamin D metabolism and Ca homeostasis in two rat models of insulin dependent diabetes.

3. To gain insight into endogenous factors involved in the modulation of steady state $1,25(OH)_2D_3$ levels and regulation of renal 1αhydroxylase activity.

This objective was achieved by examining vitamin D metabolism in vivo (chicks, rats) and in vitro (freshly isolated renal tubules) with emphasis on the role of signal transduction, (adenylate cyclase and PKC) during various physiological and pathological states: including basal conditions (normal), low Ca stress, low P stress, Mg deficiency and insulin dependent diabetes.

CHAPTER 1 - LITERATURE REVIEW

1.1. CALCIUM HOMEOSTASIS

1.1.1. EXTRACELLULAR CALCIUM HOMEOSTASIS

The extracellular Ca concentration is maintained at a constant level of 2.5 mM or 10 mg% (MacIntyre, 1986). Approximately half is ionized and the remainder is largely bound to plasma albumin. The maintenance of calcaemia is accomplished by hormonal controls of bone resorption or synthesis, intestinal Ca and phosphorus (P) absorption, and renal Ca and P reabsorption (Shahani, 1989). The resorption or synthesis of bone involves complex interactions between PTH, $1,25(OH)_2D_3$, calcitonin and other factors not well understood (Stein, 1990, Narbaitz, 1991). Intestinal absorption of Ca occurs primarily in the upper portions of the small intestine and is dependent on Ca status and requirements, the form of Ca ingested and other dietary components such as phosphate, fiber, oxalate, and phytate. The most important regulator of active Ca absorption is $1,25(OH)_2D_3$ (Shahani, 1989). Hormonal regulation of renal Ca reabsorption occurs primarily in the distal tubules and the major regulator is PTH while the relative importance of $1,25(OH)_2D_3$ is unclear (Holick, 1987).

Under conditions of Ca stress (pregnancy, lactation, low Ca intake, decreased absorption, disease), low ionized Ca results in secretion of PTH by the PTG due to liberation of stored hormone. PTH acts directly via its receptor (in kidney tubules and osteoblasts) and indirectly as a natural endogenous ionophore (MacIntyre, 1986). PTH activates both adenylate cyclase and phosphoinositide (PI) signal transduction pathways to enhance bone osteoclastic resorption, increase renal $1,25(OH)_2D_3$ synthesis and increase renal Ca reabsorption (MacIntyre, 1986). The elevated $1,25(OH)_2D_3$ in turn enhances active intestinal Ca and P absorption, Ca and P reabsorption in the distal renal tubules and enhances PTH stimulated bone resorption (DeLuca, 1984). As plasma ionized Ca returns to normal PTH levels decrease. This is achieved via Ca dependent intracellular degradation of the hormone and direct $1,25(OH)_2D_3$ inhibition of PTH synthesis and secretion (Silver et al., 1986).

Circulating $1,25(OH)_2D_3$ eventually normalizes presumably due to reduced stimulation by PTH and by $1,25(OH)_2D_3$ feedback inhibition (Slatopolsky, 1990).

1.1.2. INTRACELLULAR CALCIUM HOMEOSTASIS

In resting cells the intracellular free ionized Ca ($[Ca^{2+}]_i$) is 0.1-0.3 μM while the extracellular free Ca ion is approximately 1 mM; comprising a 10,000 fold concentration gradient of Ca^{2+} across the cell membrane. The $[Ca^{2+}]_i$ is controlled by the uptake and release of the cation across the three major membrane systems which bound the cytoplasm, as well as the preponderance and capacity of various Ca binding proteins to sequester Ca. The three major membrane systems which regulate Ca transport are the plasma membrane, the inner mitochondrial membrane and the endoplasmic reticulum (ER). Each membrane possesses distinct transport mechanisms which act in concert to regulate and modulate $[Ca^{2+}]_i$ and $[Ca]_i$ (Nicholls, 1986). Ca binding proteins (calbindins, calmodulin etc.) bind Ca in uM concentrations thereby functioning to buffer surges in $[Ca^{2+}]_i$.

At the level of the plasma membrane three mechanisms of Ca efflux exist: a Ca^{2+}/Mg^{2+}/ATPase, a Na^+/Ca^{2+} exchanger and a Ca^{2+}/H^+ exchanger. At the level of the plasma membrane there is a continuous inward Ca influx due to the electrochemical gradient as well as Ca entry through various (regulated) Ca influx channels. The mitochondrial membrane possesses a uniport carrier for Ca entry on its inner membrane and an efflux pathway exchanging Ca for Na or H. The ER contains a Ca^{2+}/ATPase for Ca uptake and an efflux pathway which can be activated by Ca, inositol trisphosphate (IP_3) or electrically (Campbell, 1987).

The steady-state $[Ca^{2+}]_i$ is determined by the Ca^{2+}/Mg^{2+}/ATPase activity which balances the continuous inward Ca leakage into the cell. The mitochondrial matrix and ER lumen adjust their Ca contents such that uptake and efflux of Ca from the cytoplasm balance. Under "normal" basal conditions the mitochondria will be largely depleted of Ca while the ER will be replete

with Ca (Nicholls, 1986).

A small increase in the permeability of the cell membrane to Ca (such as that induced by changes in $[Mg]_i$), or a small fractional release of Ca from an internal store (ER) (due to an external stimuli), will lead to a rise in $[Ca^{2+}]_i$ 10-100x that in the resting cell. Elevations in $[Ca^{2+}]_i$ are lowered back to 0.1-0.3 uM by plasma membrane Ca^{2+}/Mg^{2+}/ATPase mediated extrusion and mitochondrial uptake of Ca^{2+}, although mitochondrial uptake of Ca^{2+} is the dominant process due to the restricted V_{max} of the plasma membrane Ca^{2+}/Mg^{2+}/ATPase. Following acute $[Ca^{2+}]_i$ increases the plasma membrane Ca^{2+}/Mg^{2+}/ATPase eventually drains the excess Ca from the mitochondrial matrix (Nicholls, 1986). However, during a sustained increase (chronic) of $[Ca^{2+}]_i$, the mitochondrial matrix Ca concentration remains elevated and eventually Ca phosphate precipitation occurs (Forbe, 1971, Bunce et al., 1974).

1.2. VITAMIN D

1.2.1. VITAMIN D METABOLISM

The biologically active form of vitamin D is $1,25(OH)_2D$ (DeLuca, 1984) which functions in Ca and P homeostasis and modulates cell proliferation and differentiation (Haussler, 1986). $1,25(OH)_2D$ is a steroid hormone which interacts with a specific intracellular receptor, the vitamin D receptor (VDR), to regulate gene expression and protein synthesis; thereby influencing specific cellular functions (Pike, 1991).

$1,25(OH)_2D$ is synthesized sequentially from its parent compounds (cholecalciferol and ergocalciferol). Cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) are sterols (Deluca, 1984). Vitamin D_3 is produced in keratinocytes by the action of UV light on a precursor molecule, 7-dehydrocholesterol (an intermediate in cholesterol biosynthesis) and its synthesis is modulated by $1,25(OH)_2D$ (Esvelt et al., 1980). Vitamin D_2 is derived from ergosterol, found in plants, and differs from Vitamin D_3 by possessing a double bond between C22 and C23 and a methyl group at the C24 position (DeLuca, 1984). Vitamin D_2 and D_3 are equipotent in humans and both

must be activated via sequential hydroxylations to the active metabolite $1,25(OH)_2D$ for biological activity. Vitamin D hereafter denoted D_3, is converted to $25(OH)D_3$ in the liver by two distinct vitamin D 25 hydroxylases present in the hepatic microsomes and mitochondria. Metabolic regulation of the liver vitamin D 25-hydroxylases, and thereby circulating $25(OH)D_3$, does not appear to be strictly controlled (Turner, 1984). The $25(OH)D_3$ metabolite circulates bound to vitamin D binding protein (transcalciferin) and albumin and is converted to the active form, $1,25(OH)_2D_3$, by the action of a 25 hydroxyvitamin D 1αhydroxyase (1αhydroxylase) located in the renal proximal convoluted tubules (Akiba et al., 1980). Activity of the 1αhydroxylase and therefore circulating $1,25(OH)_2D_3$ is strictly controlled. Although extrarenal production of $1,25(OH)_2D_3$ has been reported (Reichel et al., 1991, Bikle et al., 1986, Dusso et al., 1990) circulating $1,25(OH)_2D_3$, in healthy humans, is primarily derived from the kidney, as exemplified by negligible circulating $1,25(OH)_2D_3$ in anephrectic patients (DeLuca, 1988, Slatopolsky, 1990). The half life of $1,25(OH)_2D_3$ in the circulation is four to six hours, thereafter it undergoes several catabolic reactions which render it less metabolically active, more polar and thus more excretable by the liver (DeLuca, 1984).

In addition to 1αhydroxylation, $25(OH)D_3$ can be hydroxylated at the 24 position, by a 25 hydroxyvitamin D 24 hydroxylase (24 hydroxylase), generating $24,25(OH)_2D_3$ (Kawashima et al., 1981). This metabolite is considered biologically inactive and possesses low affinity for the VDR (Haussler, 1986). Current evidence suggests $25(OH)D_3$ is converted to $24,25(OH)_2D_3$ when $1,25(OH)_2D_3$ synthesis is inhibited, indicating reciprocal regulation of these two enzymes (Turner, 1984). $1,25(OH)_2D_3$ can also be hydroxylated by the 24 hydroxylase forming $1,24,25(OH)_3D_3$ which is thereafter degraded and excreted through a series of catabolic reactions (Halloran et al., 1986, Makin et al., 1989). Thus the activity of the 24 hydroxylase also determines the catabolism of $1,25(OH)_2D_3$ and thereby influences $1,25(OH)_2D_3$ steady state levels. Consistent with its role in vitamin D catabolism, 24

hydroxylase activity has been demonstrated in renal and extra renal tissues including intestinal enterocytes and osteoblasts and correlates with the localization of the VDR (Pike, 1991). $1,25(OH)_2D_3$ increases 24 hydroxylase activity in vivo and in vitro, this effect is mediated by the VDR and is dependent upon new protein synthesis; therefore $1,25(OH)_2D_3$ may genomically induce its own catabolism (Pike, 1991). $1,25(OH)_2D_3$ also undergoes side-chain oxidation to form products such as 24-oxo-$1,25(OH)_2D_3$ and 24-oxo-$1,23,25(OH)_3D_3$ with eventual cleavage to form 1-hydroxy-23-carboxy-tetrano vitamin D_3 or calcitroic acid (Makin et al., 1989). Eventual breakdown products of calcitroic acid include D_3 glucuronides and sulfates which are excreted with bile (DeLuca, 1984). There exist several other metabolites of vitamin D_3 although to date no metabolic activity has been attributed to any of these compounds and their major function appears to be inactivation and excretion of vitamin D metabolites (Makin et al., 1989).

Thus circulating $1,25(OH)_2D_3$ (steady state levels) reflect synthesis and degradation, both of which are exquisitely controlled.

1.2.2. TARGET TISSUE EFFECTS OF VITAMIN D

The mode of action of $1,25(OH)_2D_3$ is analogous to that of steroid and thyroid hormones; the hormone interacts with a selective high affinity receptor protein, the VDR, which interacts with specific vitamin D response elements of vitamin D modulated genes to regulate their transcription, thereby altering steady state protein levels and modulating cellular functions (Haussler, 1986).

The VDR is a proteolytically sensitive intracellular protein with a species-specific molecular weight range of 48-60 kdaltons. The protein has a carboxy terminal steroid binding region and an amino terminal DNA binding region. The DNA binding region is highly basic and is composed of two finger like projections, each stabilized by a single zinc atom. The metal ions are tetrahedrally coordinated by four positionally conserved cysteine residues located at the base of each loop. The DNA binding domain of the VDR exhibits a large degree of amino acid homology to the superfamily of nuclear receptors

(steroid, thyroid, and retinoic acid receptors). The steroid binding region of the receptor extends from the DNA binding domain to the carboxyl terminus of the protein and is composed of a hydrophobic hormone binding pocket with a complex three dimensional structure. A third region of the receptor, located at the extreme carboxyl terminal portion is hypothesized to participate in the formation of either homodimeric or heterodimeric structures (Pike, 1991).

The $1,25(OH)_2D_3$-VDR complex interacts with cis-acting vitamin D responsive elements, comprised of two tandemly repeated hexanucleotide sequences separated by three base pairs, to modulate gene expression (Pike, 1991). The interaction of the receptor with $1,25(OH)_2D_3$ is characterized by an equilibruim dissociation constant of approximately 10^{-10} M (4°C). Receptor occupancy correlates with circulating $1,25(OH)_2D_3$ and reflects a simple equilibrium between extracellular $1,25(OH)_2D_3$ and unoccupied receptors, thus under normal physiologic conditions, only 10-20% of total receptor is occupied (Haussler, 1986). The VDR can be phosphorylated at serine residue(s) by protein kinase C (PKC) and casein kinase II (Haussler, 1991). PKC mediated phosphorylation at serine^{51} appears to be essential for transactivation (Pike, 1991). The phosphorylation state of the VDR may facilitate DNA binding, stabilize dimer formation or stabilize the protein to proteolytic degradation.

VDRs are located in target tissues involved in extracellular Ca homeostasis, including the intestine, bone/cartilage and kidney (Haussler, 1986). Consistent with the localization of VDRs are the traditional roles ascribed to $1,25(OH)_2D_3$ including; enhanced Ca and P absorption in the gut, synergistic modulation of Ca and P reabsorption (with PTH) in the renal tubules and coordinate remodelling of bone by osteoblasts and osteoclasts (with PTH) (Shahani, 1989). Elevations of circulating $1,25(OH)_2D_3$ thus lead to elevations of extracellular Ca and suppression of PTH (Shahani, 1990, Slatopolsky et al., 1984).

VDRs have also been located in endocrine tissues, including PTG, pancreas, pituitary gland, ovary, testes, reproductive tissue, including breast,

uterus, placenta as well as other tissues, including skin, muscle, brain, thymus, lung, liver and bone marrow (Haussler, 1986). As such the role of $1,25(OH)_2D_3$ has expanded to include effects on cell secretion, immune function and cell growth and differentiation (Pike, 1991).

The mechanism of action of $1,25(OH)_2D_3$ has been most extensively studied in the intestine. In the enterocytes of the upper small intestine $1,25(OH)_2D_3$ increases active intestinal Ca and P transport (Shahani, 1990). $1,25(OH)_2D_3$ has effects on intestinal brush-border processes (Lieberheir, 1989) ER and Golgi apparatus Ca uptake (DeRoland and Norman, 1990) alkaline phosphatase (Lucas et al., 1988) 24-hydroxylase (Tanaka and DeLuca, 1981 and PKC activity (Simboli-Campbell et al., 1991).

Of the proteins whose synthesis is modulated by $1,25(OH)_2D_3$, the best characterized are the intestinal and renal calbindins (9k and 28k) (Christakos et al., 1989). $1,25(OH)_2D_3$ modulates the expression of various bone proteins, including osteocalcin (Price and Baukol, 1980), matrix gla protein (Fraser et al., 1988), collagen (Rowe and Kream, 1982) and alkaline phosphatase (Majeska and Rodan, 1982). Other $1,25(OH)_2D_3$ modulated proteins include integral membrane binding protein (IMCAL) (Kowarski et al., 1986), mitochondrial proteins including cytochrome oxidase I and III (Kessler et al., 1986), Ca^{2+}-ATPase (Lidor and Edelstein, 1987), carbonic anhydrase in chick embryos (Narbaitz, 1991) and PKC in HL-60 cells (Martell, Simpson and Taylor, 1987).

$1,25(OH)_2D_3$ modulates the synthesis and/or secretion of a number of polypeptide hormones that include: PTH (Russell et al., 1986, Slatopolsky et al., 1990), calcitonin (Naveh-Many and Silver, 1988), prolactin (Wark and Tashijian, 1983) and insulin (Kadowski and Norman, 1984). $1,25(OH)_2D_3$ modulates cellular proliferation and differentiation in various mammalian cell lines (DeLuca, 1984), hematopoietic cells and lymphopoietic cells (Manolagas and Deftos, 1984), bone cells (Shinna et al., 1986, Stein, 1990, Nijweide et al., 1986) and epidermal keratinocytes (Dusso et al., 1990). These actions may be

related to $1,25(OH)_2D_3$'s effects on differentiation linked proto-oncogenes (c-fos and c-fms) and replication linked proto-oncogenes (c-myc and c-myb) (Simpson et al., 1987, Brelvi, Christakos and Studzinski, 1986). The immunomodulating effects of $1,25(OH)_2D_3$ may be linked to repression of interleukin II mRNA, altered levels and secretion of various cytokines (GM-CSF, gamma-interferon, TGF-beta and alpha) and immunoglobulins (Manolagas, Hustmyer and Yu, 1990). In addition, $1,25(OH)_2D_3$ regulates the stability of its own receptor to proteolysis (McCain et al., 1983) and alters VDR turnover rate in vitro (Costa et al., 1985).

$1,25(OH)_2D_3$ also has rapid non-genomic actions (liponomic) on its target cells. Vitamin D has been shown to increase de novo synthesis of phosphatidyl choline in chick duodenal mucosal cells and rat renal brush border membranes (Matsumoto, Fontaine and Rasmussen, 1981, Tsutsumi et al., 1985). The hormone also stimulates the incorporation of fatty acids into the phosphatidyl choline membrane fraction leading to an increase in polyunsaturated fatty acids, particularly arachidonic acid and linoleic acid (Kurnik, Huskey and Hruska, 1987). Alterations in the proportion of unsaturated to saturated fatty acids (in the acyl side chains) of membrane phospholipids have been shown to influence membrane fluidity, ion transport and Na+/K+ ATPase activity (Koutouzov et al., 1982).

Taken in unison $1,25(OH)_2D_3$ has effects on secreted and intrinsic membrane proteins, cytoplasmic proteins and protooncogene products as well as membrane lipids. These target tissue effects in turn modulate transepithelial Ca transport, $[Ca]_i$ and extracellular Ca, cell secretion, cell proliferation and differentiation.

1.2.3. RENAL VITAMIN D HYDROXYLATION

$1,25(OH)_2D_3$ is synthesized from $25(OH)D_3$ by the renal 1αhydroxylase. The 1αhydroxylase is a mixed function oxidase, located on the inner mitochondrial membrane of the epithelial cells of the proximal convoluted tubule, which catalyzes the hydroxylation of $25(OH)D_3$ at the 1α position on

the steroid ring. The reaction involves the transfer of reducing equivalents from NADPH by a flavoprotein (renoredoxin reductase) to a non-heme-iron protein (renoredoxin) which then transfers electrons to a specific cytochrome P450 (P450) which in turn catalyzes the hydroxylation of $25(OH)D_3$, using molecular oxygen (O_2), at the 1α position (Turner, 1984, Ghazarian, 1990). The displaced hydrogen is found in H_2O and the reaction requires the presence of Mg^{2+} ions (Gray et al., 1972). In a similar reaction, in the kidney, $25(OH)D_3$ may be 24 hydroxylated by the 24 hydroxylase.

It is not clear which, if any, components are shared between the 1α and 24 hydroxylases. Ghazarian (1990) has suggested that post translational cleavage of the P450 determines whether it functions in 1α or 24-hydroxylation. Warner (1982) presented evidence that one P450 (regulated by different factors) catalyzed both 1α and 24 hydroxylations. However, monoclonal antibodies generated against purified bovine (Bort and Crivello, 1988), chick (Mandel et al., 1990) and porcine (Gray and Ghazarian, 1989) renal mitochondrial P450s recognize distinct proteins which can catalyze either 1α or 24 hydroxylation in vitro (Ghazarian, 1990). Further support for this hypothesis is provided by Kung et al. (1988) who demonstrated differential regulation of 24 and 1αhydroxylation by P450 and protein synthesis inhibitors. Ohyama, Hayashi and Okuda (1989) recently reported the purification and characterization of a rat kidney mitochondrial P450 which demonstrates only 24 hydroxylase activity, while Ghazarian et al. (1991) has isolated a chick kidney mitochondrial P450 with both 1αhydroxylase and 24 hydroxylase activity; raising the possibility of two distinct P450s with potential 24 hydroxylase activity. It remains to be clarified if the two proteins possess similar biochemical localization and regulation. In agreement with this hypothesis, Kawashima et al. (1981) demonstrated constitutive 24 hydroxylase activity in the renal proximal convoluted tubule while the renal proximal straight tubule exhibited only $1,25(OH)_2D_3$ inducible 24 hydroxylase activity. These data suggest the existence of two distinct 24 hydroxylases; one

constitutive and one inducible. It remains to be clarified if the constitutive 24 hydroxylase protein will demonstrate 1αhydroxylase activity or cDNA sequence similarity. Both the rat kidney 24 hydroxylase (Okuda, 1991) and chick kidney 1α/24 hydroxylase (Ghazarian, 1991) have been cloned and demonstrate unique cDNA sequences. The sequence discrepancies may simply reflect species differences or demonstrate unique P450s. A definitive explanation awaits purification and cloning of a rat kidney mitochondrial P450 that demonstrates 1αhydroxylase activity.

1.2.4. REGULATION OF RENAL HYDROXYLATION OF 25(OH)D_3

Synthesis of 1,25$(OH)_2D_3$ reflects stimulation and inhibition of 1αhydroxylase activity, steady state 1αhydroxylase enzyme levels, cofactors (Mg, NADPH) and substrate availability (25$(OH)D_3$). Net renal synthesis of 1,25$(OH)_2D_3$ reflects production and degradation.

On a physiological level, Ca and P represent two major determinants of 1,25$(OH)_2D_3$ synthesis, precisely controlling circulating 1,25$(OH)_2D_3$ to the mineral needs of the organism (Shahani, 1990). Additional factors such as: Mg ions, insulin, growth hormone and insulin like growth factor I (IGF-1), pH, sex steroids (estrogen, prolactin), glucocorticoids, metal ions (cadmium, lead etc), calcitonin, and vitamin D status have all been shown to influence circulating 1,25$(OH)_2D_3$ (Turner, 1984, Carpenter, 1990). However the mechanism whereby these factors regulate circulating 1,25$(OH)_2D_3$ and influence 1αhydroxylase activity remains poorly defined.

By examining current knowledge on the regulation of steroidogenesis, a model for 1,25$(OH)_2D_3$ synthesis may be derived. Steroidogenesis is regulated by factors affecting basal enzyme activity, substrate availability and steady state enzyme levels. Both acute and chronic regulation of steroidogenesis, by elevations of intracellular cAMP, have been described. Elevations of intracellular cAMP acutely increase steroid synthesis rates by increasing substrate (cholesterol) availability and by inducing the synthesis of a short-lived protein which can stimulate steroidogenesis within 15 minutes by

affecting the rate-limiting step, the side-chain cleavage of cholesterol to pregnenolone (Waterman, Mason and Simpson, 1988, Purvis et al., 1973). Chronic cAMP regulation of steroidogenesis may (directly or indirectly) induce expression or activity of steroid hydroxylase inducing proteins (SHIPs) which regulate steroid hydroxylase gene expression by binding to cis-acting elements in the genome (Waterman, Mason and Simpson, 1988). Additional regulatory pathways have been highlighted by Suh and Amsterdam (1990) who demonstrated phorbol ester induction of protein kinase C (PKC) activity opposed cAMP induced steroidogenesis and John et al. (1987) who reported cAMP independent regulation of steroidogenesis by fetal growth factors.

Analogous to steroidogenesis, several factors known to regulate $25(OH)D_3$ 1αhydroxylation (1αhydroxylation) such as PTH and calcitonin, alter kidney cAMP metabolism as well. Both acute and chronic stimulation of $1,25(OH)_2D_3$ production by agonists which stimulate cAMP synthesis have been described. Reconstitution experiments suggest that acute regulation of 1αhydroxylation (by PTH and forskolin) is achieved via reversible phosphorylation of renoredoxin; a 1αhydroxylase enzyme component (Ghazarian, 1990). De-phosphorylation of renoredoxin is associated with stimulation of 1αhydroxylation activity (Siegel et al., 1986) whereas phosphorylation of renoredoxin (on serine and threonine residues) is associated with 1αhydroxylase inhibition (Nemani et al., 1989) and enhanced 24 hydroxylase activity (Mandel et al., 1990). This data suggests the existence of a phosphoprotein which activates the 1αhydroxylase by dephosphorylating renoredoxin (Siegel al., 1986). Ghazarian (1990) has suggested this putative phosphoprotein phosphatase is a substrate for cAMP-dependent protein kinase, evidenced by dephosphorylation of renoredoxin and stimulation of 1αhydroxylation by forskolin and other activators of adenylate cyclase and cAMP production (Armbrecht et al., 1984a,b). However evidence exists that several mitochondrial phosphatases and kinases exist which are involved in the phosphorylation and dephosphorylation of phosphoserine and

phosphothreonine-containing proteins (Shenolikar and Ingebritsen, 1984). The activity or expression of these phosphatases may be regulated by a number of agents including heat stable proteins, inhibitor 1 and inhibitor 2, Ca-calmodulin and Mg ions (Li, Price and Tabarini, 1985). For example Mg is a selective regulator of protein phosphatase 2C which has been shown to preferentially dephosphorylate P450 enzymes involved in cholesterol metabolism. Nemani et al. (1989) recently demonstrated dephosphorylation of 1αhydroxylase renoredoxin by this phosphatase. Thus acute phosphorylation/de-phosphorylation regulation of $25(OH)D_3$ hydroxylation likely occurs via both cAMP dependent and independent mechanisms.

Chronic elevation of cAMP has been correlated with enhanced $1,25(OH)_2D_3$ net synthesis (Korkor et al., 1987). This chronic cAMP mediated stimulation of 1αhydroxylation is dependent upon the presence of insulin and new protein synthesis (Henry, 1981, Korkor et al., 1987) implying the possible involvement of cAMP response elements in target genes, analogous to that of adrenal steroid hydroxylases. However this issue cannot be resolved until the proteins of the 1αhydroxylase complex have been cloned, sequenced and characterized.

Henry and Luntao (1989) demonstrated that incubation of primary cultures of chick renal tubules with 12-O-tetradecanoyl phorbol 13-acetate (TPA) (a phorbol ester which activates PKC) but not an inactive phorbol ester analogue, resulted in decreased net synthesis of $1,25(OH)_2D_3$ and increased net synthesis of $24,25(OH)_2D_3$ after four hours. Although PKC activation by TPA has been well documented (Leach and Blumber, 1989) no changes were observed in phorbol ester binding sites after four hours in these experiments. However, Mandla, Boneh and Tenenhouse (1990) demonstrated acute stimulation of net $24,25(OH)_2D_3$ synthesis by 1.6μM TPA which was preceded by activation of PKC.

Simultaneous incubation of tubules cultures with TPA and PTH or forskolin (demonstrated stimulators of 1αhydroxylase activity) abolished the

stimulatory effects characteristically observed with these agonists (Henry and Luntao, 1989). Since similar observations have been made in steroidogenic cell cultures by Suh and Amsterdam (1990), this suggests a common mechanism for steroidogenetic and vitamin D hydroxylase regulation by PKC.

Boneh, Mandla and Tenenhouse (1989) demonstrated that TPA increased mitochondrial PKC activity and phosphorylation of several mitochondrial substrates. Although PKC phosphorylates its substrates on serine and threonine residues (Leach and Blumberg, 1989) and phosphorylation of renoredoxin occurs on serine and threonine residues, the possibility that renoredoxin is a substrate for PKC has not been addressed.

It is not clear whether PKC affects the activity of one or both hydroxylase moieties. The data presented by Henry and Luntao (1989) support the concept that 1αhydroxylation exhibits greater sensitivity to agonist stimulation of PKC than 24 hydroxylation. A comparable decrease in net synthesis of $1,25(OH)_2D_3$ but no increase in $24,25(OH)_2D_3$ was obtained using the natural PKC stimulator diacylglycerol (DAG) and 24 hydroxylase stimulation was only observed using high (1.6μM) TPA concentrations in freshly isolated mouse tubules (Mandla et al., 1990). The concept of separate vitamin D hydroxylases should be considered. Both α and β^1 isozymes of PKC are expressed in kidney (Kosaka et al., 1988) and the β^1 isozyme has been shown to be exquisitely sensitive to DAG (Jaken, 1989). Thus it is possible that modulation of 1α and 24 hydroxylation are mediated by different PKC isozymes. Resolution of these questions awaits future experimentation.

The physiological relevance of these results requires demonstration of modulation of vitamin D metabolism by PKC activation in vivo. Both PTH and $1,25(OH)_2D_3$ activate PKC in kidney and modulate 1αhydroxylase activity. However further work is needed to integrate the actions of PTH and $1,25(OH)_2D_3$ in relation to renal PKC activity and determine their physiological relevance to the regulation of vitamin D metabolism.

1.2.4.a. CALCIUM AND $1,25(OH)_2D_3$ SYNTHESIS

Ca is a major determinant of steady state $1,25(OH)_2D_3$ and under normal circumstances Ca restriction is associated with elevated 1αhydroxylase activity (Boyle, Gray and DeLuca, 1971). The mechanism by which Ca restriction increases $1,25(OH)_2D_3$ net synthesis may include direct effects of ionized Ca (Bushinsky et al., 1985). However, the major effect appears to be mediated indirectly via stimulation of PTH secretion (Rosen and Chesney, 1983).

Manipulation of serum Ca by Ca infusion or EDTA without simultaneous PTH infusion indicated that changes in extracellular Ca can regulate $1,25(OH)_2D_3$ production independently of PTH (Matsumoto et al., 1987). In isolated mitochondria, both stimulatory and inhibitory effects of media Ca concentration upon 1αhydroxylase activity have been reported (Bikle, Murphy and Rasmussen, 1975). A biphasic dose response has been demonstrated, with stimulation occurring as Ca concentration is increased until maximum 1αhydroxylase activity is observed, then activity decreases to basal as Ca concentration is increased further. Postulated mechanisms for a Ca stimulatory effect may include increasing the availability of reducing equivalents for 1αhydroxylase activity by increasing the activity of intramitochondrial Krebs' cycle dehydrogenases (Hansford, 1985) indirectly increasing available energy to the mitochondria, altering the conformation of various protein components involved in enzyme activity (Kay, McCubbin and Sykes, 1987) or by activating calmodulin, which has been shown to enhance 1αhydroxylase activity (Taft et al., 1986). Inhibitory effects of Ca may be elicited through toxicity by intramitochondrial accumulation of Ca (Bunce et al., 1974) or by activating PKC or other factors which inhibit 1αhydroxylation (Ghazarian and Yanda, 1985). Elevated $[Ca^{2+}]_i$ is also associated with activation of PKC (Leach and Blumberg, 1989) and activators of PKC such as phorbol esters decrease net synthesis of $1,25(OH)_2D_3$ (Henry and Luntao, 1989).

Secretion of PTH by the PTG is exquisitely sensitive to the prevailing

Ca concentration (Brown and Chen, 1989) such that decreased extracellular Ca enhances PTH secretion while increased extracellular Ca represses PTH secretion (Slatopolsky et al., 1990). Both in vivo and in vitro experiments have directly implicated PTH as a major regulator of 1αhydroxylase activity and thus circulating $1,25(OH)_2D_3$. Hyperparathyroidism is associated with elevated circulating $1,25(OH)_2D_3$ and enhanced $1,25(OH)_2D_3$ production (Rosen and Chesney, 1983) while hypoparathyroidism is associated with both decreased circulating and net synthesis of $1,25(OH)_2D_3$ (Turner, 1984, DeLuca, 1984). PTH secretion and $1,25(OH)_2D_3$ synthesis are increased in rats fed diets deficient in either Ca or vitamin D (Fox, Kollenkirchen and Watters, 1991, Boyle et al., 1971) and parathyroidectomized rats have markedly decreased renal 1αhydroxylase activity (Ikeda et al., 1987, Turner, 1984).

Both acute and chronic stimulatory effects of PTH on 1αhydroxylase have been reported. PTH rapidly stimulates adenylate cyclase activity and elevates intracellular cAMP and cAMP dependent protein kinase activity in renal slices (Armbrecht et al., 1984a). Analogous to classical steroid hydroxylase regulation (Waterman, Mason and Simpson, 1988) elevated cAMP levels in renal proximal tubules have been correlated with acute and chronic increases in 1αhydroxylase activity. Kremer and Goltzman (1982) have reported that 15 minute incubation with PTH stimulated 1αhydroxylase activity in guinea pig renal slices. Siegal, Wongsurawat and Armbrecht (1986) demonstrated that dephosphorylation of the renoredoxin component of the 1αhydroxylase enzyme system occurred with 5 minutes exposure to PTH and was associated with increased 1αhydroxylase activity in a reconstituted system. These authors suggested that the putative cAMP/protein kinase A responsive phosphoprotein phosphatase which dephosphorylates renoredoxin is modulated by PTH (Nemani et al., 1989).

Elevated cAMP has also been correlated to chronic stimulatory effects of PTH on 1αhydroxylation which require new protein synthesis (Armbrecht et al., 1984b, Korkor et al., 1987). Chronic stimulation of 1αhydroxylation by

PTH in chick renal tubule cultures required the presence of insulin (Henry, 1981). The chronic regulation of 1αhydroxylase by cAMP and the role of cAMP response elements in target genes (Waterman, Mason and Simpson, 1988) cannot be resolved until the genes of the 1αhydroxylase complex have been cloned and characterized and antibodies to these proteins generated.

PTH has also been demonstrated to have acute effects on renal PI metabolism (Hsuska et al., 1987) although the relevance to vitamin D metabolism has yet to be resolved (for review refer to Welsh et al., 1991).

1.2.4.b. PHOSPHORUS AND $1,25(OH)_2D_3$ SYNTHESIS

P is the second major ion involved in the regulation of net $1,25(OH)_2D_3$ synthesis (Tanaka and DeLuca, 1973, Carpenter, 1990). The action of P on the 1αhydroxylase is unclear and may be direct or through the actions of growth factors such as IGF-1 (Gray et al., 1987).

In vitro studies with isolated renal tubules and mitochondria have not consistently demonstrated acute direct regulation of enzyme activity by P, although inhibition has been demonstrated by addition of P salts to mitochondrial preparations (Henry and Norman, 1976). Considering the postulated role for protein phosphorylation (Ghazarian, 1990) in the regulation of $1,25(OH)_2D_3$ biosynthesis, further studies should be conducted to elucidate potential direct actions of changes in intracellular P on 1αhydroxylase activity. In addition the role of alterations in P transport by the renal proximal tubules in the regulation of the 1αhydroxylase activity awaits further investigation.

In vivo studies have suggested a chronic role for extracellular P in the regulation of circulating $1,25(OH)_2D_3$ and 1αhydroxylase activity (Tanaka and DeLuca, 1974). P-deficient rats have elevated serum $1,25(OH)_2D_3$, hypercalcemia and reduced serum PTH levels (DeLuca, 1984, ibid, 1988). P depletion has been shown to increase 1αhydroxylase activity in chicks (Bikle et al., 1975) and guinea pigs (Simboli, 1988) and decrease $1,25(OH)_2D_3$ catabolism in guinea pigs (Simboli, 1988). Unlike hypocalcemia, PTH is not involved in hypophosphatemic activation of 1αhydroxylase, since P depletion

results in elevated circulating $1,25(OH)_2D_3$ in parathyroidectomized rats (Turner, 1984). However, growth hormone is required for the response to hypophosphataemia which is diminished in rats following hypophysectomy (Gray, 1981). IGF-1 mediates many of the trophic effects of growth hormone, is itself controlled by growth hormone (Harp, Goldstein and Phillips, 1991) and IGF-1 receptors are present in proximal tubules (Hammerman and Rogers, 1987). Thus IGF-1 has been implicated in the enhancement of 1αhydroxylase activity secondary to hypophospataemia (Gray, 1983). Infusion of IGF-1 did not elevate plasma $1,25(OH)_2D_3$ in normal or diabetic rats however, suggesting IGF-1 does not directly stimulate 1αhydroxylase activity but may be permissive for the hypophosphataemic effect (Gray, 1987). Much future work is required to unravel the mechanism of P regulation of vitamin D metabolism.

1.2.4.c. MAGNESIUM AND VITAMIN D

The influence of Mg on net $1,25(OH)_2D_3$ synthesis is unclear, although inappropriately normal circulating $1,25(OH)_2D_3$ has been recorded in clinical Mg deficiency and decreased 1αhydroxylase activity has been reported in experimental Mg deficiency (Coburn et al., 1973, Rude et al., 1985). In an isolated mitochondrial system, 1αhydroxylase activity displayed an absolute requirement for Mg (Gray et al., 1972). Mg is required for maintenance of membrane integrity, G protein interactions and adenylate cyclase activity (Gunther, 1981, Rude and Oldham, 1987) therefore Mg may influence 1αhydroxylase activity, ligand membrane receptor binding, receptor/G protein coupling to adenylate cyclase or adenylate cyclase activity. Thus Mg may influence $1,25(OH)_2D_3$ synthesis directly or may affect signal transduction mediated stimulation of 1αhydroxylase activity. However, Carpenter et al. (1987) demonstrated refractoriness of basal 1αhydroxylase activity, measured in isolated rat mitochondria, to changes in extramitochondrial Mg. Therefore a definitive assessment of the actions of Mg on 1αhydroxylase activity awaits further examination.

1.2.4.d. INSULIN AND $1,25(OH)_2D_3$ SYNTHESIS

A role for insulin in the regulation of vitamin D metabolism has been derived from observations of reduced circulating $1,25(OH)_2D_3$ and decreased 1αhydroxylase activity associated with insulin deficiency (Wongsurawat et al., 1983, Schneider et al., 1977) and correction of this defect by precise insulin therapy (Hough et al., 1983). Henry (1981) demonstrated that 24 hour incubation with insulin was required to permit chronic PTH mediated stimulation of 1αhydroxylase activity in primary cultures of chick renal tubules. However insulin did not alter basal 1αhydroxylase activity in these studies. Although renal proximal tubules possess insulin receptors (Yagil, Frank and Rabkin, 1988) no further studies on the direct actions of insulin on $25(OH)D_3$ hydroxylation have been conducted.

1.2.4.e. MEASUREMENT OF NET SYNTHESIS OF $1,25(OH)_2D_3$

Despite growing interest in the regulation of $1,25(OH)_2D_3$ biosynthesis, a thorough comprehension of its biochemistry and molecular biology remains scant. High basal 1αhydroxylase activity and low substrate requirement in avian (Booth, Tsai and Morris, 1985) and guinea pig kidney preparations (Delvin and Dussault, 1985) have facilitated measurement of in vitro 1αhydroxylase activity in these species, whereas rodents exhibit low basal 1αhydroxylase activity and require high substrate concentration to permit demonstration of 1αhydroxylase activity. Therefore much of the current information on the regulation of vitamin D metabolism has been generated using renal preparations from chicks and guinea pigs or rodents which have been subjected to various dietary or surgical manipulations to elevate basal 1αhydroxylase activity.

Vitamin D biosynthesis has been studied in renal homogenates (Engstrom et al., 1984), renal slices (Kremer and Goltzman (1982), mitochondria (Delvin and Dussault, 1985, Simboli, 1988) and fresh or cultured renal tubule preparations (Shain, 1972, Henry, 1981) from chicks and guinea pigs fed either control, low Ca, low P or vitamin D deficient diets.

Unfortunately, use of these species is frustrated by the general lack of bioassays and specific purified active peptides (in particular, PTH) which complicate attempts to examine signal transduction and conduct parallel in vivo studies with these animals. Caution must be used when extrapolating information generated in these species, particularly with respect to the disparity in Ca metabolism in birds vs mammals. For example, birds have higher mineral requirements due to egg laying capabilities, lower circulating $1,25(OH)_2D_3$, lower 24 hydroxylase activity, lower transcalciferin levels (Turner, 1984) and striking differences in renal anatomy and renal physiology (King, 1975). Experimental difficulties using these two species involve disparities in growth rates (chicks), difficulties with animal housing space (chicks), experimental cost (guinea pigs) and lack of an existing experimental data base (guinea pigs). Since the most commonly used experimental animal for diabetic and dietary deficiency studies is the rodent, and the existing data bases consist predominantly of information on these models, much effort has been directed towards development and validation of an in vitro 1αhydroxylase assay from rat or mouse renal tissue.

Assays in mammalian systems other than the guinea pig (rat, mouse) have been hampered by technical difficulties including: competition for substrate by transcalciferin (Ghazarian et al., 1978), inhibition by serum factors (Botham et al., 1976), elevated 24 hydroxylase activity (Vieth and Fraser, 1979) and production of alternate metabolites which comigrate with $1,25(OH)_2D_3$ on conventional straight phase HPLC (Brown and DeLuca, 1985). These problems have necessitated the use of renal preparations from vitamin D deficient, low Ca fed or thyroparathyroidectomized animals to elevate basal 1αhydroxylase activity. In addition, strategies to overcome the presence of inhibitors have included: extensive renal perfusion (Lobaugh, Almond and Drezner, 1986), high substrate concentrations (Vieth and Fraser, 1979, Tanaka and DeLuca, 1981) protease treatment (Ghazarian et al., 1983), enzyme purification (Nemani et al., 1989) and extensive product isolation and

verification (Lobaugh, Almond and Drezner, 1986) to obtain reliable and measurable activity.

With these techniques in vitro 1αhydroxylase activity has been sucessfully measured in renal homogenates from vitamin D deficient and vitamin D replete mice and rats (Lobaugh and Drezner, 1982, Vieth and Fraser, 1979), renal mitochondria from vitamin D deficient rats (Warner and Tenenhouse, 1985), renal slices from vitamin D deficient, low Ca fed and thyroparathyroidectomized rats (Wongsurawat et al., 1983, ibid, 1984, ibid, 1990, Ikeda et al., 1985), a reconstituted 1αhydroxylase system from control rats (Nemani et al., 1989), fresh preparations of suspended kidney cells from vitamin D deficient rats (Turner et al., 1982), freshly purified renal proximal tubules from low Ca, vitamin D deficient and control fed rats (Favus and Langman, 1986), and primary cultures of mouse renal tubules (Korker et al., 1987). However, caution must be exercised when interpreting data obtained from animals previously subjected to dietary or surgical manipulations. Vitamin D status modulates 1αhydroxylase stimulation by PTH in vivo (Booth, Tsai and Morris, 1985), and $1,25(OH)_2D_3$ modulates protein kinase A isozyme expression and activity (Liu, 1984). In addition, $1,25(OH)_2D_3$ modulates PKC activity (Simboli-Campbell et al., 1991) and gene expression (Simpson et al., 1987), which has been associated with $25(OH)D_3$ hydroxylase regulation (Henry and Luntao, 1989).

Although basal 1αhydroxylase activity has been demonstrated in various renal preparations, data on signal transduction modulation of 1αhydroxylase activity remains scant and inconsistent. Evidence has accumulated concerning the effects of species, diet or surgical regime on signal transduction modulation of $25(OH)D_3$ hydroxylase activity. For example, PTH stimulation of $1,25(OH)_2D_3$ production is not consistently observed at similar doses or incubation times. Indeed in vitro incubation with PTH (or in vitro stimulation of adenylate cyclase activity) has not been consistently associated with elevations in 1αhydroxylase activity. Acute stimulation of 1αhyroxylase activity

by PTH has been demonstrated in control fed guinea pig slices (3x basal activity, 15 minutes, $1x10^{-10}$ M PTH) (Kremer and Goltzman, 1982), in a reconstituted 1αhydroxylase system derived from renal slices from control rats (2x basal activity, 5 minutes $1x10^{-7}$ M PTH) (Siegal, Wongsurawat and Armbrecht, 1986) and in freshly isolated renal proximal tubules from low Ca fed rats (1.8x basal activity, 10 minutes, $1x10^{-7}$ M PTH), (Ro et al., 1990). Low doses of PTH ($1x10^{-7}$ M) have been associated with chronic 1αhydroxylase stimulation (four to six hours) in primary renal cultures of chick and mouse tubules (Henry, 1981, Korkor et al., 1987, Fukase et al., 1982 [D deficient]). In these studies four to six hours of incubation was necessary to achieve only a modest stimulation of 1αhydroxylase activity by PTH (1.3 to 1.8x basal stimulation). Further, inhibition of 1αhydroxylase activity was observed at higher PTH doses (greater than $1x10^{-8}$ M) in these studies. In contrast, Armbrecht et al. (1984b), reported that higher doses of PTH resulted in chronic 1αhydroxylase stimulation in vitamin D deficient, low Ca parathyroidectomized rat renal slices. Forskolin also enhanced 1αhydroxylase activity in renal slices from vitamin D deficient, low Ca parathyroidectomized rats. However neither PTH nor forskolin stimulated 1αhydroxylase activity in renal slices from control rats (Armbrecht et al., 1984a) and did not consistently inhibit 24 hydroxylase activity in control mice (Mandla, Martel and Tenenhouse, 1991). PTH did not increase 1αhydroxylase activity or decrease 24 hydroxylase activity in primary chick renal cultures, unless cultures were pretreated with $1,25(OH)_2D_3$. Stimulation of 24 hydroxylase by PKC activators was only observed subsequent to overnight incubation with $1,25(OH)_2D_3$ in chick primary cultures (Henry and Luntao, 1989). Taken in unison, these data suggest that dietary regime, surgical manipulations and the model system not only have profound effects on basal 1αhydroxylase activity but also influence the signal transduction pathways which mediate stimulation and inhibition of the vitamin D hydroxylases. Therefore interpretation of data on in vitro vitamin D hydroxylase activity and regulation must be viewed with respect to this information.

Measurement of 1αhydroxylase activity has traditionally been accomplished using radioactive substrate (^{3}H-25(OH)D_3) with quantification of newly synthesized 1,25$(OH)_2D_3$ by scintillation detection following column chromatography and HPLC purification of product. However basal (control) 1αhydroxylase activity in rodents is too low to permit measurement using this technique. In addition, this method may overestimate 1αhydroxylase activity due to reliance on recovery of total activity as an estimation of product recovery, and comigration of 19-nor-10-oxo-25-hydroxyl [^{3}H] vitamin D_3 (which can be produced in vitro from 25(OH)D_3 and comigrates with 1,25$(OH)_2D_3$ on conventional straight phase HPLC). To overcome these problems, measurement of 1αhydroxylase activity in rodents has been accomplished by using high substrate concentrations and a radioreceptor detection assay, which permits quantitation of picogram (pg) quantities of newly synthesized 1,25$(OH)_2D_3$. This assay is conducted in kidney preparations which have been thoroughly perfused with fresh buffer and subjected to protease digestion (to reduce interference by inhibitors and transcalciferin and decrease the nonspecific protein content). Unfortunately extensive isolation and rigorous product validation are required to verify 1,25$(OH)_2D_3$ authenticity and maintain assay reproducibility with this technique. Thus this method is very time consuming although it does permit estimation of 1αhydroxylase activity in preparations from control animals (Lobaugh, Almond and Drezner, 1986) and overcomes many of the drawbacks encountered using radioactive substrate. Finally, this assay uses supraphysiological substrate concentrations (80 μM), which may not reflect true enzyme rates, thus caution must be exercised when interpreting results obtained with this system. However, recently Favus and Langman (1986) described an improved in vitro 1αhydroxylase assay system which combines the use of purified rat renal proximal tubules with a radioreceptor assay for 1,25$(OH)_2D_3$. In this system lower substrate concentrations (5 μM) were used and assay sensitivity was sufficient to permit measurement of 1,25$(OH)_2D_3$ synthesis in preparations from control animals.

Use of fresh tissue preparations may be hampered by problems with tissue quantity and assay reproducibility and is contingent on rigorous adherence to experimental protocols and biological variability. While use of primary cultures of renal tubules overcomes many of these problems, 1αhydroxylase activity is inherently low in this system, 24 hydroxylase activity is absent unless stimulated by incubation with $1,25(OH)_2D_3$ (Henry, 1981) and this system requires the use of serum free medium for the demonstration of $1,25(OH)_2D_3$ synthesis (Korkor et al., 1987). Further, effects of diet and disease cannot be studied in cultured cells. Freshly prepared tissue more closely reflects in vivo enzyme activity and regulation, thereby it allows the study of the effects of pathological conditions on vitamin D hydroxylase regulation. Since isolated renal tubules maintain membrane receptor integrity, adenylate cyclase and PI systems, this experimental system is ideal for studying the role of signal transduction in the regulation of vitamin D metabolism. Thus freshly purified rat renal tubules were prepared and assayed as described by Favus and Langman (1986) after modification and validation, for the rat studies described in this thesis.

1.3. MAGNESIUM

1.3.1. MAGNESIUM HOMEOSTASIS

The human body contains 1000 nmoles of Mg with 60% located in the skeleton, 39% intracellularly (27% skeletal muscle; 6-7% in other cells) and only 1% in the extracellular fluid (Shils, 1988). Bone Mg is distributed within the surface pool (hydration shell or on the crystal surface - 30%) and the mineral crystal (integral bone Mg - 70%). Plasma Mg is composed of 55% free, 13% complexed (citrate phosphate and other ions) and 32% protein bound. The normal total plasma concentration of Mg is 0.7 - 1.0 mmol/l (2.0 - 2.5 mg/dl) (Wester, 1987).

There are three exchangeable Mg pools in the body 1) A quick turnover pool which consists mainly of extracellular Mg and bone surface Mg (bone surface Mg rapidly equilibrates with plasma Mg). 2) A medium turnover pool

of $[Mg]_i$ and 3) a slow turnover pool which consists mainly of that portion of skeletal Mg which is an integral part of the bone crystal (Shils, 1988).

Mg homeostasis is achieved via a balance between absorption of dietary Mg and excretion. Bone resorption contributes Mg to the plasma pool whereas bone formation removes Mg from the plasma pool. Under steady state conditions incorporation of Mg into tissues, including bone, equals the release of Mg during tissue turnover, thus Mg balance is maintained when ingestion and absorption equals excretion (Wester, 1987). Under conditions of growth (young animals) incorporation of Mg into tissues (primarily bone) must exceed breakdown and resorption, thus ingestion must exceed excretion to maintain balance. Since 60% of the body's Mg is located in bone, growing animals have relatively high Mg requirements and are therefore more susceptible to Mg deficiency than older animals.

Mg is absorbed predominantly in the small intestine (both the upper and lower proximal portions). There exists a nonsaturable simple diffusion pathway in the jejuneum and a saturable carrier mediated transport system in the ileum, which is saturated at low intraluminal Mg concentrations (Hardwick et al., 1991). Intestinal absorption of Mg is inefficient and except in extreme Mg deficiency is dependent upon the intestinal Mg concentration rather than the body Mg status (Carney et al., 1980). Factors shown to influence the efficiency of Mg absorption include the Ca, P, phytate and protein content of the diet. $1,25(OH)_2D_3$ may also modulate intestinal absorption of Mg but the current data is contradictory and inconclusive (Hardwick et al., 1991).

The kidney is the major excretory pathway for Mg and constitutes the major control point for effecting Mg balance (Quamme, 1989). Thus when Mg intake is low renal excretion of Mg is negligible. Normally only three to five% of filtered Mg is excreted in urine each day (80% of serum Mg is ultrafiltrable). 20 - 30% of the filtered Mg is reabsorbed by the proximal tubules (which have a low permeability to Mg) in conjunction with sodium (Na) transport. 65% of filtered Mg is reabsorbed in the distal nephron and the thick ascending limb

of the loop of Henle by an active transport mechanism. Several factors influence tubular reabsorption of Mg including: extracellular fluid expansion, osmotic agents, alcohol, loop diuretics and chronic excess glucocorticoids (Quamme and Dirks, 1983, Quamme, 1989).

1.3.2. INTRACELLULAR MAGNESIUM HOMEOSTASIS

The majority of an organism's Mg is located intracellularly (Shils, 1988). Total $[Mg]_i$ concentration is 10 - 30 nM, however most of this Mg is bound to negatively charged groups by outer sphere complexes, therefore the free $[Mg^{2+}]_i$ concentration is only 0.5 - 1.0 nM in muscle tissue and 0.1 - 0.5 nM in non muscle tissue. 90% of $[Mg]_i$ is compartmentalized with 45% in microsomes, 23% in the mitochondria (4% outer membrane, 50% intermembraneous space bound to two major proteins, 5% inner membrane and 41% matrix), 15% in the nucleus and the balance, 14%, in the cytosol. The nuclear and microsomal fractions of Mg are bound to nucleic acids while 90% of cytosolic Mg is bound to ATP and other Mg binding ligands (such as the negatively charged membranes and the surfaces of polyamines) (Grubb and Maguire, 1982). This bound cytosolic Mg represents an $[Mg^{2+}]_i$ reserve such that large reductions in extracellular Mg do not cause appreciable changes in free $[Mg^{2+}]_i$ (Gunther, 1986).

Cellular Mg levels are maintained by coupling Mg influx to efflux. There exists at least two pathways for Mg transport across the plasma membrane. The first is an electroneutral Na+/Mg2+ antiport which can be inhibited by amiloride and is ATP dependent while the second is a Mg2+/Ca2+ exchanger. Mg influx is mediated by a H+-ATPase. Current knowledge has been derived using Mg^{2+} sensitive electrodes however the recent development of Mg^{2+} specific fluorescent dyes should yield more precise information on transcellular Mg transport, intracellular distribution and ascertain whether $[Mg^{2+}]_i$ is dynamically regulated by signal transduction mediated stimuli, similarly to $[Ca^{2+}]_i$ (Gunther and Vorman, 1986, ibid, 1985, Gunther, 1986, Fry, 1986).

1.3.3. PHYSIOLOGICAL FUNCTIONS OF MAGNESIUM

Many of the biological effects of Mg are caused by its ability to form (weak) chelates (Shils, 1988). The major physiological roles of Mg are stabilization of membranes and chelation of intracellular ATP. Mg stabilizes membranes by binding to phospholipids and decreasing their mobility within the membrane, thereby decreasing membrane fluidity and membrane permeability. This physiochemical effect of Mg on membranes may, in turn, affect specific transporters, membrane bound enzymes, general membrane integrity and ion fluxes. Thus, alterations in extracellular Mg result in increased cytosolic free $[Ca^{2+}]_i$ through cellular depolarization, either by influencing Ca channels directly or by affecting phospholipid metabolism. This results in an increase in plasma membrane permeability for Ca. Decreased plasma membrane bound intracellular Mg would indirectly lead to increased plasma membrane bound intracellular Ca and thereby increased total $[Ca]_i$. In addition, Mg ions have both direct and indirect (via modulation of $[Ca]_i$) effects on smooth muscle contractility (Gunther, 1981).

$[Mg^{2+}]_i$ is an essential prosthetic group in at least 300 enzymatic reactions in intermediary metabolism and functions by influencing enzyme kinetics or thermodynamics (Altura, Durlach and Seelig, 1987). Mg may reduce the high negative charge of the substrate ATP^{-4} thus increasing its affinity for an enzyme. Alternately Mg may bind directly to an enzyme and cause a conformational change in the molecule by polarizing R-O bonds, thus facilitating the reaction by changing the reaction equilibrium. The substrate in a given reaction may chelate Mg which changes the thermodynamic threshold of the reaction favoring product formation (Gunther, 1981). Enzyme reactions (and/or metabolic pathways) known to involve Mg include; 1) hydrolysis and transfer of phosphate groups 2) thiokinases (of fatty acid degradation) 3) alkaline phosphatase and pyrophosphatase 4) adenylate cyclase (Rude and Oldham, 1987) and renal vitamin D hydroxylation (Gray et al., 1972). Mg dependent enzymatic reactions are dependent upon $\log[Mg^{2+}]_i$

concentration, such that large changes in free $[Mg^{2+}]_i$ would be required to alter the rate of a Mg dependent catalytic reaction (Gunther. 1981).

Physiological processes known to involve Mg include 1) activation of amino acids and protein synthesis by effects on ribosomal aggregation and binding of messenger RNA to 70s ribosomes 2) the synthesis and degradation of DNA and 3) the contractability of cardiac and smooth muscle (Gunther, 1981).

1.3.4. MAGNESIUM DEFICIENCY

Human Mg deficiency is characterized by hypomagnesaemia, hypocalcaemia, hypokalaemia and, if of sufficient duration, hypoparathyroidism. Clinical symptoms include neuromuscular changes, tremors, gross muscle spasms with marked personality changes, anorexia, nausea and apathy (Flink, 1981). A similar profile is observed in experimental Mg deficiency for all species except the rat, which does not exhibit hypocalcemia unless fed low dietary Ca.

The aetiology of the disturbed Ca homeostasis, most notably the progressive hypocalcaemia, in Mg deficiency, is unknown. The initiating factor appears to be failure of the normal heterionic exchange of bone Ca for Mg in the bathing medium at the labile bone mineral surfaces. It has been postulated that this initial failure of Ca release from bone is due primarily to a physiocochemial effect of Mg (Shils, 1988). Under normal conditions Mg exchanges with Ca in the hydration shell; this exchange is thought to be purely physicochemical, since fetal mouse bones incubated in low Mg media exchanged Ca for Mg irrespective of whether the bones were living or dead (Johannesson and Raisz, 1983). This physicochemical exchange would favor retention of Ca and release of Mg during hypomagnesaemia; the result being decreased bone Mg, and increased bone Ca. As plasma Mg levels decrease, in the early stages of Mg deficiency, osteoclast and possibly osteoblast/osteocyte responsiveness to PTH declines, leading to reduced active bone resorption (Jones, Schwartz and Krook, 1980). Alterations in vitamin D metabolism may

also occur in Mg deficiency and contribute to the progression of the hypocalcaemia (Fuss et al., 1985, Rude et al., 1985). Vitamin D metabolism has major effects on bone metabolism and functions synergistically with PTH to effect bone resorption (DeLuca, 1984). Thus alterations in vitamin D metabolism could potentiate skeletal resistance to PTH. Thereafter the characteristic hypocalcemia develops. Several reports suggest that a transient rise in circulating PTH in response to the hypocalcaemia may occur (Anast and Forst, 1983, Rayssiguier et al., 1982). As hypomagnesaemia progresses, the hypocalcaemic condition is perpetuated with the advent of further PTH target tissue resistance. Chronic hypomagnesaemia eventually results in decreased circulating PTH, despite adequate PTG hormonal reserves. Thus chronic severe Mg depletion would be characterized by unresponsive bone, hypocalcaemia, low circulating PTH, hypokalaemia and Na retention (Flink, 1981).

Administration of Mg to Mg deficient subjects increases circulating Mg, which is followed by a rapid rise in PTH. Restoration of circulating Ca and potassium (K) is much slower than Mg (Shils, 1988). The lag time between restoration of normal plasma Mg and correction of hypocalcaemia and hypokalemia may reflect changes in tissue and bone Mg content required for responsiveness to PTH and/or $1,25(OH)_2D_3$.

On a cellular level the consequences of Mg deficiency include increased intracellular Na and Ca and decreased K and Mg. Changes in Na and K are possibly due to increases in plasma membrane permeability and effects on the Na^+/K^+ ATPase. The changes in ion concentrations are quickly followed by decreases in protein, DNA and RNA synthesis. The increase in $[Ca]_i$ appears to be due to increased binding of Ca to intracellular membranes and to increased cellular Ca uptake (George and Heaton, 1975). In chronic Mg deficiency prolonged cellular Ca uptake results in deposition of Ca phosphate crystals within the mitochondria with potential compromise of specific mitochondrial functions (Forbe, 1971, Bunce et al., 1974).

1.3.5. MAGNESIUM DEFICIENCY AND VITAMIN D METABOLISM

The role of $1,25(OH)_2D_3$ in the pathogenesis of the hypocalcaemia of Mg deficiency is unclear. In the classical response to hypocalcaemia, PTH acts on the renal proximal tubules to stimulate $1,25(OH)_2D_3$ synthesis which in turn increases active Ca transport and in conjunction with PTH enhances renal reabsorption of Ca and P and elevates bone resorption (MacIntyre, 1986). Since competent bone resorption and renal reabsorption of Ca require the presence of adequate circulating $1,25(OH)_2D_3$ and intact $1,25(OH)_2D_3$ target tissue responsiveness, alterations in vitamin D metabolism might be pivotal to the perpetuation (and possibly the initiation) of hypomagnesaemic hypocalcaemia.

Both lower and inappropriately normal circulating $1,25(OH)_2D_3$ have been recorded in clinical Mg deficiency (Ralston et al., 1983, Rude et al., 1985) while decreased $1,25(OH)_2D_3$ synthesis (Coburn et al., 1973) and a blunted increase in circulating $1,25(OH)_2D_3$ in response to low dietary Ca has been described in experimental Mg deficiency (Welsh and Weaver, 1988).

Since PTH is a major regulator of 1αhydroxylase activity, the changes in vitamin D metabolism during Mg deficiency have been attributed to primary PTG/PTH alterations (Shils, 1989). Impaired PTH secretion has been reported in chronic human Mg deficiency (Suh et al., 1973) and PTH secretion in vitro decreases sharply as media Mg concentration is reduced below 1 mM (Habener and Potts, 1976, Takatsuki, 1980). However, although PTG failure and hypoparathyroidism have been reported in chronic Mg deficiency (Brown and Chen, 1989), circulating PTH is often increased in acute human Mg deficiency (Anast and Forte, 1983, Rayssiguier et al., 1982). Animal studies have indicated PTG hypertrophy in acutely Mg deficient chicks (Welsh, Schwartz and Krook, 1981) and serum PTH was increased comparably in Mg deficient and control rats exposed to a low Ca diet (Welsh and Weaver, 1988). No reductions in circulating PTH were reported in hypocalcaemic calves (Rayssiguier et al., 1977) or dogs (Levi et al., 1974) with acute Mg deficiency. Thus, the acute alterations in vitamin D metabolism, during Mg deficiency may not be directly attributed to low PTH.

The effects of PTH are mediated via a specific membrane receptor coupled (via a G protein) to adenylate cyclase generation of cAMP (Slatopolsky et al., 1990). PTH receptors have been localized to bone cells (osteoblasts) and kidney cells (proximal and distal tubules) (Slatopolsky et al., 1990). Since Mg ions are required for the maintenance of membrane integrity, G protein interactions and adenylate cyclase activity (Rude and Oldham, 1987, Gunther, 1981) Mg deficiency may influence PTH binding to its receptor, receptor/G protein coupling to adenylate cyclase or adenylate cyclase activity. The ultimate effect of these perturbations could be decreased PTH stimulated cAMP synthesis and blunting of target tissue responses which could explain low or inappropriately normal circulating $1,25(OH)_2D_3$ in Mg deficiency. The cofactor role of Mg in 1αhydroxylase activity (Gray et al., 1972) offers an alternate explanation for target tissue resistance to PTH during Mg deficiency. Finally, Mg deficiency is associated with elevated $[Ca]_i$ which specifically accumulates within the mitochondria (Sarkar, 1988), the site of the 1αhydroxylase. Elevations of intramitochondrial Ca could compromise the functioning of the 1αhydroxylase (Bunce et al., 1974) preventing PTH stimulation.

Evidence for skeletal and renal target tissue resistance to PTH in Mg deficiency has indeed been reported. Johannesson and Raisz (1983) demonstrated skeletal refractoriness to PTH in fetal bone cultures incubated in low concentrations of media Mg. Freitag, Martin and Conrades (1979) demonstrated decreased cAMP synthesis by tibiae from Mg deficient animals in response to in vitro PTH perfusion. Renal resistance to PTH was demonstrated by Forbes and Parker (1980) who demonstrated impaired urinary cAMP generation in response to parathyroid hormone extract (PTE) injection in Mg deficient rats. The defective renal PTH cAMP responsiveness was not evident in vitro or if the animals were consuming low dietary Ca suggesting Mg deficiency per se was not responsible for the PTH resistance. Target tissue resistance to PTH was present in chronic Mg deficiency in the

presence of hypocalcaemia but not in acute Mg defiency before onset of hypocalcaemia (Breitenbach et al., 1973). These results suggest that hypomagnesaemia per se is not responsible for the apparent target tissue resistance to PTH in Mg deficiency.

Reddy and Sivakumar (1976) reported that massive doses of vitamin D failed to alleviate severe hypocalcemia and rickets in Mg deficient patients despite normal circulating PTH. Treatment with oral Mg corrected all clinical manifestations of the disorder. Rude et al. (1985) reported that circulating $1,25(OH)_2D_3$ was significantly lower in chronically Mg deficient humans, than in controls, despite normal or elevated PTH in 50% of the patients. Mg therapy elevated PTH and $1,25(OH)_2D_3$ in most patients, although the changes in $1,25(OH)_2D_3$ occurred more slowly than changes in PTH. Fuss et al. (1985) confirmed low $1,25(OH)_2D_3$ despite normal PTH while the administration of Mg elevated both $1,25(OH)_2D_3$ and PTH. However, urinary cAMP levels were elevated both before and after Mg supplementation suggesting a post receptor defect in PTH signal transduction. Thus the issue of PTH target tissue resistance in Mg deficiency requires clarification.

Most human studies of Mg deficiency are complicated by secondary factors (primarily alcoholism or malnutrition) which may induce metabolic derangements making it difficult to discern the role of Mg per se. Animal studies on primary Mg deficiency and vitamin D metabolism are scarce but do support the clinical data. Coburn et al. (1973) reported, in abstract form, reduced 1αhydroxylase activity in kidney homogenates from vitamin D deficient Mg depleted chicks compared to vitamin D deficient control chicks. At commencement of the present studies this was the sole information concerning vitamin D metabolism and Mg deficiency in a species known to exhibit hypomagnesaemic hypocalcaemia. In 1988, Welsh and Weaver demonstrated that Mg deficient rats fed low dietary Ca increased plasma $1,25(OH)_2D_3$ in response to elevated PTH but had significantly lower $1,25(OH)_2D_3$ and were hypocalcaemic compared to low Ca fed controls.

Mg deficiency may also influence vitamin D metabolism by altering target tissue responsiveness to $1,25(OH)_2D_3$. Since the effects of $1,25(OH)_3D_3$, at the bone and kidney require PTH (DeLuca, 1984) impaired secretion and/or action of PTH could alter bone and kidney target tissue responsiveness to $1,25(OH)_2D_3$. In addition, decreased plasma Mg may modify target tissue responsiveness to $1,25(OH)_2D_3$ by altering $[Ca]_i$ concentration. Changes in $[Ca]_i$ have been demonstrated to influence intestinal and renal calbindin expression independently of $1,25(OH)_2D_3$ (Clemens et al., 1989, Theofan and Norman, 1986). In a case study, Ralston et al. (1983) reported severe chronic Mg deficiency with concomittant hypocalcaemia despite normal plasma $1,25(OH)_2D_3$. Mg therapy normalized serum PTH, elevated the $1,25(OH)_2D_3$ and corrected the hypocalcaemia. In 1987, Carpenter et al., observed significant hypocalcaemia in Mg and vitamin D deficient rats despite elevated plasma $1,25(OH)_2D_3$. Welsh and Weaver (1988) reported significant hypocalcaemia in Mg deficient rats challenged with a low Ca regime despite significantly elevated $1,25(OH)_2D_3$. Bone resorption is reduced in Mg deficiency, despite hypocalcaemia, resulting in retention of Ca in bone (Jones, Schwartz and Krook, 1980). Decreased bone turnover, suggested by reduced circulating osteocalcin, was reported in Mg deficient rats with normal circulating $1,25(OH)_2D_3$ (Carpenter et al., 1988). In vitro, $1,25(OH)_2D_3$ stimulated ^{45}Ca release from bone (0.8 mM Mg) was prevented when media Mg was reduced to low levels (0.25 mM) (Johannesson and Raisz, 1983). Finally intestinal calbindin D28 levels and active Ca transport were not increased in response to hypocalcaemia in Mg deficient chicks (Rayssiguier et al., 1982). Taken in unison, current evidence suggests target tissue resistance to $1,25(OH)_2D_3$ exists in Mg deficiency.

1.4. INSULIN DEFICIENCY

1.4.1. DIABETES MELLITUS

Diabetes Mellitis is clinically and etiologically a heterogeneous disorder. Insulin dependent diabetes is defined as an absolute deficiency of insulin while

non insulin dependent diabetes is defined as a condition of insulin resistance.

Diabetes is characterized by hyperglycemia, increased catabolism of triacyglycerol (with resultant elevations of plasma fatty acids and ketones), increased protein catabolism and increased gluconeogenesis which if not controled can lead to ketoacidosis. Hyperglycemia leads to glomerular hyperfiltration, glycosuria, calciuria, magnesuria and Na retention.

Chronic diabetes is associated with renal hypertrophy, atherosclerosis, small vessel and neural disease, increased susceptibility to infection, blindness and alterations in Ca homeostasis. Clinical and experimental insulin dependent diabetes is associated with osteopenia (altered bone growth, osteoporosis and increased bone fractures) (Einhorn et al., 1988) and alterations in extracellular Ca homeostasis (Ishida et al., 1983). The aetiology of the disturbed Ca homeostasis remains obscure, however, decreased circulating $1,25(OH)_2D_3$ in experimental and clinical diabetes (Schneider et al., 1977, Gertner et al., 1979, Frazer et al., 1981, Nyomba et al., 1986) decreased net synthesis of $1,25(OH)_2D_3$ in experimental diabetes (Hough et al., 1983) and compromised $1,25(OH)_2D_3$ target tissue effects (decreased intestinal calbindin D9k and alkaline phosphatase activity [Stone et al., 1991a], decreased active Ca transport [Nyomba et al., 1989], decreased osteocalcin synthesis [Verhaeghe et al., 1989], impaired bone turnover [Verhaeghe et al., 1990], decreased osteoid maturation and mineralization [Goodman and Hori, 1984], and impaired induction of renal Calbindin D28k by $1,25(OH)_2D_3$ [Schedl et al., 1984]) suggests that this altered Ca homeostasis may result from altered vitamin D metabolism. The origin of the disturbed vitamin D metabolism has yet to be resolved but may be due to insulin deficiency, hyperglycemia or physiological and/or biochemical alterations induced by these states.

1.4.2. DIABETES AND VITAMIN D METABOLISM

The defect in vitamin D metabolism associated with insulin dependent diabetes has been proposed to reside at the level of $1,25(OH)_2D_3$ synthesis. This hypothesis is supported by reduced renal 1αhydroxylase activity measured

indirectly in streptozotocin (STZ) diabetic rats (Spencer et al., 1980) or directly in renal homogenates from vitamin D deficient STZ diabetic rats (Hough et al., 1983). However, although the metabolic clearance rate of tritiated $1,25(OH)_2D_3$ was similar in STZ diabetic and control rats (Spencer et al., 1980) elevated 24 hydroxylase activity in renal slices of STZ diabetic rats fed a low Ca diet (Wongsurwat et al., 1983) implies that enhanced clearance of $1,25(OH)_2D_3$ might exist in diabetic animals fed certain diets. Thus it remains to be established if the vitamin D defect associated with insulin dependent diabetes resides only at the level of $1,25(OH)_2D_3$ synthesis.

Reduced circulating $1,25(OH)_2D_3$ and decreased 1αhydroxylase activity in insulin dependent diabetic animals is normalized by precise in vivo insulin therapy (Hough et al., 1983, Spencer et al., 1980, Wongsurawat and Armbrecht, 1985). Wilson et al. (1982) demonstrated that 12 hours after insulin injection, circulating $1,25(OH)_2D_3$ in diabetic rats was doubled, and increased further to within the normal range within 36 hours. Therefore the effect of insulin on vitamin D metabolism is relatively fast, however it is not yet clear whether insulin mediates its effects on vitamin D metabolism directly or indirectly.

Steady state $1,25(OH)_2D_3$ levels reflect factors which influence both synthesis and catabolism. Insulin deficiency could influence $1,25(OH)_2D_3$ synthetic rates by: directly affecting renal tubule metabolic integrity and thereby cofactor levels, influencing steady state levels of one or more 1αhydroxylase component(s), modulating signal transduction pathways known to affect 1αhydroxylase activity; or indirectly by: influencing substrate availability, altering the presence of 1αhydroxylase stimulators or inhibitors or inducing physiological changes which impair vitamin D metabolism. Insulin deficiency could affect factors known to influence vitamin D catabolism. Factors influencing the catabolism of $1,25(OH)_2D_3$ are poorly understood although $1,25(OH)_2D_3$ catabolism is inducible and has been correlated with the presence of the VDR and the activity of the 24 hydroxylase enzyme (McDonnell et al., 1987, Henry and Norman, 1984).

While it is well established that insulin deficiency has profound metabolic and cellular effects (Wang and Taub, 1991, Bachel et al., 1986), it does not appear that the aberrant vitamin D metabolism apparent in insulin dependent diabetes can be attributed to compromised renal tubular integrity. This is best exemplified by studies which demonstrated increases in circulating $1,25(OH)_2D_3$ when STZ diabetic animals were placed on a low Ca diet (Wilson et al., 1982). In addition, renal 24 hydroxylase activity is not impaired by STZ diabetes (Wongsurawat et al., 1983). The 24 hydroxylase enzyme requires a metabolic milieu (cofactors, substrate) comparable to the 1αhydroxylase system for optimal activity. However, an examination of the direct consequences of insulin deficiency on renal cellular metabolism with respect to vitamin D metabolism would clarify this issue.

Analogous to its known trophic effects on other enzyme systems (Meisler and Howard, 1989, O'Brien and Granner, 1990), insulin may be required to maintain steady state levels of one or more components of the 1αhydroxylase system. Wongsurawat and Armbrecht (1985) reported that synthesis of $1,25(OH)_2D_3$ by renal slices from STZ diabetic and control rats, that had been thyroparathyroidectomized, was identical. Henry et al. (1981) reported that 24 hours incubation with insulin did not increase basal 1αhydroxylase activity in primary cultures of avian renal tubules. These results argue against a direct trophic role for insulin on 1αhydroxylase. Future studies will be necessary to investigate this issue further.

Insulin deficiency may result in compromised PTG function and reduced circulating PTH. Hypoparathyroidism could account for decreased 1αhydroxylase activity and reduced circulating $1,25(OH)_2D_3$ evident in diabetes. However, several reports on human and experimental diabetes have demonstrated significantly increased or normal circulating PTH levels in diabetes (Schedt et al., 1978, Schneider et al., 1974, Wongsurawat et al., 1983).

It has been suggested that decreased $1,25(OH)_2D_3$ production during diabetes is secondary to decreased Ca needs, due to decreased growth rates

combined with increased Ca intake due to hyperphagia (Nyomba et al., 1986). However, this theory is not compatible with reports of reduced intestinal Ca transport, hypocalcaemia and normal or elevated circulating PTH in human and experimental diabetes.

Hypomagnesaemia has been reported in insulin dependent diabetes (Mooradian and Morley, 1987) and insulin has been shown to modulate cellular Mg homeostasis (Paolisso et al., 1990). Perturbations in Ca, PTH and vitamin D metabolism have been demonstrated in Mg deficiency and hypomagnesaemia contributes to the aberrant vitamin D metabolism associated with insulin dependent diabetes (Sagesse et al., 1991). To date, no animal studies have focused on the possible relevance of Mg deficiency to the aberrant vitamin D metabolism in diabetes.

Alternately, insulin may be required for 1αhydroxylase stimulation by factors such as PTH. While significantly decreased circulating $1,25(OH)_2D_3$ and reductions in net synthesis of $1,25(OH)_2D_3$ have been consistently observed in diabetes, these observations have been from animals in which endogenous PTH secretion had been manipulated. Thus, Hough et al. (1983) demonstrated synthesis of $1,25(OH)_2D_3$ in vitamin D deficient STZ diabetics was 80% lower than 1αhydroxylase activity in vitamin D deficient controls and Wongsurawat et al. (1983) demonstrated 75% lower $1,25(OH)_2D_3$ synthesis in STZ diabetic rats fed low Ca diets as compared to low Ca fed controls. Both vitamin D deficiency and low dietary Ca induce relative hyperparathyroidism. Other studies suggest that the relative differences in $1,25(OH)_2D_3$ synthesis between diabetics and controls diminishes under basal conditions (ie. normal circulating PTH). Ikeda et al. (1987) demonstrated STZ diabetics fed control diets had 1αhydroxylase activity 40% lower than control animals, whereas STZ diabetics fed low Ca diets had 1αhydroxylase activity 75% lower than control animals fed low Ca . The differences in 1αhydroxylase activity between control and diabetics was not evident if rats were thyroparathyroidectomized (where negligible circulating PTH exists) (Wongsurwat et al., 1985, Ikeda et al., 1987).

Thus defective synthesis of $1,25(OH)_2D_3$ during diabetes becomes apparent only when basal activity is stimulated. In support of this hypothesis, Henry et al. (1981) demonstrated that insulin was required for the PTH induced stimulation of net $1,25(OH)_2D_3$ synthesis in primary renal avian cultures but did not influence basal activity.

Evidence exists for the involvement of insulin in both acute and chronic PTH treatment enhancing $1,25(OH)_2D_3$ synthesis. Armbrecht et al. (1991) demonstrated that PTH failed to dephosphorylate the renoredoxin component of the 1αhydroxylase in renal slices from STZ diabetic rats, although dephosphorylation was evident in slices from non-diabetic controls. Since dephosphorylation of renoredoxin may be a critical determinant of 1αhydroxylase activation, reduced 1αhydroxylase activity in diabetes could be related to interactions between insulin and PTH at the level of protein phosphorylation. Korkor et al. (1987) and Henry (1981) reported that insulin was required for chronic stimulation of 1αhydroxylase by PTH. However the biochemical events integrating PTH stimulation of $1,25(OH)_2D_3$ production and the phosphorylation state of mitochondrial renoredoxin are unknown. It remains unclear if the phosphorylation state of renoredoxin is associated with acute or chronic modulation of PTH mediated 1αhydroxylase stimulation. Thus, it is unclear if both acute and chronic stimulation of 1αhydroxylation require insulin. In vivo data indicates that despite diabetes low dietary Ca or PTH infusion into thyroparathyroidectomized rats can significantly enhance $1,25(OH)_2D_3$ synthesis without altering circulating insulin or glucose (Wongsurawat et al., 1983, Ikeda et al., 1987). Therefore it is doubtful that all facets of PTH induced 1αhydroxylase stimulation are compromised by insulin deficiency.

Diabetes may affect PTH stimulation of 1αhydroxylase activity at the plasma membrane level (PTH-receptor-G_s-adenylate cyclase) or distal to cAMP generation. Sulimovici et al. (1981) demonstrated less nephrogenous cAMP synthesis in response to in vivo PTH in STZ diabetics compared to controls.

However, no differences were observed in cAMP synthesis of renal slices from diabetic and control animals exposed to PTH in vitro (Wongsurwat et al., 1983, Kung and Rao, 1988). Further, Wongsurawat and Armbrecht (1985) demonstrated that PTH reduced renal tubule reabsorption of phosphate and elevated serum Ca (both cAMP mediated effects) similarly in STZ diabetic and control rats. These results suggest that diabetes affects PTH stimulation of 1αhydroxylase distal to adenylate cyclase generation of cAMP at the plasma membrane. Clarification of the available data, however, requires a systematic examination of the mechanism of PTH induced 1αhydroxylase stimulation and the effects of diabetes.

PTH may activate PKC (Hruska et al., 1987, Nishizuka, 1988) and stimulation of PKC activity is associated with decreased 1αhydroxylase activity (Henry and Luntao, 1989). In this respect, it is intriguing that elevated PKC activity has been reported in renal basolateral membranes from chronically diabetic rats (Hise and Mehta, 1988). Tenenhouse and Henry (1985) demonstrated increased PKC activity in kidneys of hyp mice which also exhibit elevated 24 hydroxylase activity (Tenenhouse, Yip and Jones, 1988) offering an explanation for the increased vitamin D metabolic clearance (Jones, Yip and Tenenhouse, 1989) and abberrant 1αhydroxylase activity observed in this animal model (Eicher et al., 1976). Thus, similarily in diabetes, the failure of PTH to dephosphorylate renoredoxin and achieve comparable stimulated 1αhydroxylase activity, may be associated with enhanced PKC activity. A systematic examination of the role of PKC in renal $25(OH)D_3$ hydroxylation in a mammalian system will be required to explore these suggestions. Specifically, direct evidence of modulation of 1αhydroxylase activity by PKC and a thorough examination of renal PKC activity in diabetes are required.

Phosphate restriction increases 1αhydroxylase activity and elevates circulating $1,25(OH)_2D_3$ levels independently of PTH (Carpenter, 1990). Matsumoto et al. (1986) demonstrated that STZ diabetic rats failed to increase circulating $1,25(OH)_2D_3$ in response to phosphate restriction, whereas control

rats exhibited significantly elevated plasma $1,25(OH)_2D_3$ in response to phosphate restriction. Thus insulin deficiency appears to blunt stimulation of 1αhydroxylase by both PTH and phosphate restriction.

Insulin deficiency results in impaired weight gain and abnormalities in the growth hormone axis: specifically, decreased serum and tissue IGF-1 and decreased steady state IGF-1 mRNA levels (Clemmons and Underwood, 1991). Since growth hormone and IGF-1 have been implicated in enhancement of $1,25(OH)_2D_3$ production secondary to phosphate restriction (Gray, 1987) abnormalities in these factors may be related to compromised vitamin D metabolism during diabetes.

Associated with compromised overall growth rate in diabetes are tissue specific growth abnormalities. For example, adaptive increases in intestinal and renal mass have been reported in acutely diabetic rats despite reduced body weights (Flyvbjerg et al., 1990, Seyer-Hensen, 1977). The significance of renal hypertrophy to aberrant vitamin D metabolism in diabetes has not been examined although Almond et al. (1989) reported a negative correlation between renal 1αhydroxylase activity and renal hypertrophy following unilateral nephrectomy. Further, increased renal mass following unilateral nephrectomy was correlated with increased renal membrane PKC activity (Caramelo et al., 1988). The relevance of these observations to the defective $1,25(OH)_2D_3$ synthesis in diabetes merits further scrutiny.

CHAPTER 2 - MATERIALS AND METHODS

2.1. ANIMALS, DIETS AND HOUSING

2.1.1. CHICK STUDIES

Experiments were conducted in two week old white Leghorn chicks (Agriculture Canada, Ottawa). The animals were housed in groups of six to ten in thermostatically-controlled battery brooders with raised wire mesh floors in an air-conditioned room with an automatic 12-hour light/dark cycle. In all experiments, chicks were fed a practical-type chick starter diet (Scott et al., 1976) for the first few days before being switched to the experimental regime. Previous analysis has shown this starter diet contains 0.1925% Mg (Welsh, 1980). Chicks received deionized water ad libitum and were fed one of four semi-purified soy-based diets: control (0.1% Mg/1.5% Ca); Mg-deficient (0.015% Mg/1.5% Ca); low Ca (0.1% Mg/0.2% Ca); and Mg-deficient/low Ca (0.015% Mg/0.2% Ca). All diets contained 0.9% P and 2.2 IU vitamin D_3/g diet. The experimental diets were prepared from a low Mg/low Ca basal diet (Table 2.1, appendix 1) which was a modification of the diet of Scott et al. (1976). The modifications were as follows: a Mg-free mineral mixture was prepared (Table 2.2, appendix 1) omitting $CaCO_3$ and $CaHPO_4.2H_2O$ to reduce the Ca content. A basal diet was then prepared with this mineral mix and aliquots were analyzed for Mg, Ca and P. The amount of $MgSO_4.7H_2O$ required for the desired final concentration of Mg was calculated and added. The amount of Ca as $CaHPO_4.2H_2O$ and $CaCO_3$ required for the desired final concentration of Ca was calculated and added. KH_2PO_4 was then added to the low Ca diets to maintain the P content at 0.9% in all diets. The resulting diets were then re-ashed and re-analyzed to ensure that these levels had been obtained.

The level of Mg in the Mg deficient diets was set at 0.015%, since this level sustains growth, but results in characteristic Mg deficiency signs and plasma Mg depletion within 14 days (Chou et al., 1979). The level of Ca in the low Ca diets was set at 0.2% to induce the characteristic response to a low Ca stress (Welsh, 1980).

In one study, before dietary challenge, chicks were subcutaneously implanted (under halothane anaesthesia) with Alzet osmotic mini pumps (Model 2001) delivering either 7ng/hr $1,25(OH)_2D_3$ (dissolved in 5% ethanol/95% propylene glycol) or vehicle (5% ethanol/95% propylene glycol).

In all studies, food intake was monitored daily and matched to the experimental group with the lowest intake.

At the end point of each experiment, animals were fasted overnight and blood was removed by cardiac puncture under halothane anaesthesia.

2.1.2. RAT STUDIES

2.1.2.a. GENERAL STUDIES

Male Sprague Dawley rats weighing 50-150 g (Charles River, Montreal, Quebec) were housed in groups of two to six in plastic cages and fed ad libitum one of several semi-purified diets (US Biochemicals, Cleveland, OH) with free access to tap water or deionized water. Dietary compositions were as follows: Control - American Institute of Nutrition (AIN): 0.6% Ca/0.4% P, low Ca: 0.013% Ca/0.34% P ($CaCO_3$ and $CaHPO_4.2H_2O$ reduced, P content adjusted with KH_2PO_4), low P: 0.6% Ca/0.04% P and rachitogenic diet: 0.6% Ca/0.4% P omitting vitamin D. Vitamin D deficiency was induced by placing weanling rats on vitamin D deficient diets for five to six weeks in the absence of UV light. Ca restriction was induced in 120-140 g rats by feeding a low Ca diet for one to two weeks. P depletion was induced in 120-140 g rats by feeding a low P diet for one to two weeks. In all studies, 120-140 g animals who received AIN semi-purified diets for two to fourteen days served as controls. Animals resided in air conditioned facilities and (except for those on vitamin D deficient diets) were maintained on a normal 12 hour dark/light cycle. All rats were sacrificed by decapitation.

2.1.2.b. DIABETIC STUDIES

2.1.2.b.1. STREPTOZOTOCIN INDUCED DIABETES

Male weanling Sprague Dawley rats (Charles River, Montreal, Quebec) were individually housed in stainless steel hanging cages and fed control AIN

semi-purified diet as described. To induce diabetes, rats weighing 120-130 g were injected intraperitoneally with 85mg/kg STZ in citrate buffer (pH 4.5); control rats received citrate buffer injection. Except for the diabetes induction time courses, only animals which developed diabetes within two to three days (as assessed by urinary glucose > 55 mmol) were used for studies. In the induction time course studies, all animals were used and exclusions were made later based on serum glucose measurements. With this protocol, diabetic animals grew at a reduced rate but did not lose weight or develop ketonuria (assessed with Clinistix [Miles Laboratories]) during the experimental period. Untreated animals were sacrificed within 14 days of induction of diabetes. In some experiments, diabetic animals were treated every morning with NPH insulin (Eli Lilly, Indianapolis, Indiana); the amount given was adjusted as necessary to produce early morning trace glucosuria (<2.8 mmol). In mineral restriction studies, animals were fasted the night before sacrifice.

2.1.2.b.2. SPONTANEOUS DIABETES

Spontaneously diabetic male BB Wistar rats were obtained from Dr. P. Thibert, of the Health Protection Branch, Health and Welfare Canada, Ottawa. Animals were received in groups of five to six one day after detection of diabetes by glucosuria. The mean age of the animals on receipt varied from 45 to 60 days. Control animals were age-matched male Wistar rats bred from the same Wistar colony from which the diabetic rats were originally bred in 1967. Animals were housed in individual stainless steel, wire-bottom cages with free access to Purina lab chow and tap water. All rats were weighed daily; BB rats were tested for glucosuria and ketonuria daily with Clinistix. Animals were routinely used within seven days of receipt; rats who exhibited ketonuria were excluded. In one experiment, BB diabetic rats were treated with NPH insulin as described for STZ induced diabetic rats.

2.2. BLOOD PARAMETERS

2.2.1. PREPARATION

2.2.1.a. PLASMA

Plasma was obtained by collecting blood into 10 ml heparinized plastic

centrifuge tubes (Monovette Sarstedt), spinning at 2,500g (10 minutes) in a refrigerated centrifuge and collecting the supernatant.

2.2.1.b. SERUM

Serum was obtained by collecting blood into 15 ml glass tubes, allowing the blood to fully clot, spinning at 2,500g (10 minutes) in a refrigerated centrifuge and collecting the supernatant.

2.2.2. BLOOD MINERALS

2.2.2.a. CALCIUM AND MAGNESIUM

Ca and Mg in plasma or serum were determined in duplicate by atomic absorption spectrophotometry (Instrumentation Laboratories Model 951) with an air-acetylene flame in the presence of 2% lanthanum in 5% HCL (Sunderman and Caroll, 1965). Ca and Mg standards were prepared, in duplicate, at the same time, from $CaCO_3$ and $MgNO_3$ reference solutions (1000 ppm ± 1%) (Certified Atomic Absorption Standards, Fisher Scientific).

2.2.2.b. INORGANIC PHOSPHORUS

Inorganic P was determined in serum and plasma by a modification of the colorimetric method of Fiske and Subbarow (1925) as described in Sigma Technical Bulletin No. 670 (1976). Samples were treated with 20% (w/v) trichloroacetic acid and microcentrifuged to remove protein and lipid P. Diluted supernatant was mixed with ammonium molybdate in acid solution to form phosphomolybdate. A mixture of sodium bisulfite, sodium sulfite, and 1-amino-2-napthol-4 sulfonic acid was used to reduce the phosphomolybdate to a phosphomolybdenum blue complex, the intensity of which was measured spectrophotometrically at 660nm. Standard solutions (1-6 µg/ml [ppm]) of monobasic potassium phosphate were used.

2.2.3. VITAMIN D METABOLITES

2.2.3.a ISOLATION OF METABOLITES

Vitamin D metabolites were isolated as originally described by Reinhardt and Hollis (1986). Vitamin D metabolites were extracted from plasma or serum (0.25-1.0 ml diluted to 1 ml with saline) with an equal volume

of acetonitrile, vortexed and centrifuged (1500g, 10 minutes) and the supernatant decanted into 1/2 volume of 0.4 M K_2HP0_4, pH 10.6. This extract was applied directly to a C-18 Sep-Pak affinity chromatography column (Waters Scientific Ltd., Miss., Ont.) pre-washed with 5 ml hexane, 5 ml chloroform, 5 ml methanol, and 5 ml distilled water. Excess salt was removed by washing the cartridge with 5 ml distilled water, and polar lipids were removed by washing the cartridge with 2.5 ml methanol:water (70:30). The vitamin D metabolites were then eluted with 4 ml acetonitrile and dried under a stream of nitrogen. Isolation of 25(OH)D_3 and 1,25$(OH)_2D_3$ was achieved by applying the C18 extract to a silica Sep-Pak adsorption chromatography column (Waters Scientific Ltd., Miss., Ont.) in 1.0 ml hexane:isopropanol (96:4) previously pre-washed with 5 ml methanol, 5 ml chloroform, 5 ml hexane and 5 ml hexane:isopropanol (96:4). The column was washed with 9.0 ml of hexane:isopropanol (96:4) to elute 25(OH)D_3, 7.0 ml hexane:isopropanol:chloroform (91:7:2), and 9.0 ml hexane:isopropanol (84:16) to elute 1,25$(OH)_2D_3$ (Figure 2.1, appendix 1). These eluants were subsequently dried under a stream of nitrogen and quantitatively analyzed (see section 2.C.2a and 2.C.2b). Further purification of 24,25$(OH)_2D_3$, from C-18 Sep-Pak eluants, was achieved by straight phase HPLC on Zorbax-SIL 5 µm (NEN-DUPONT) eluted with hexane:isopropanol:methanol (91:7:2) (Figure 2.2, appendix 1) (Jones, 1980). For all vitamin D metabolite analyses, radiotracer (2,000-3,000 cpm) of the appropriate metabolite (Amersham) was added to the original sample for estimation of % recovery.

2.2.3.b. QUANTITATION OF 24,25$(OH)_2D_3$ AND 25(OH)D_3

2.2.3.b.1. PREPARATION AND STANDARDIZATION OF COMPETITIVE PROTEIN BINDING ASSAY

Serum isolated from vitamin D deficient male Spague Dawley rats was used as the source of transcalciferin for the competitive protein binding assays (Shephard and Deluca, 1980). Briefly, at sacrifice, blood was collected and pooled from 10 to 20 animals and serum was prepared and frozen in 50 to 500

µl aliquots. To test the serum for $25(OH)D_3$ and $24,25(OH)_2D_3$ binding, several dilutions (1:1,000-1:6,000) of serum in 50 mM sodium phosphate (pH 7.4) were incubated with $25(OH)D_3$ (0 - 6.4 ng) and 6,000 cpm of the appropriate tritiated tracer; 500 ng of $25(OH)D_3$ was used for measurement of non specific binding. The binding curve with a sensitivity down to 0.1 ng/tube was chosen as the optimal serum dilution to be used for all subsequent assays with each prepared batch. Inter assay and intra assay variabilities were 13.2% and 6.7%, respectively, for assay of $25(OH)D_3$ and 14.7% and 17.7%, respectively, for $24,25(OH)_2D_3$.

2.2.3.b.2. QUANTITATION OF $25(OH)D_3$ AND $24,25(OH)_2D_3$

$25(OH)D_3$ and $24,25(OH)_2D_3$, isolated from plasma or serum samples (see section 2.2.C.1) were quantitated in extracts reconstituted with 200 µl of absolute ethanol as described by Shephard and Deluca (1980) with minor modifications. Briefly, duplicate 50 µl samples were aliquoted into borosilicate glass assay tubes, and a third 50 µl sample was aliquoted into a scintillation vial for recovery assessment. A parallel quality control sample, of known concentration, was isolated and assessed with each assay. Dilution curves using standard pure $25(OH)D_3$ or $24,25(OH)_2D_3$ (Biomol Inc., Plymouth Meeting, PA, Hoffman LeRoche) were prepared as described in section 2.C.2.a. For each assay, 6,000 cpm of the appropriate tritiated tracer in 25 µl ethanol and 0.5 ml of the appropriately diluted rat serum was added to each tube. After vortexing, tubes were covered and incubated at 4 °C overnight. The following morning 0.2 mls of dextran-coated charcoal suspension (5% charcoal, 0.5% dextran suspended in 50 mM sodium phosphate, pH 7.4) was added to all tubes on ice, tubes were vortexed and allowed to stand for 30 minutes. Following centrifugation at 1,500g (20 minutes, 4 °C) supernatants were counted in scintillation vials.

Standard curves were prepared by plotting specific bound cpm versus the standard concentration on semilog paper. The amounts of $25(OH)D_3$ and $24,25(OH)_2D_3$ in each sample tube were determined by relating the bound cpm

to the standard curve and correcting for original volumes and % recovery. Recoveries were calculated as:

$$\% \text{ recovery} = \frac{(\text{cpm-background})100}{(50\mu l/200\mu l)(\text{initial cpm added-background})}$$

$$\text{ng/ml} = \frac{\text{ng in sample tube}}{(50\mu l/200\mu l)(\text{ml of plasma or serum})(\% \text{ recovery})}$$

2.2.3.c. QUANTITATION OF $1,25(OH)_2D_3$

2.2.3.c.1. PREPARATION AND STANDARDIZATION OF CALF THYMUS RADIORECEPTOR ASSAY

Fresh thymus glands, obtained from male calves aged 2-6 months, were used as a source of VDR (Reinhardt et al., 1982). Once removed thymus glands at slaughter were immediately placed in ice-cold phosphate buffered saline (PBS) on ice and transported to the lab. The tissue was cleaned of membrane and fatty tissue, cut into small pieces, washed with additional ice cold PBS, frozen in liquid nitrogen and stored at -20 °C or -70 °C. Cytosol was prepared by mincing freshly thawed tissue with scissors and homogenizing (20% wt/vol) in a buffer containing 50 mM K_2HP0_4, 5 mM dithiothreitol, 1 mM EDTA, and 400 mM KCl and 10 mM $Na_2MoO_4.2H_2O$, pH 7.4. The tissues were homogenized by 5x30 second bursts with a Polytron (Brinkman) using power setting six. Tissue was cooled on ice during and in between homogenization bursts. The homogenate was centrifuged at 20,000g (15 minutes) to remove nuclear, mitochondrial and particulate fractions. The supernatant was subsequently recentrifuged at 100,000g (60 minutes) and cytosol was collected and pooled. The VDR was semi-purified by slow addition of pulverized solid $(NH_4)_2S0_4$ to 35% saturation. The cytosol/salt mixture was stirred for 30 minutes at 4 °C. The suspension was then aliquoted in 5 to 15 ml aliquots in 15 ml Sorval glass centrifuge tubes and spun at 20,000g (20 minutes). After discarding the supernatant, tubes were inverted and drained, purged with nitrogen, frozen in liquid nitrogen and stored at -20 °C to -70 °C. Prior to being used in the $1,25(OH)_2D_3$ radioreceptor assay, a thymus cytosol pellet was

thawed, reconstituted in 4-7 x its original volume in ice cold assay buffer (50 mM K_2HP0_4, 5 mM dithiothreitol, 1.0 mM EDTA and 150 mM KCl (pH 7.4) and its protein concentration determined. The correct dilution of reconstituted receptor used in the assay was determined empirically for each new batch of receptor by comparing standard curves generated for various dilutions of receptor. Once determined for a batch, each subsequent assay utilized the same dilution. Dilutions of authentic pure $1,25(OH)_2D_3$ (Biomol, Pymouth Meeting, PA) (1-64 pg/50 µl ethanol) were utilized for the standard curve, with 800 pg $1,25(OH)_2D_3$ for nonspecific binding determination. Interassay and intrassay variabilities were 14% and 15.3%, respectively.

2.2.3.c.2. QUANTITATION OF $1,25(OH)_2D_3$

Samples were reconstituted and aliquoted as described for analysis of $25(OH)D_3$ and $24,25(OH)_2D_3$. A parallel quality control sample, of known concentration, was isolated and assessed with each assay. Tritiated $1,25(OH)_2D_3$ (4,000-6,000 cpm in 25 µl ethanol) and 500 µl of thymus cytosol solution were added to each tube and gently vortexed. Samples and standards were incubated at 20 °C for one hour with gentle rocking. After incubation, tubes were transfered to an ice bath and 200 µl of dextran-coated charcoal suspension (as described in section 2.C.2b, except boric acid solution [pH 8.5] was utilized as the buffer) was added. After vortexing, tubes were incubated for 30 minutes (room temperature) then centrifuged at 1,500g (20 minutes). Supernatants were transfered to scintillation vials and counted. $1,25(OH)_2D_3$ in samples was calculated from standard curves after correction for recovery as described for $25(OH)D_3$ and $24,25(OH)_2D_3$ (section 2.C.2b).

2.2.4. GLUCOSE

Glucose was analyzed using a reagent kit (Sigma Diagnostic Procedure 510) based upon the coupled enzymatic reaction of glucose oxidase and peroxidase (Raabo and Terkildsen, 1960). Serum or plasma was added to a mixture containing glucose oxidase, peroxidase and o-dianisidine. The reaction was allowed to proceed to completion in 60 minutes at room temperature, and

the absorption was measured spectrophotometrically at 450 nm, which was proportional to the glucose concentration. A 25 µl aliquot of control serum and a 10 µl aliquot of diabetic serum (in duplicate) was used for analysis.

2.2.5. BIOACTIVE PEPTIDES

2.2.5.a. PTH

Serum PTH was quantified using a commercially available radioimmunoassay kit (RIA) (Immunonuclear [Incstar Corp], Stillwater, Minn.) containing an antibody sensitive to the 44-68 (midmolecule) region of rat PTH. The Immunonuclear RIA mmPTH is a disequilibrium procedure using delayed tracer addition to increase sensitivity. In this assay, sample or standard (rat mmPTH) and first antibody (Chicken anti-mmPTH serum) were combined and incubated for 15 minutes at room temperature. Tracer ^{125}I-mmPTH was then added, followed by a second incubation for two hours, also at room temperature. Phase separation was achieved in 15 minutes with a precipitated complex of second antibody (Goat anti-chicken precipitating complex) carrier and PEG. Samples were subsequently centrifuged 1,500g (10 minutes), supernatants discarded and the pellets counted in a gamma (γ) counter. The amount of mmPTH in each sample was determined from standard curves by relating the precipitated counts to the standard rat mmPTH curve and correcting for original volumes. Duplicate 100 µl serum aliquots (when possible) were used for assay.

2.2.5.b. INSULIN

Serum insulin was quantified using a commercially available RIA kit (Incstar Corp., Stillwater, Minn.) containing an antibody sensitive to rat insulin (INCSTAR Cat. 20118) which is an equilibrium assay with an initial polyethylene glycol (PEG) precipitation step included to remove endogenous insulin antibodies and other interfering substances prior to assay. Sample or standard (rat insulin) first antibody (guinea pig anti-rat-insulin) and tracer (^{125}I-Insulin) were combined and incubated for 16-20 hours at 2-8 °C. Following incubation, a pre-precipitated second antibody complex (normal guinea pig

serum, pre-precipitated with goat anti-guinea pig serum and polyethylene glycol, diluted in BSA-borate buffer) was added. The samples were then incubated for 15-25 minutes at room temperature, centrifuged at 1,500g (10 minutes), the supernatant decanted and the pellet counted in a γ counter. The amount of rat insulin in each sample was determined from standard curves by relating the precipitated counts to the standard rat insulin curve and correcting for original volumes. µunits/ml were converted to ng/ml by using the conversion factor of 25 µunits/ng of insulin. Duplicate 100 µl serum aliquots (when possible) were used for assay.

2.2.6. ALKALINE PHOSPHATASE ACTIVITY

Plasma alkaline phosphatase activity was determined on samples diluted 1:5 by colorimetric determination of the amount of p-nitrophenol formed when phosphate ions are split from p-nitrophenyl phosphate (Neafsey and Schwartz, 1977). Complete buffered substrate (freshly prepared) contained p-nitrophenyl phosphate (0.215g/100ml), Mg_2Cl (0.0407g/100ml) and bovine serum albumin (BSA) (0.05%) in 0.5M 2-amino, 2-methyl 1,3 propanediol buffer (pH 10.3). 5 mMolar working standards of p-nitrophenol were prepared from a 10 mMolar stock solution at the time of the assay. One ml aliquots of substrate were pipetted into tubes into which aliquots of samples, deionized water or p-nitrophenol standard were added. The tubes were mixed and after exactly 20 minutes incubation (37 °C), 3 mls of 0.15N NaOH was added to stop the reaction. Enzyme blanks, in which the NaOH was added before addition of sample, were included with each sample. The absorption of p-nitrophenol at 410 nM was measured spectrophotometrically after 15 minutes at room temperature. Enzyme activity was expressed as µmoles of p-nitrophenol formed per ml of serum per hour, after correction for enzyme blanks.

2.3. BONE ANALYSIS

2.3.1. GRAVIMETRIC ANALYSES

Whole chicken tibiae, or whole rat femora were cleaned of soft tissue and weighed to the nearest 0.1 mg on an analytical balance (Mettler H72) after

drying in a Blue-M hot air oven overnight at 100 °C.

2.3.2. MINERAL ANALYSIS

2.3.2.a. BONE ASHING

Preweighed whole bones were placed in weighed porcelain crucibles and ashed overnight at 600 °C in a Blue M muffle furnace. After cooling, ash was further digested by gentle heating on a hot plate with two additions of 0.5 ml nitric acid (BDH AnalaR). After the nitric acid was evaporated, samples were re-ashed for one hour at 600 °C. After weighing, the final ash was dissolved in 3 ml of concentrated hydrochloric acid (BDH AnalaR) and left overnight to solubilize. The acid solution was quantitatively transferred to a volumetric flask, brought to volume with deionized water, and stored for mineral analysis (Welsh et al., 1981). Recoveries, estimated by standard addition, ranged from 85-90% for the ashing procedure.

2.3.2.b. CALCIUM AND MAGNESIUM ANALYSES

Solubilized ash was diluted to obtain Ca concentrations of 1-3 ppm and Mg concentrations of 0.1-0.3 ppm. Ca and Mg were then analyzed by atomic absorption spectrophotometry as described for serum/plasma (section 2.B.1).

2.3.2.c. PHOSPHORUS ANALYSIS

Solubilized ash was diluted (1:2,500-5,000) to obtain P concentrations of 1-6 ppm. Inorganic P was then analyzed by the method of Fiske and Subbarow as described for serum (section 2.B.2.).

2.3.3. ALKALINE PHOSPHATASE ASSAY

The knee joint epiphysis of chick tibiae was removed from freshly excised bone, weighed and frozen in liquid nitrogen. To prepare supernatants, the tissue was crushed in liquid nitrogen with a mortar and pestle, suspended in 10 ml ice cold 25 mM carbonate buffer (pH 8.0) and centrifuged at 1,500g (10 minutes). The supernatant was diluted 1:5 and analyzed for protein and alkaline phosphatase as described in section 4.C. and 3.C.2. respectively and expressed as μmoles of p-nitrophenol formed per g of bone per hour, after correction for enzyme blanks.

2.3.4. HISTOLOGICAL ANALYSIS

2.3.4.a. BONE DECALCIFICATION

Bones were removed, cleaned of tissue and immediately placed in 10-20 volumes of 10% phosphate buffered formalin for storage before decalcification. To decalcify bones, they were removed from buffered formalin, rinsed with deionized water and placed into 10-20 volumes of 10% EDTA and shaken vigorously at room temperature in a water bath shaker. Fresh EDTA solution was substituted weekly and shaking continued until bones were decalcified, as determined by testing supernatant for the presence of Ca by the oxalate test (Humasen, 1967). Briefly, to one ml of supernatant was added one ml of 5% ammonium hydroxide and one ml of 5% ammonium oxalate. If the suspension did not turn cloudy after 30 minutes the bones were considered sufficiently decalcified.

2.3.4.b. TISSUE FIXING AND EMBEDDING

Bones were removed from EDTA solution and rinsed twice with deionized water. Longitudinal sections at the epiphyseal plate and cross sections at mid-diaphysis were cut with a scalpel. The pieces were then placed in 70% ethanol (absolute ethanol:deionized H_2O). The day before embedding, samples were placed in 80% ethanol for 6 hours and 95% ethanol overnight. The following morning, tissues were immersed in absolute ethanol for 1, 0.5 and 1 1/2 hours (with fresh substitutions of ethanol in between) two xylene baths of 1 1/2 hours each and two paraffin solutions (in a hot air oven) of 1 1/2 and 1 hours duration. Paraffin blocks of the samples were prepared to allow for simultaneous cutting of longitudinal and cross sections of each bone. 6μ sections were prepared with a disposable microtome blade, and sections were stained with hematoxylin and eosin by standard procedures (Welsh et al., 1981).

2.4. GENERAL ANALYSES

2.4.1. BODY WEIGHTS

Body weights were measured in the morning (unless otherwise indicated) with a top loading balance.

2.4.2. FOOD INTAKES

Food intakes were assessed by subtraction of previously weighed food trays and was measured daily in the morning.

2.4.3. PROTEIN ANALYSIS

Protein content was assessed by the coomassie blue dye binding method of Bradford (1976). Briefly, when coomassie blue G-250 is dissolved in acid at pH below one it turns red-brown, but when bound to protein, the blue color is restored due to a shift in the pK_a of the bound coomassie blue. For protein analysis, 5 mls of coomassie blue/acid mixture was added to 10-100 µl of sample and standards (bovine serum albumin 10-60 µg/ml) and absorbance was measured spectrophtometrically at 595 nm.

2.5. INTESTINAL ANALYSIS

2.5.1. PREPARATION OF MUCOSA

The first 10 cm of the small intestine distal to the stomach was removed, flushed with ice-cold PBS, split longitudinally, blotted dry and gently scraped with a glass slide.

2.5.2. GRAVIMETRIC ANALYSIS

Intestinal mucosa was transferred to weigh boats and weighed on an analytical balance to the nearest 0.1 mg.

2.5.3. DETECTION OF CALBINDIN D-28K

2.5.3.a. SAMPLE PREPARATION

Intestinal mucosa was homogenized in 5x weight/volume 0.01 M Tris, 0.15 M NaCl, 5 mM mercaptoethanol, 1 mM phenylmethyl sulfonyl fluoride (PMSF) (pH 7.4) and post nuclear fractions (PNF) were prepared by centrifugation at 1,500g (5 minutes). The supernatant was subsequently heat treated at 65 °C (15 minutes) and centrifuged at 27,000g (15 minutes) as

described by Christakos et al. (1979).

2.5.3.b. GEL ELECTROPHORESIS

Supernatant proteins were separated on 15% SDS-PAGE using the Bio-Rad Mini-Protean II electrophoresis system. Denatured samples (200 ng) in 40 µl PKC lysis buffer A (hypotonic medium 1 mM $NaHCO_3$, 5 mM $MgCl_2$, 100 µm PMSF, pH 7.5) and 15% SDS loading buffer (62.5 mM Tris-HCl, 10% glycerol, 5% 2-b-mercaptoethanol, 0.00125% (w/v) bromophenol blue and 2% (w/v) SDS, pH 6.8 in deionized water) were loaded into each lane. Gels were run at 130 volts for approximately one hour. Low molecular weight (14-97 kD) markers (Bio-RAD Low MW standards) were run with each gel.

2.5.3.c. IMMUNOBLOTTING

Proteins were transferred from the polyacrylamide gel to nitrocellulose filters (Bio-RAD 0.45 µ Transblot transfer medium) using a semi-dry transfer apparatus (Tyler Research, Edmonton, Alberta) (500 mAmps, 30 minutes). Filters were pre-equilibrated in transfer buffer (25 mM Tris, 192 mM glycine and 20% (w/v) methanol). After transfer, the nitrocellulose filters were rinsed in deionized water and stained for five minutes with Ponceau red solution (0.2% (w/v) Ponceau Red S, 3.0% (w/v) trichloroacetic acid). To visualize protein bands, filters were rinsed in deionized water, the lanes appropriately marked in pencil and the molecular weight marker lanes sliced out and stored. The nitrocellulose filters were then destained in PBS for 10 minutes and stored at -20 °C in sealed Parafilm bags. Efficiency of transfer was assessed by staining the gel with coomassie blue (25% (v/v) isopropanol, 10% (v/v) glacial acetic acid, 0.5% (w/v) coomassie blue dye) followed by destaining overnight by shaking in 40% MeOH/10% HOAc.

For immunoblotting, the method of Cadrin et al. (1990) was used. Filters were first shaken for two hours in blocking solution (PBS containing 0.5% skim milk). The filters were then washed six times in PBS/Tween (PBS containing 0.1% Tween 20) and incubated for one hour at 37 °C with mouse monoclonal anti-calbindin D-28K (Sigma), diluted 1:200 with blocking solution. The filters

were then washed six times in PBS/Tween and incubated for one hour at room temperature, with gentle shaking, with goat anti-mouse IgG-biotinylated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 1:1000 in blocking solution. After incubation, the samples were washed six times in PBS/Tween and incubated with strepavidin peroxidase diluted in PBS/skim milk for 20 minutes at room temperature with shaking. Finally, the filters were washed three times with PBS/Tween, three times with PBS and color was developed by immersing in a solution of 0.05% (w/v) 4 mg/ml of 4-chloro-1-naphthol, 16.6% (v/v) methanol in PBS, (pH 7.4) to which H_2O_2 was added for a final concentration of 0.01% (v/v). After full color development, the filters were washed with deionized water, dried and photographed. To check for non-specific binding of goat anti-mouse antibodies, parallel samples were incubated with blocking solution instead of primary antibody.

2.6. KIDNEY ANALYSES

2.6.1. WHOLE KIDNEY

2.6.1.a. GRAVIMETRIC ANALYSIS

Whole kidneys were cleaned of fat and connective tissue, blotted dry and weighed on an analytical balance to the nearest 0.1 mg.

2.6.1.b. DNA DETERMINATION

DNA was measured by the fluorometric method of Downs and Wilfinger (1983) using calf thymus DNA as standard. Briefly, tissue samples were sonicated for up to 15 seconds at setting 8-10 and 5 mg tissue protein was diluted to one ml in assay buffer (100 mM NaCl, 10 mM EDTA and 10 mM Tris, pH 7.0) and incubated at 37 °C (10 minutes). 200 µl homogenized sample was diluted with one ml assay buffer and centrigued 2,500g (30 minutes, 4 °C). 100 µl supernatant and 3 ml Hoechst solution (2 mM EDTA, 50 mM Na_2HPO_4, 2 M NaCl, pH 7.4) were mixed and read in a fluorometer at 350 nm and 455 nm for excitation and emission, respectively.

2.6.1.c. MEMBRANE ADENYLATE CYCLASE ACTIVITY

2.6.1.c.1. MEMBRANE PREPARATION

Renal plasma membranes were isolated as described by Martz et al. (1985). Briefly, whole kidneys were minced and homogenized in 25% w/v ice-cold SET buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) using a hand homogenizer. The homogenate was centrifuged at 300g (10 minutes) and the pellet resuspended in SET buffer and recentrifuged at 1,500g (10 minutes). The pellet was resuspended in 2.0 M sucrose and centrifuged at 14,600g (10 minutes). The resulting supernatant was diluted in 7x v/v ice cold deionized water and recentrifuged at 39,000g (10 minutes). The membrane pellet was washed several times in SET buffer and finally resuspended in fresh SET to 5 mg protein/ml, frozen in liquid nitrogen and stored at -70 °C.

2.6.1.c.2. RENAL MEMBRANE ADENYLATE CYCLASE ACTIVITY

Adenylate cyclase activity was determined from the conversion of γ^{32}P-ATP to ^{32}P-cAMP as described by Forte et al. (1982). Briefly, renal membranes (50 µg in 20 µl of Tris buffer, pH 7.5) were incubated with 60 µl of incubation mix (50 mM Tris-HCl, 1.5 mM $MgCl_2$, 12 mM creatine phosphate, 13.3 units/ml creatine phosphokinase, 266 µg/ml BSA, 16 mM caffeine, $1x10^{-6}$ M GTP, 0.1 mM cAMP, 0.1 mM ATP, ^{3}H-cAMP and γ^{32}P-ATP) and deionized water or test agent for a final volume of 100 µl. The mixture was incubated at 30 °C (20 minutes) after which 100 µl of stopping solution (0.2% SDS, 1.4 mM cAMP and 40 mM ATP, pH 7.5) and 800 µl of deionized water were added and the samples vortexed. Samples were boiled for 10 minutes to denature the proteins, cooled on ice and poured onto prewashed DOWEX columns. The DOWEX columns were washed with 2 ml deionized water and the eluant discarded. The samples were eluted onto prewashed alumina columns with an additional 2.5 ml deionized water. The alumina columns were washed with 1 ml imidazole and the eluant discarded. The sample fraction containing ^{32}P-cAMP was eluted into scintillation vials with 3.6 mls imidazole. Samples were counted for ^{3}H and ^{32}P and corrected for % recovery of ^{3}H-cAMP. Activity was

expressed as pmoles cAMP formed/min/mg membrane protein. Basal activities were assayed in the presence of vehicle and maximal activity with 10^{-4} M Forskolin. The effect of n1-34 bovine PTH (MW 4109) was assessed in a dose response with 10^{-6} M GTP at 0.05, 0.1, 0.25, 0.50, 1.0, 2.5, 5.0 and 10.0 µg/ml PTH (corresponding roughly to $5x10^{-9}$, $1x10^{-8}$, $2.5x10^{-8}$, $5x10^{-8}$, $1x10^{-7}$, $2.5x10^{-7}$, $5x10^{-7}$, $1x10^{-6}$ and $5x10^{-6}$ M PTH) (Figure 2.3, appendix 1). Conditions were optimized for GTP and Mg concentrations and the effect of Ca concentration was assessed (Figures 2.4, 2.5a,b, 2.6a,b, 2.7a,b, appendix 1).

2.6.2. MIXED RENAL TUBULES

2.6.2.a. PREPARATION OF MIXED RENAL TUBULES

2.6.2.a.1. MIXED RENAL TUBULES FROM CHICKS

Kidneys were removed from 3-6 week old chicks, cleaned, cut into fine pieces and washed in ice cold oxygenated buffer (15 mM Tris-HCl, 120 mM NaCl, 4 mM KCl, 1.6 mM $MgSO_4$, 2 mM $NaH_2PO_4.2H_2O$, 10 mM glucose and 1.2 mM $CaCl_2$, pH 7.4). Kidneys were digested with 160 mg/100 ml type I collagenase (Sigma) and 100 mg/100 ml hyaluronidase (Sigma) in the same buffer (5-10 ml per set of kidneys) with shaking at 37 °C (30 minutes) until an even suspension was formed (Larkins et al., 1974). The suspension was immediately sieved through a 100 µm nylon mesh, diluted 5x with ice cold buffer and centrifuged at 50-100g (60 seconds). After washing several times in ice cold buffer, the pellet was resuspended (2-4 mg tubule protein/ml) in the same buffer supplemented with 0.1 % essential fatty acid free BSA (Sigma).

2.6.2.a.2. MIXED RENAL TUBULES FROM RATS

Preparation of rat renal tubules was modified from the procedure of Vinay et al. (1981). Briefly, kidneys were bissected with a scalpel and the medulla was discarded. The cortex was minced and washed in ice cold oxygenated isolation buffer (115 mM NaCl, 3.2 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, 2.4 mM $MgSO_4$, 25 mM $NaHCO_3$). The tissue was then digested with 160 mg/ml collagenase (Sigma type 1) and 100 mg/100 ml hyaluronidase (Sigma) in isolation buffer (5-10 mls per set of kidneys) containing 10%

essential fatty acid free BSA (Sigma). Suspensions were incubated with vigorous shaking for 20 minute intervals after which time the suspension was filtered through a tea strainer, the filtrate diluted with 4x volume ice cold buffer and the undigested kidney reincubated with fresh enzyme mixture until complete digestion was achieved. The filtrate was centrifuged at 100g (60 seconds) and washed several times to remove cellular debris and digesting enzymes. The final pellet was resuspended (2-4 mg tubule protein/ml) in K1 culture media (50% Dulbecco's MEM, 50% Hams F12 supplemented with transferrin (5 µg/ml) and cortisol ($5x10^{-8}$ M) and where appropriate, insulin (5 µg/ml) (a gift of Eli Lilly, Indianapolis, IN) and 0.1% essential fatty acid free BSA.

2.6.2.b. VIABILITY OF MIXED RENAL TUBULES

2.6.2.b.1. TRYPAN BLUE

Tubule integrity was examined by microscopic examination and viability by incubation of tubule suspension with 0.15% trypan blue for 1-20 minutes. Suspensions with 90-95% of the cells excluding the dye were considered intact and viable. Using this protcol, chick tubule suspensions were viable for 6-8 hours following isolation and rat tubule suspensions were viable for 20-26 hours (at 37 °C in an incubator) following isolation.

2.6.2.b.2. GLUCOSE METABOLISM

A second criteria of viability and biochemical activity of tubule suspensions was the linearity of glucose uptake measured as the production of $^{14}CO_2$ from ^{14}C-Glucose (Amersham) as described by Shain and Barnea (1971). Briefly, 1.5 ml aliquots of tubule suspension (4-6 mg tubule protein/ml) were pipetted into the outer chamber of specially constructed erlenmeyer flasks with open central wells. Into the central well was pipetted 200 µl of fresh hyamine solution (Sigma) and a 2.1 cm piece of fluted filter paper was placed into the well and allowed to absorb the hyamine. 10 µl of ^{14}C-Glucose was added to the outer cell suspension in each erlenmeyer, which was flushed for 20-30 seconds with 95% O_2/5% CO_2, sealed with a rubber stopper and incubated at 37 °C with

gentle shaking. To stop the reaction, 0.2 mls of 1 N sulfuric acid (BDH, AnalaR) was added through the rubber stopper using a needle and syringe. After an additional 20 minutes the filter paper and 0.5 mls of outer fluid were removed, placed into separate scintillation vials with scintillation cocktail and counted. Values were expressed as cpm $^{14}CO_2$ produced per minute. Using this protocol avian and rat tubules were viable in a sealed environment for at least 120 minutes (Figure 2.8a,b, appendix 1).

2.6.2.b.3. NADH PENETRATION ASSAY

A third criteria for the viability of tubule suspensions was the NADH penetration assay. Renal tubules (2-4 mg tubule protein/ml) were suspended in 1 ml modified Krebs/Henseleit buffer (6.9g NaCl, 0.3g KCl, 0.13g KH_2PO_4, 0.292g $MgSO_4.7H_2O$, 0.374g $CaCl_2.2H_2O$, 2.0g $NaHCO_3$/l, 25 mM HEPES, pH 7.4) containing 1.3 mM pyruvate and 0.2 mM NADH. The decrease in absorbance at 340 nm indicated the activity of lactate dehydrogenase to which NADH was accessible. Addition of 100 µl of 5% Triton X-100 resulted in cell lysis and made NADH accessible to 100% of the lactate dehydrogenase. The ratio of the rate of NADH oxidation before lysis to that after lysis is expressed as the % NADH penetration (Amador et al., 1963).

2.6.2.c. ASSESSMENT OF MIXED RENAL TUBULE ORIGIN

The renal tubule origin of the tubule suspensions was assessed by the adenylate cyclase response to PTH and vasopressin (Figure 2.9, appendix 1) and the ability to metabolize $25(OH)D_3$ to $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ (Ormstad et al., 1981).

2.6.2.d. 25(OH)D HYDROXYLATIONS

2.6.2.d.1. AVIAN 1αHYDROXYLASE ACTIVITY

Avian 1αhydroxylase activity was measured in freshly isolated mixed renal tubules as described by Shain (1972). Suspensions of tubules in Tris buffer, pH 7.4 (refer to section 6.B.1a) were adjusted to 0.5 mg tubule protein/ml in 3 mls of buffer, flushed for 20-30 seconds with 95% O_2/5% CO_2, covered and preincubated for 30 minutes in a shaking water bath at 37 °C. Net

$1,25(OH)_2D_3$ synthesis was determined by addition of 250 ng cold authentic pure $25(OH)D_3$ (Biomol) (repurified and quantitated as described by Suda et al., 1969 [Figure 2.10, appendix 1]) in ethanol and incubation for 4-5 minutes. The reaction was terminated by addition of 3x chloroform:methanol 1:2 (v/v), vortexing and placing on ice. The reaction was stopped immediately after substrate addition for zero time tubes. The mixtures were homogenized, sonicated and lipids were extracted by the method of Bligh and Dyer (1959). Further purification and quantification of $1,25(OH)_2D_3$ in incubation mixtures was as described under sections 2.C.1. and 2.C.3b. with further purification of $1,25(OH)_2D_3$ achieved using straight phase Zorbax-SIL 5 µm HPLC (NEN DUPONT, Wilmington, DE), flow rate 2.0 mls/min, Hexane:Isopropanol:Methanol (91:7:2) for comparison (Jones, 1980). Recoveries of $1,25(OH)_2D_3$, assessed by addition of ^3H-$1,25(OH)_2D_3$ to incubation mixtures after the reactions were terminated, were between 45-60%. Net synthesis of $1,25(OH)_2D_3$ was expressed as net $1,25(OH)_2D_3$ produced/min/mg tubule protein after correction for blanks. The reaction was optimized for substrate concentration, incubation time and protein. The apparent Vmax for the system was 1111 fmoles/min/mg low Ca chick tubule protein, while the Km was 100 nM (Figure 2.11, appendix 1). The reaction was linear for approximately 8 and 4-5 minutes in control and low Ca chick tubules, respectively and was proportional to protein concentration up to and including 4 mg tubule protein (Figures 2.12, 2.13, 2.14, appendix 1). A significant correlation ($r = 0.77$) between in vitro $1,25(OH)_2D_3$ production and circulating $1,25(OH)_2D_3$ was observed when data from individual animals was analyzed (Figure 3.1.2.7). Product verification was as described under section 6.C.4.

2.6.2.d.2. MAMMALIAN 24 HYDROXYLASE ACTIVITY

Mammalian 24 hydroxylase activity in mixed suspensions of renal tubules (prepared as described in section 6.B.1b) was measured as described by Turner et al. (1980) with modifications [for diabetic studies measurements were made in Percoll purified proximal tubule suspensions]. Briefly, one ml

aliquots of tubule suspension (2-4 mg tubule protein/ml) in K1 culture medium were preincubated in 25 ml siliconized erlenmeyers at 37 °C for 10-30 minutes, after which substrate (1,000 nM ^{3}H-25(OH)D_3 [100,000 cpm]) and test solution or vehicle were added. Net production of 24,25$(OH)_2D_3$ was determined after incubation for up to 120 minutes at 37 °C. After reactions were stopped by the addition of equivolume acetonitrile, samples were vortexed, homogenized and transferred to an ice bath. Reaction mixtures in which substrate was incubated with buffer in the absence of tubules, or in the presence of denatured tubules (kept on ice) served as background controls. The 24,25$(OH)_2D_3$ produced in incubation mixtures was extracted and purified after the addition of equivolume potassium phosphate buffer (pH 10.4) on C18 Sep-Paks as described under section 2.C.1. Quantitation was achieved following HPLC separation [25x4.6 cm Zorbax-SIL 5 µm HPLC column] (NEN-DUPONT, Wilmington, DE) flow rate 2.0 ml/min, with: hexane:isopropanol:methanol (91:7:2) as solvent and scintillation counting of the eluant comigrating with authentic pure 24,25$(OH)_2D_3$ (Biomol, Plymouth Meeting, PA) or ^{3}H-24,25$(OH)_2D_3$ (Amersham Corp. Canada) and calculated after correction for recovery and estimation of specific activity by:

Rate of Net Synthesis of 24,25$(OH)_2D_3$

= fmoles 24,25$(OH)_2D_3$/min/mg tubule protein

$$\frac{\text{(cpm 24,25(OH)}_2\text{D}_3\text{ peak - cpm blank) X subst conc (fmols)}}{\text{tubule protein conc (mg) X incub period (minutes)}}$$

Recovery of total counts was 40-65%. The assay was optimized for substrate concentration, incubation time and tubule protein concentration. The apparent Vmax for the system was 500 fmoles 24,25$(OH)_2D_3$ produced per minute per mg of tubule protein, while the Km was estimated to be 1.25 µM (Figure 2.15, appendix 1). The reaction was linear for 60 minutes and proportional to protein concentrations up to and including 6 mg tubule protein (Figures 2.16, 2.17, appendix 1). Product comigrated with authentic 24,25$(OH)_2D_3$ pure standard (Biomol) or ^{3}H-24,25$(OH)_2D_3$ (Amersham) on straight phase Zorbax-SIL 5µm Hexane:Isopropanol:Methanol [91:7:2] HPLC (Jones et al., 1983).

2.6.2.e. RENAL TUBULE ADENYLATE CYCLASE ACTIVITY

2.6.2.e.1. SAMPLE PREPARATION

Mixed tubules were prepared and resuspended (2-4 mg tubule protein/ml) in K1 tissue culture medium without BSA. 10 mls of suspension was transferred to a 250 ml tissue culture flask, flushed with 95% O_2/5% CO_2 and the intracellular ATP pool was labeled by the addition of 0.125 uCi 2-8 ^{3}H-adenine (ICN, Irvine, CA) with gentle shaking in a 37 °C water bath for 4 hours. The suspension was centrifuged at 1,500g (5 minutes) and the tubules resuspended in fresh K1 medium.

2.6.2.e.2. MEASUREMENT OF ADENYLATE CYCLASE ACTIVITY

Adenylate cyclase activity was determined as described by Aasheim et al. (1989) in 1 ml aliquots of tubule suspensions after ^{3}H-adenine prelabelling. In all experiments, 0.1 M 1-methyl-3-isobutyl-xanthine (IBMX) (Sigma) was added to prevent degradation of cAMP by cyclic nucleotide phosphodiesterase. The appropriate agents or vehicle were added and the tubules were incubated for up to 60 minutes at 37 °C in a shaking water bath. Reactions were terminated after 0.5-60 minutes by centrifugation at 4 °C at 200g (3 minutes). Supernatants were discarded and the pellets washed with cold PBS. Final pellets were resuspended in 0.5 mls of ^{14}C lysis buffer (150 mM NaCl, 10 mM Na_2HPO_4, 1 mM EDTA, 0.1 mM cAMP, 0.1 mM MIX, 1% nonionic phosphate detergent P-40 and 0.001 uCi ^{14}C-cAMP to monitor ^{3}H-cAMP recovery). The tubes were vortexed and after 30 minutes at 4 °C were spun at 200g (5 minutes). Supernatant were applied to freshly washed DOWEX 50WX-4 (200 mesh) columns. The ATP fraction was eluted and discarded with 4 mls distilled water and the cAMP was eluted onto prewashed alumina columns with an additional 7 mls distilled water. The cAMP was eluted off the alumina columns with 5 mls of 50 mM TRIS buffer (pH 7.5), collected in scintillation vials and counted for quantitation of ^{3}H-cAMP and ^{14}C-cAMP. Enzymatic activity was calculated as pmoles cAMP/unit time/mg tubule protein calculated from the specific activity of the ^{3}H-cAMP after estimation of product recovery (assessed

from recovery of ^{14}C-cAMP counts).

2.6.2.f. PROTEIN KINASE C ACTIVITY

2.6.2.f.1. MEMBRANE AND CYTOSOL PREPARATION

Renal tubules were isolated and homogenized (2-4 mg tubule protein) in 5x v/v PKC lysis buffer B (50 mM Tris-HCl, 5 mM EDTA, 10 mM EGTA, 0.3% w/v beta-mercaptoethanol, 10 mM benzamidine, 50 µg/ml PMSF, 100 µg/ml leupeptin, 5 mM levamisole hydrochloride, 1 mM sodium vanadate and 10 mM ouabain). PNF were prepared by centrifuging at 1,500g (5 minutes) and the resultant supernatant recentrifuged at approximately 100,000g (15 minutes) in a microcentrifuge at 4 °C in eppendorf tubes. The resultant supernatants were removed and saved (crude cytosolic fraction) and the crude membrane pellets were resuspended in 100-200 µl PKC lysis buffer B.

2.6.2.f.2. PROTEIN KINASE C ASSAY

PKC activity was determined in tubule membrane (10 µg protein) and cytosol (25 µg protein) using the Amersham PKC enzyme assay system (RPN 77) PKC activity was linear with protein up to and including 25 µg membrane protein and up to and including 50 µg cytosolic protein [refer to Figure 2.18, appendix 1). The assay detects the incorporation of γ^{32}P-ATP into the threonine group of a PKC synthetic peptide. Reactions were performed in 50 mM Tris-HCl buffer (pH 7.5) in the presence of 1 mM Ca acetate, 0.67 mole % phosphatidyl-L-serine, 2 µg/ml phorbol 12-myristate 13-acetate (to maximally stimulate PKC activity), 2.5 mM dithiothreitol and 75 µm PKC specific peptide and was initiated by addition of 3.75 mM Mg acetate, 12.5 µm ATP, 10 uCi/ml γ^{32}P-ATP and gentle mixing. The tubes were incubated at 25 °C (15 minutes) and the reaction terminated by the addition of 100 µl stopping reagent (dilute acidic reaction-quenching reagent). Phosphorylated peptide was separated from free γ^{32}P-ATP using peptide-specific binding papers, and the degree of peptide phosphorylation was detected by ^{32}P scintillation counting of acid (5 % v/v acetic acid) washed papers. PKC enzyme activity was expressed as pmoles phosphate transferred per minute per 25 mg cytosolic or 10 mg membrane

protein after correction for enzyme blanks and calculation of ^{32}P-ATP specific activity. Enzyme blanks consisted of parallel assay incubations containing PKC buffer in lieu of tissue protein. Specificity for the PKC peptide in this system was demonstrated by assaying in the presence of PKC buffer B in lieu of peptide reagent.

2.6.3. PROXIMAL RENAL TUBULES

2.6.3.a. PREPARATION OF PURIFIED RENAL PROXIMAL TUBULES

Proximal tubules were purified from mixed renal tubules using Percoll (Pharmacia Fine Chemicals, Baie d'Urfe, Quebec) gradient centrifugation as described by Vinay et al. (1981) with modifications as described by Gesek et al. (1987). Suspensions of mixed renal tubules were pelleted (100g, 1-2 minutes) and resuspended in 30 ml ice-cold freshly oxygenated 45% Percoll with various salts added to obtain a final solution with osmolality of 300 mosmol/kg (pH 7.4): 28 mM HCO_3, 140 mequivalents/l sodium, 1.7 mequivalents/l chloride, 4.9 mequivalents/l potassium, 1.2 mM P and 2 mM Ca. The final concentration of the various salts was identical to the original isolation buffer (refer to section 6.B.1b.) The Percoll solution was spun at 12,200g (30 minutes) at 4 °C to establish a density gradient between 1.05 and 1.35. The density gradient was monitored using density marker beads (Pharmacia). After centrifugation, viable tubules separated into 4-6 separate bands with the lower bands (4-6) corresponding to the convoluted and straight proximal tubule enriched fractions and upper bands (1-2) to distal tubule enriched fractions. The desired bands were removed by pipet, resuspended in 10-20x v/v isolation buffer and washed several times to remove Percoll before use.

2.6.3.b. VIABILITY OF PURIFIED RENAL PROXIMAL TUBULES

2.6.3.b.1. TRYPAN BLUE

The viability of purified proximal tubules was indicated by the ability to form a gradient on Percoll and was routinely determined by trypan blue exclusion and light microscopy examination (refer to section 6.B.2a.). Proximal

enriched tubules remained viable in K1 medium in a tissue culture incubator (37 °C) for up to 26 hours and could be successfully cultured.

2.6.3.b.2. GLUCOSE METABOLISM

The viability of proximal tubules was also assessed by their ability to metabolize $^{14}CO_2$ from ^{14}C-glucose as described for mixed renal tubules (refer to section 6.B.2b.). By this criteria, purified proximal tubules were viable in a sealed environment at 37 °C for at least 90 minutes (Figure 2.8b, appendix 1).

2.6.3.c. CHARACTERISTICS OF PURIFIED PROXIMAL TUBULES

The origin and identity of purified proximal tubules was assessed by the following criteria as described by Gesek et al. (1987) and Vinay et al. (1981); a) light microscopy identification of proximal enriched fractions: morphology of proximal and distal nephron segments are distinguishable from one another without staining. Proximal tubules are longer and often convoluted with large tubule diameters whereas distal tubules are shorter, with much thinner tubule diameters. b) differential sensitivity to agonists (distal tubule nephron segments exhibit enhanced sensitivity to arginine vasopressin while proximal tubule nephron segments exhibit enhanced sensitivity to PTH) (Figure 2.19, appendix 1) c) measurement of proximal tubule marker enzyme activity as compared to distal tubule marker enzymes (distal tubule nephron segments exhibit high glycolytic activity exemplified by; higher hexokinase activity in mixed tubules, while proximal tubule nephron segments exhibit high gluconeogenic activity exemplified by; higher fructose-1,6 bisphosphatase and glucose-6-phosphatase activity in purified proximal tubules) (Figures 2.20, 2.21, appendix 1) d) the ability to metabolize $25(OH)D_3$ to $1,25(OH)_2D_3$ (refer to section 6.C.4.).

2.6.3.d. $25(OH)D_3$ HYDROXYLATION IN PROXIMAL TUBULES

Net synthesis of $1,25(OH)_2D_3$ was determined in 1 ml suspensions of purified proximal tubules (2-4 mg tubule protein/ml) in K1 medium containing 0.1% essential fatty acid free BSA and (depending upon the experiment) 5 µg/ml insulin in 25 ml siliconized erlenmeyer flasks, as described by Favus and

Langman (1986) with major modifications. Tubules were pre-equilibrated to 37 °C for 10-30 minutes after which the reaction was initiated by addition of 5 µm re-purified cold $25(OH)D_3$ (Biomol) and vehicle or test solution and incubated at 37 °C for the desired time. The reaction was terminated by addition of equivolume acetonitrile, vortexed and transferred to an ice bath. One ml of phosphate buffer (pH 10.4) and 2,000-3,000 cpm ^3H-$1,25(OH)_2D_3$ were added and the suspension hand homogenized. Reaction blanks consisted of buffer without tubules to which substrate was added and tubule suspensions which were denatured (by solvents or boiling) immediately following substrate addition. The $1,25(OH)_2D_3$ was initially isolated as described in section 2.C.1. and further purified by straight phase HPLC [Zorbax-SIL, 25x4.6cm (NEN DUPONT Instruments, Wilmington, DE) flow rate 2.0 mls/min; Hexane:Isopropanol:Methanol (91:7:2)]. The fraction comigrating with authentic pure $1,25(OH)_2D_3$ (Biomol, Hoffman LaRoche) and ^3H-$1,25(OH)_2D_3$ (Amersham) was collected and quantified by radioreceptor assay (refer to section 2.C.3a,b.) The assay was optimized for substrate concentration, incubation time and protein concentration. The apparent Vmax for the system was 21.98 fmoles/min/mg proximal tubule protein isolated from vitamin D deficient rats and 66.67 fmoles/min/mg proximal tubule protein isolated from low Ca fed rats, while the Km was estimated to be 1 µmolar irrespective of dietary regime (Figures 2.22, 2.23, appendix 1). The hydroxylation reaction was linear for 20-30 minutes for tubules isolated from vitamin D deficient rats, 20 minutes for tubules isolated from low Ca fed rats and at least 120 minutes for tubules isolated from control rats and was proportional to protein concentration up to and including 10 mg control tubule protein (Figures 2.24, 2.25, 2.26, 2.27, appendix 1). In vitro $1,25(OH)_2D_3$ production correlated significantly ($r=0.80$) with circulating $1,25(OH)_2D_3$ when data from individual animals was analyzed (Figure 2.28, appendix 1).

Product verification was achieved by preparative scale isolation of the fraction containing the putative $1,25(OH)_2D_3$ and subsequent comigration of

product with authentic pure standard $1,25(OH)_2D_3$ or ^3H-$1,25(OH)_2D_3$ on two HPLC systems [normal phase Zorbax-SIL 5 µm Hexane:Isopropanol:Methanol [91:7:2], 2 ml/min (Figure 2.29, appendix 1) [Jones, 1980] and normal phase Zorbax-SIL 5 µm Methylene Chloride:Isopropanol [19:1] 1 ml/min (data not shown) [Brown and Deluca, 1985])], demonstration of UV absorbance spectra analysis typical of a vitamin D metabolite (absorbance maximum 265 nm no visible absorbance at 315 nm (ascribed to the 10-oxo-19-nor-25(OH)D_3 compound) (Figure 2.30, apprendix 1), and affinity for the vitamin D receptor in the radioreceptor assay (Korkor et al., 1987).

2.6.4. PRIMARY CULTURE OF RAT RENAL TUBULES

2.6.4.a. PREPARATION OF CULTURES

Primary cultures of both mixed and proximal renal tubules were successfully established using methods described by Korkor et al. (1987) and Bell et al. (1988) with minor modifications. Suspensions of rat tubules were prepared as described in section 6.B.1b. and 6.C.1., cells were sedimented at 100g (5 minutes), resuspended in K1 medium (supplemented with 10% BSA, 5 µg/ml insulin, 5 µg/ml transferrin, $5x10^{-8}$ hydrocortisone, 25 ng/ml prostaglandin E_1 and 1% fungizone (antibiotic/antimycotic [50 µg/ml gentamycin, 50 units/ml penicillin]), 4-5 v/v, and gently aspirated up and down. 5 mls tubule suspension was plated per 100 mm plastic culture dish. The primary cultures were maintained in a humidified 5% CO_2/95% air incubator at 37 °C. 24 hours after plating, the medium was removed and replaced with serum-free K-1 medium. Culture medium was changed routinely every 2 days. Cells grew in discrete clusters of closely packed cells, which appeared to migrate out from the initial attached tubule fragments. Cultures were 90% confluent by 5-7 days, and yielded approximately 1-1.25 mg protein per dish, at which time they were used for experimentation.

2.6.4.b. PRELIMINARY CHARACTERIZATION OF PRIMARY CULTURES

Primary cultures of rat tubules were sensitive to rat $1x10^{-7}$ 1-34 PTH

and 10 mU/ml arginine vasopressin; exhibited by enhanced basal adenylate cyclase synthesis of cAMP in the presence of IBMX (Figure 2.31, appendix 1), linearly metabolized $25(OH)D_3$ to $1,25(OH)_2D_3$ for at least 3 hours (Figure 2.32, appendix 1) and exhibited increased basal $1,25(OH)_2D_3$ net synthesis in response to $1x10^{-7}$ 1-34 PTH in the presence of IBMX (Figure 4.2.1.a.2).

2.7. STATISTICAL ANALYSIS

Data comparisons between three or more treatment groups were by one-way ANOVA. Newman Keuls analysis was used for determination of differences between means when a significant F value was obtained with ANOVA. Data comparisons betwen two treatment groups were made with Student's t test for unpaired or paired data, as appropriate. In all cases, differences were considered significant if a p value less than 0.05 was obtained. ANOVA, Newman Keuls, t tests and calculations of Pearson's correlation coefficients were done with the True Epistat computer program. Data are expressed as mean ± standard error unless otherwise indicated.

CHAPTER 3 - MAGNESIUM DEFICIENCY AND VITAMIN D METABOLISM

3.1. OBJECTIVES AND RATIONALE

Changes in extracellular or $[Mg]_i$, induced by Mg deficiency, may influence the metabolism of vitamin D by directly affecting net synthesis or actions of $1,25(OH)_2D_3$ or by indirectly affecting PTH target tissue responsiveness. Alterations in vitamin D metabolism may be related to the aberrant Ca homeostasis of Mg deficiency.

At commencement of these studies little information existed on vitamin D metabolism in experimental Mg deficiency. Clinical data was contradictory and often hampered by conflicting factors. In general, those studies previously published on vitamin D metabolism in experimental Mg deficiency were conducted in rats, which do not exhibit hypocalcaemia during Mg deficiency. The only exception being one abstract on 1αhydroxylase activity in vitamin D deficient hypomagnesaemic hypocalcaemic chicks. While skeletal responsiveness to PTH had been extensively investigated, the effect of Mg deficiency on renal responsiveness to PTH required clarification, particularly with respect to vitamin D metabolism. The relevance of alterations in vitamin D metabolism to the hypomagnesaemic hypocalcaemia was unclear.

Thus the objectives of the Mg deficiency studies were:

1. To monitor systematically vitamin D metabolism (synthesis and actions) and Ca homeostasis during the induction of Mg deficiency.
2. To investigate renal and skeletal responsiveness to PTH in Mg deficiency.
3. To determine if alterations in the metabolism of $1,25(OH)_2D_3$ were involved in the pathogenesis of the hypocalcaemia of Mg deficiency.

3.2. ANIMAL MODEL

The young growing chick was chosen for these studies on vitamin D metabolism in Mg deficiency based on the following criteria:

1. The Mg deficient chick exhibits hypocalcaemia.
2. Considerable experimental data exists on Ca homeostasis and Mg deficiency in the chick.
3. The young growing chick has high mineral requirements, thus Mg deficiency develops rapidly.
4. The measurement and regulation of avian renal 1αhydroxylase activity has been extensively studied facilitating measurement and comprehension of experimental data.

3.3. EXPERIMENTAL PROTOCOLS

3.3.1. VITAMIN D METABOLISM AND RENAL RESPONSIVENESS TO PTH IN VITRO DURING MAGNESIUM DEFICIENCY IN CHICKS

In the first set of experiments, vitamin D metabolism and Ca homeostasis were assessed during the development of Mg deficiency in young growing chicks. Mg status was evaluated by plasma and bone Mg concentrations. Ca homeostasis was assessed by plasma and bone Ca and P concentrations. Indices of vitamin D metabolism were plasma $1,25(OH)_2D_3$, $24,25(OH)_2D_3$ and $25(OH)D_3$ and renal tubule basal 1αhydroxylase activity. Target tissue responsiveness to $1,25(OH)_2D_3$ was assessed by monitoring plasma and bone alkaline phosphatase activity. Renal responsiveness to PTH in vitro included stimulation of adenylate cyclase in renal membranes and 1αhydroxylase activity in freshly isolated renal tubule suspensions.

3.3.2. VITAMIN D METABOLISM AND PTH RESPONSIVENESS IN VIVO DURING MAGNESIUM DEFICIENCY IN CHICKS

In the second set of experiments, the effect of low dietary Mg on target tissue responsiveness to PTH and $1,25(OH)_2D_3$ in vivo was evaluated. This was achieved by exposing chicks to low dietary Ca, which stimulates production of both PTH and $1,25(OH)_2D_3$ in normal animals. Mg status was monitored by

plasma and bone Mg concentrations while Ca homeostasis was assessed by plasma and bone Ca and P concentrations. Indices of PTH and vitamin D actions were circulating $1,25(OH)_2D_3$, plasma Ca, bone mass and bone mineral content.

3.3.3. VITAMIN D AND THE PATHOPHYSIOLOGY OF THE HYPOCALCAEMIA OF MAGNESIUM DEFICIENCY

In the third set of experiments, the effect of $1,25(OH)_2D_3$ supplementation was assessed in Mg deficient chicks. Mg status was assessed by plasma and bone Mg levels while Ca and bone homeostasis were assessed by plasma and bone Ca and P and bone histology. Circulating $1,25(OH)_2D_3$ and intestinal calbindin-28K levels were measured as indices of vitamin D metabolism.

3.4. VITAMIN D METABOLISM AND RENAL RESPONSIVENESS TO PTH IN VITRO DURING MAGNESIUM DEFICIENCY IN CHICKS

3.4.1. EXPERIMENTAL PROTOCOL

Two week old white leghorn chicks were fed and housed as described under "Materials and Methods". They received deionized water ad libitum and were fed one of three soy-based diets as follows: control (0.1% Mg; 1.5% Ca), Mg deficient (0.013% Mg; 1.5% Ca) or low Ca (0.1% Mg; 0.2% Ca). The low Ca group was included as a positive control group in which the classical response to low Ca stress would be demonstrated. The control and low Ca groups were pair-fed to the Mg deficient group, which consistently had the lowest food consumption. Four studies were performed: three time course experiments (14-16 days duration) in which five to eight animals from each group were sacrificed at intervals of three to four or seven to eight days, and a 14 day study with 12 chicks on the Mg deficient and control diets and seven chicks on the low Ca diet. At sacrifice, blood was collected into heparinized syringes by cardiac puncture under halothane anaesthesia; plasma was stored at -70°C for subsequent mineral, alkaline phosphatase activity and vitamin D metabolite analysis. Left tibiae were removed and analyzed for dry weight and mineral

content. Right tibiae were removed, cleaned and the epiphysis excised and flash frozen in liquid nitrogen for alkaline phosphatase activity.

In one of the time course experiments, renal tubules were isolated from two to four birds per time point per group for measurement of basal and PTH stimulated 1αhydroxylase activity. In the 14 day study, renal membranes were prepared from individual birds from each dietary group for measurement of adenylate cyclase activity (basal, PTH and forskolin stimulated).

3.4.2. RESULTS

BODY WEIGHT AND GROWTH

Mg deficient chicks grew at comparable rates as their control and low Ca fed counterparts. Due to the pair feeding regime there were no significant differences in body weights between any of the groups at any time during the experimental period (data not shown).

PLASMA MINERALS

Mg deficient birds consistently developed significant hypomagnesaemia by day four and significant hypocalcaemia by day 11, as compared to birds fed either control or low Ca diets (Figures 3.4.2.1 and 3.4.2.2). No changes in plasma Mg were observed in control or low Ca fed chicks. Plasma Ca was significantly lower in low Ca than in control chicks from day eight on, but, in contrast to the Mg deficient chicks, the magnitude of the difference was less and plasma Ca did not progressively decrease. Mg deficient birds developed significant hyperphosphatemia (by day 15), while chicks fed low Ca developed significant hypophosphatemia (by day eight), compared to control values (Figure 3.4.2.3).

CIRCULATING VITAMIN D METABOLITES

Although there was a trend towards lower plasma $25(OH)D_3$ in Mg deficient chicks, as compared to controls, the difference was not significant (Table 3.4.2.1). Plasma $25(OH)D_3$ levels in low Ca chicks were, however, significantly lower than in control chicks. $24,25(OH)_2D_3$, a metabolite of vitamin D which is not effective in increasing plasma Ca, was slightly lower

in the low Ca chicks than in the Mg deficient chicks, which in turn was slightly lower than in control chicks (Table 3.4.2.1). These differences were not statistically significant. Plasma $1,25(OH)_2D_3$ was significantly elevated in chicks fed low dietary Ca but was not increased in chicks fed low dietary Mg. Time course data (Figure 3.4.2.4) demonstrated that $1,25(OH)_2D_3$ was elevated in low Ca chicks within four days. In contrast, despite the development and progression of hypocalcaemia in the Mg deficient chicks, circulating $1,25(OH)_2D_3$ was not elevated at any time point studied.

ISOLATED RENAL TUBULES

Renal tubule viability, assessed by trypan blue exclusion and metabolism of glucose to CO_2, was comparable between tubules isolated from Mg deficient and control chicks (Figure 3.4.2.5). Net synthesis of $1,25(OH)_2D_3$ measured in renal tubules isolated from hypocalcaemic Mg deficient and control chicks on day 12 was comparable. In contrast, net synthesis of $1,25(OH)_2D_3$ measured in renal tubules isolated from low Ca chicks was significantly elevated (two fold) in comparison to that measured in tubules from Mg deficient and control chicks (Figure 3.4.2.6). Similar results were obtained for days four and 15 (data not shown). A significant correlation ($r = 0.77$) between 1αhydroxylase activity and plasma $1,25(OH)_2D_3$ was observed for all dietary groups at all times (days 4, 12 and 15) (Figure 3.4.2.7). Acute exposure of renal tubules isolated from Day 12 Mg deficient and control chicks to PTH comparably stimulated 1αhydroxylase activity (1.3 fold) at the dose tested ($1x10^{-7}$M PTH) (Figure 3.4.2.8).

PTH RESPONSIVENESS

Basal and forskolin stimulated adenylate cyclase activity was not significantly affected by Mg deficient or low Ca diets (Table 3.4.2.2). A dose dependent stimulation of adenylate cyclase activity by PTH was observed for renal membranes isolated from Mg deficient, control and low Ca fed chicks. No significant differences between efficacy of PTH to stimulate adenylate cyclase activity was observed between the three groups (Figure 3.4.2.9).

BONE MINERAL CONTENT

Bone Mg content decreased significantly in Mg deficient chicks (Table 3.4.2.3), and by day 15 was only 40% of control values. Bone Ca, P and total ash content were significantly elevated in the Mg deficient chicks but were significantly decreased in the low Ca group compared to controls (Table 3.4.2.3). Similar results were obtained for Mg, Ca and P analysis per gram ash (data not shown).

ALKALINE PHOSPHATASE ACTIVITY

Plasma alkaline phosphatase activity was not significantly different between control, Mg deficient and Low Ca fed animals (Figure 3.4.2.10). Bone epiphyseal alkaline phosphatase activity was not significantly different between control and Mg deficient chicks (Figure 3.4.2.11).

FIGURE 3.4.2.1 Plasma Mg in chicks pair-fed Control, Mg Deficient or Low Ca diets for up to 15 days.
Data points are mean ± SEM, n = 7-21 chicks. Plasma Mg was significantly lower in chicks fed Mg deficient diets from day 4 on, compared to controls or low Ca fed chicks.

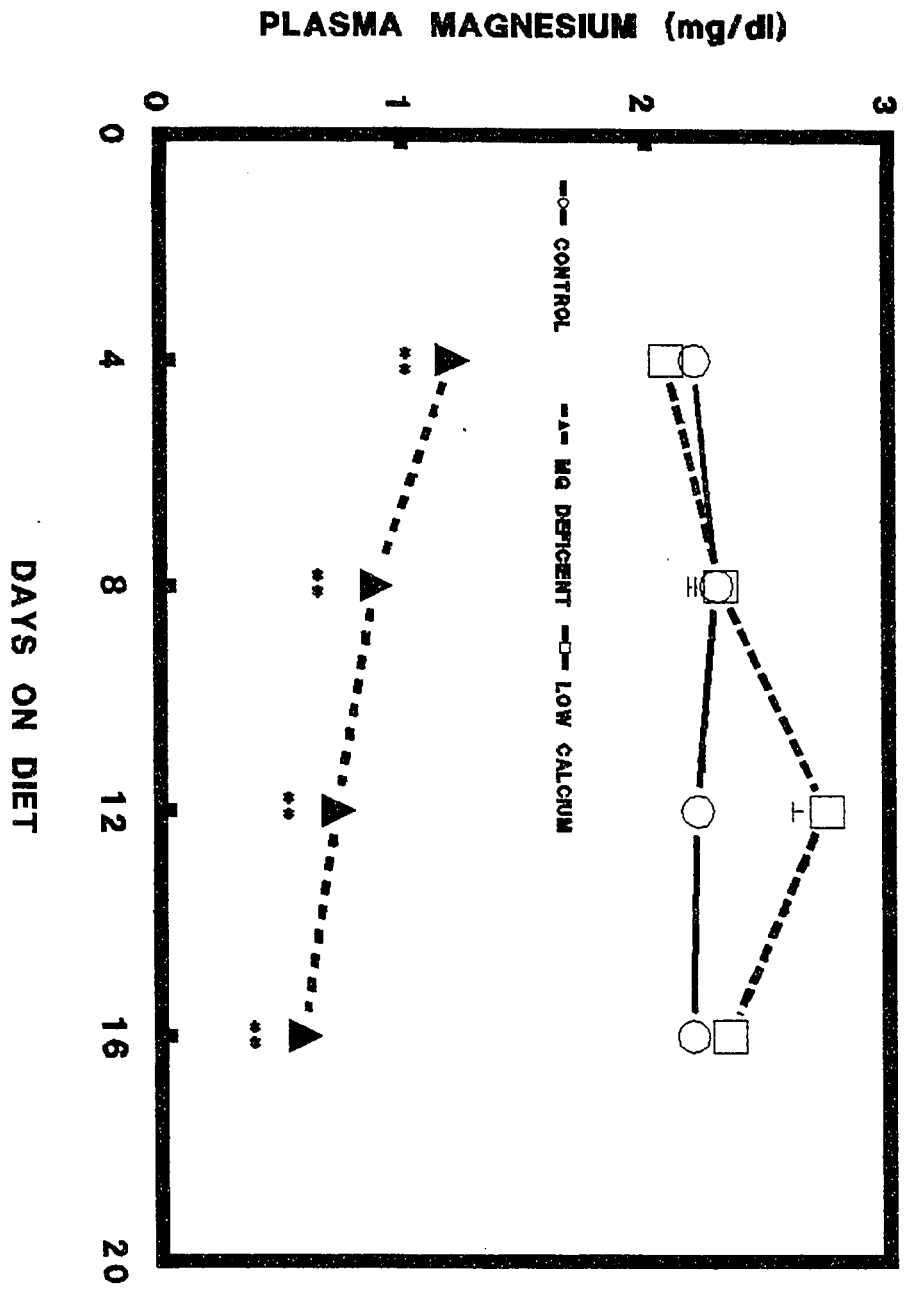

PLASMA MAGNESIUM (mg/dl)
0
1
2
3
0
4
8
12
16
20
DAYS ON DIET
CONTROL
MG DEFICIENT
LOW CALCIUM
**
**
**
**

FIGURE 3.4.2.2 Plasma Ca in chicks pair-fed Control, Mg deficient or Low Ca diets for up to 15 days.
Data points are mean ± SEM, n = 7-21 chicks. Plasma Ca was significantly lower in low Ca fed chicks (from days 8) and in Mg deficient chicks (from day 11-12), compared to control fed chicks.

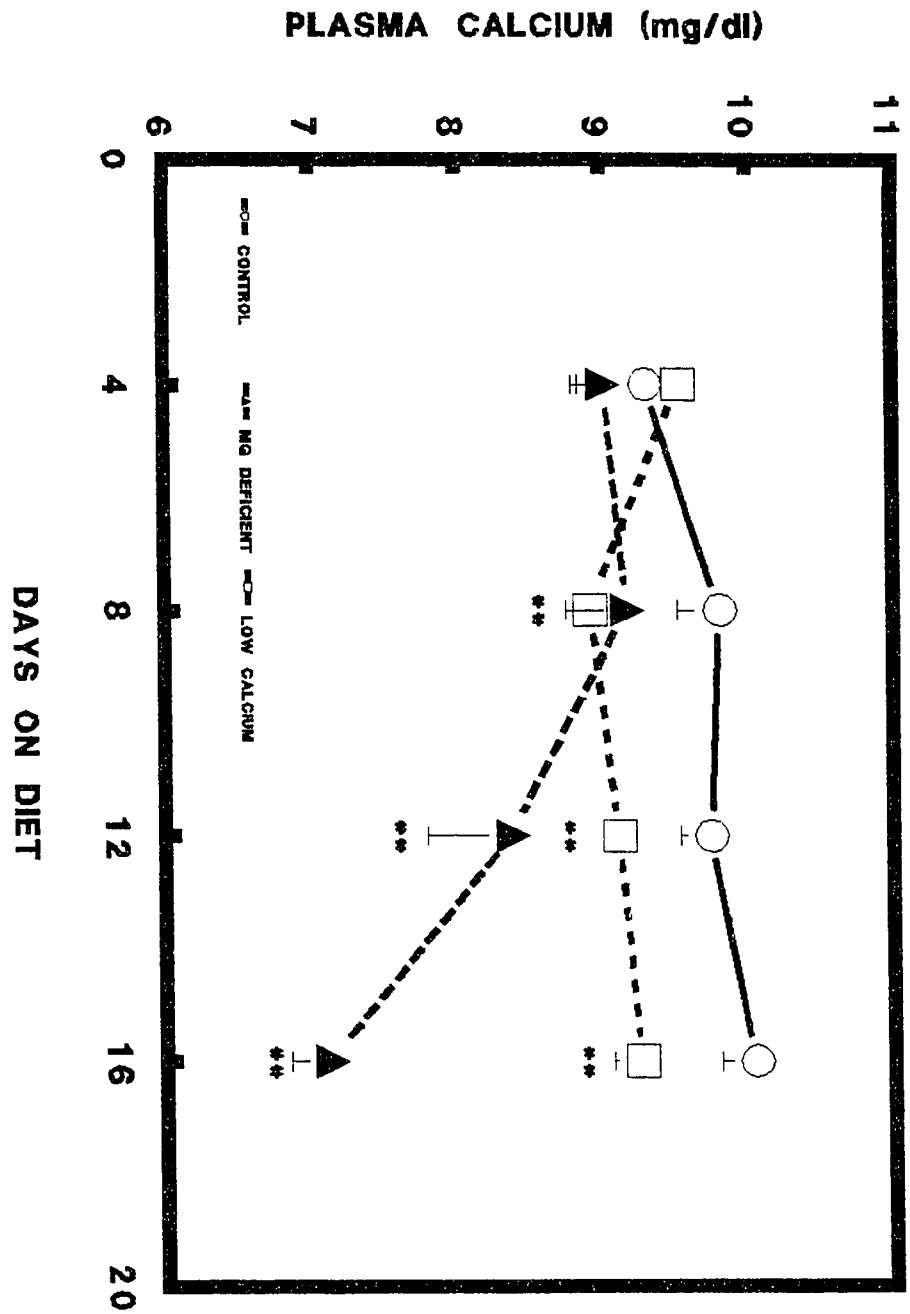

PLASMA CALCIUM (mg/dl)
6
7
8
9
10
11
0
4
8
12
16
20
DAYS ON DIET
CONTROL
NO DEFICIENT
LOW CALCIUM
**
**
**
**
**

FIGURE 3.4.2.3 Plasma P in chicks pair-fed Control, Mg deficient or Low Ca diets for up to 15 days.
Data points are mean ± SEM, n = 7-21 chicks. Plasma P was significantly higher in Mg deficient chicks (by day 15) and significantly lower in low Ca fed birds (by day 8).

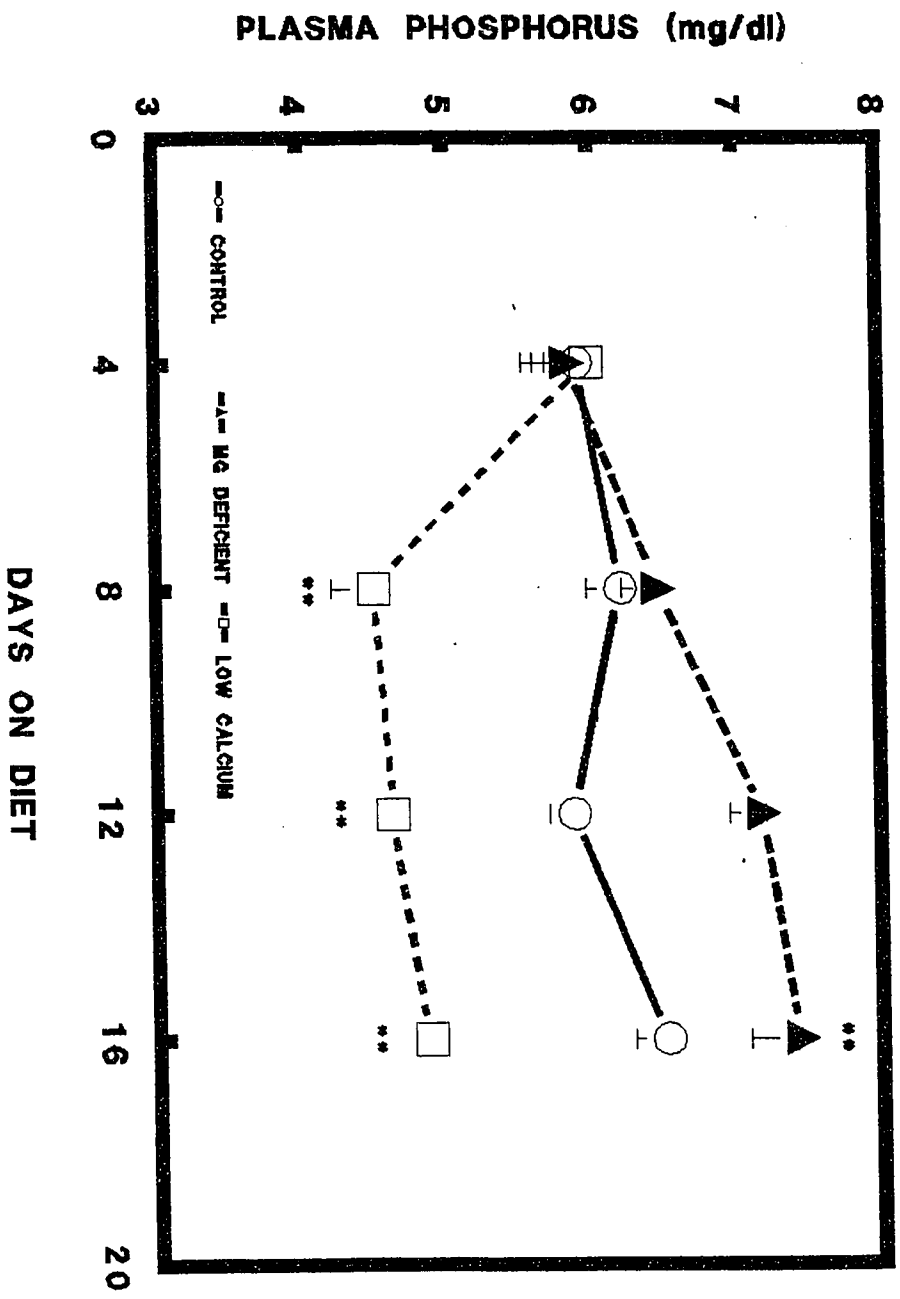

PLASMA PHOSPHORUS (mg/dl)
3
4
5
6
7
8
0
4
8
12
16
20
DAYS ON DIET
=○= CONTROL
=△= MG DEFICIENT
=□= LOW CALCIUM
**
**
**
**

TABLE 3.4.2.1

Plasma 25(OH)D_3 and 24,25$(OH)_2D_3$ in chicks pair-fed Control, Low Ca or Mg deficient diets for 15 days. Data are means ± SEM, n = 6-20.

	25(OH)D (ng/ml)	24,25$(OH)_2$D (ng/ml)
Control	9.6 ± 0.8[a]	6.8 ± 0.8
Low Ca	7.5 ± 1.1[b]	4.6 ± 0.4
Mg Deficient	9.0 ± 0.7[a]	4.1 ± 0.8

[a,b]Means with different letters in their superscripts are significantly ($p < 0.05$) different by one way ANOVA, followed by Newman Keuls.

FIGURE 3.4.2.4 Plasma $1,25(OH)_2D_3$ in chicks pair-fed Control, Mg deficient or Low Ca diets for up to 15 days.
Data points are mean ± SEM, n = 7-19 chicks. No significant difference in circulating $1,25(OH)_2D_3$ was observed between controls and Mg deficient chicks, while plasma $1,25(OH)_2D_3$ was significantly increased in low Ca fed chicks from day 4 on.

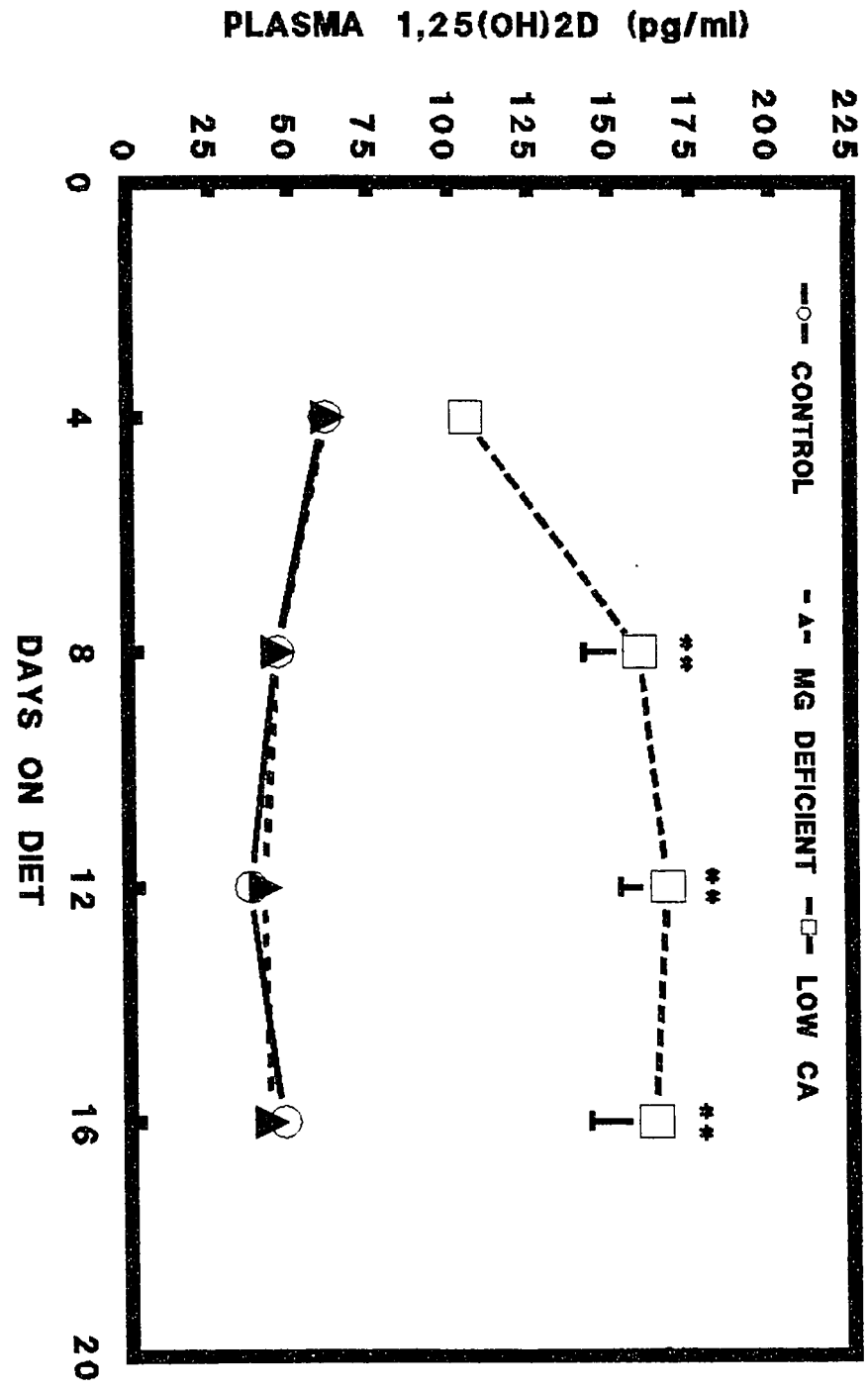

PLASMA 1,25(OH)2D (pg/ml)
225
200
175
150
125
100
75
50
25
0
0
4
8
12
16
20
DAYS ON DIET
—○— CONTROL
- -△- - MG DEFICIENT
—□— LOW CA
**
**
**

FIGURE 3.4.2.5 Renal tubule viability (assessed as glucose oxidation) in tubules from chicks pair-fed Control or Mg deficient diets for 15 days.

Data are mean ± SEM, of duplicates or triplicates from 3 different experiments. No significant differences in tubule viability were observed between control and Mg deficient chicks.

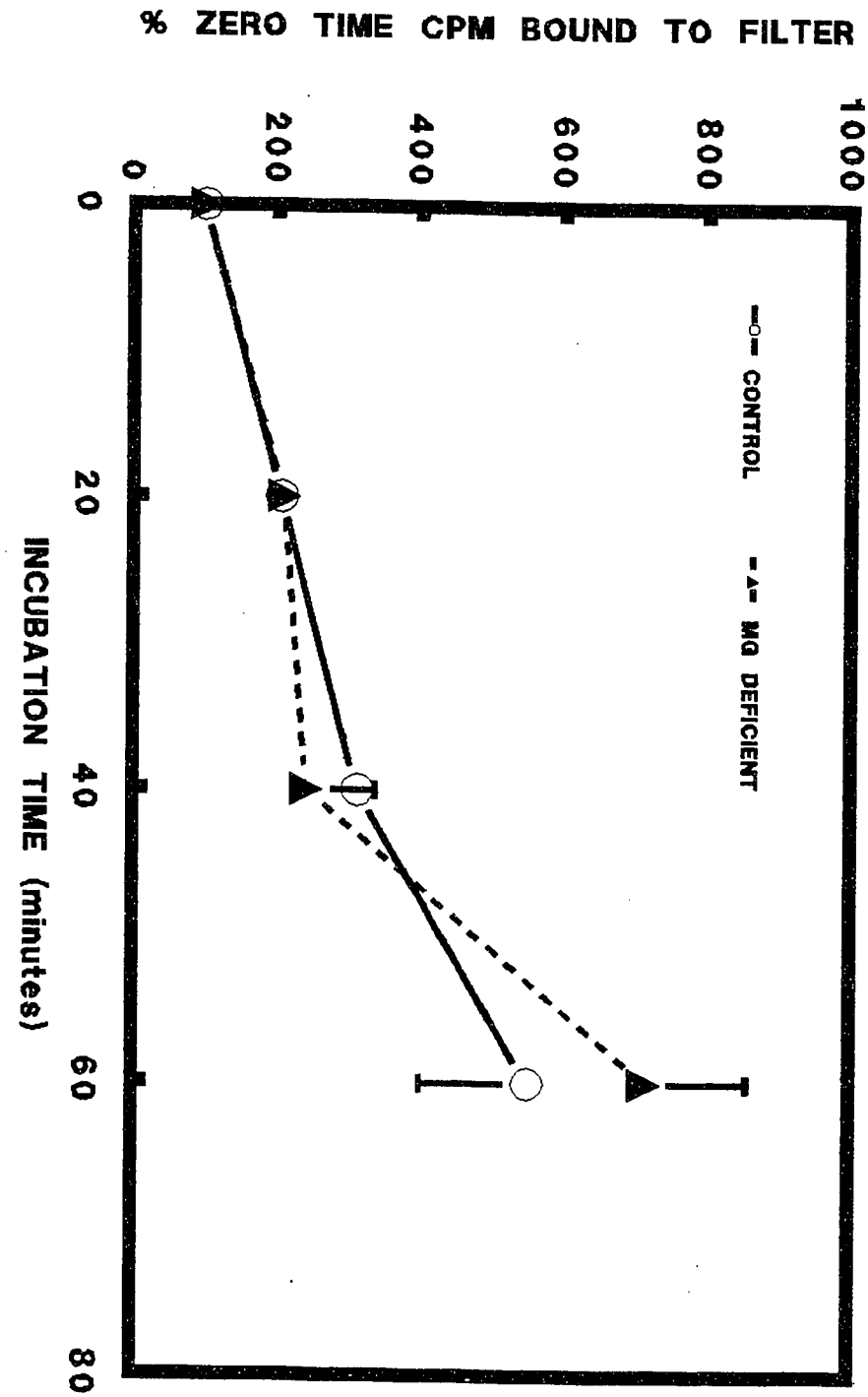

% ZERO TIME CPM BOUND TO FILTER
0
200
400
600
800
1000
0
20
40
60
80
INCUBATION TIME (minutes)
CONTROL
MG DEFICIENT

FIGURE 3.4.2.6 Net synthesis of $1,25(OH)_2D_3$ (1αhydroxylase activity) in renal tubules isolated from chicks pair-fed Control, Mg deficient or Low Ca diets for 12 days.

Individual data points of means of duplicates are shown, horizontal bars indicate group means. There were no significant differences between 1αhydroxylase activity in tubules isolated from control and Mg deficient chicks. 1αhydroxylase activity was significantly higher in tubules isolated from low Ca fed chicks as compared to tubules isolated from either control or Mg deficient chicks.

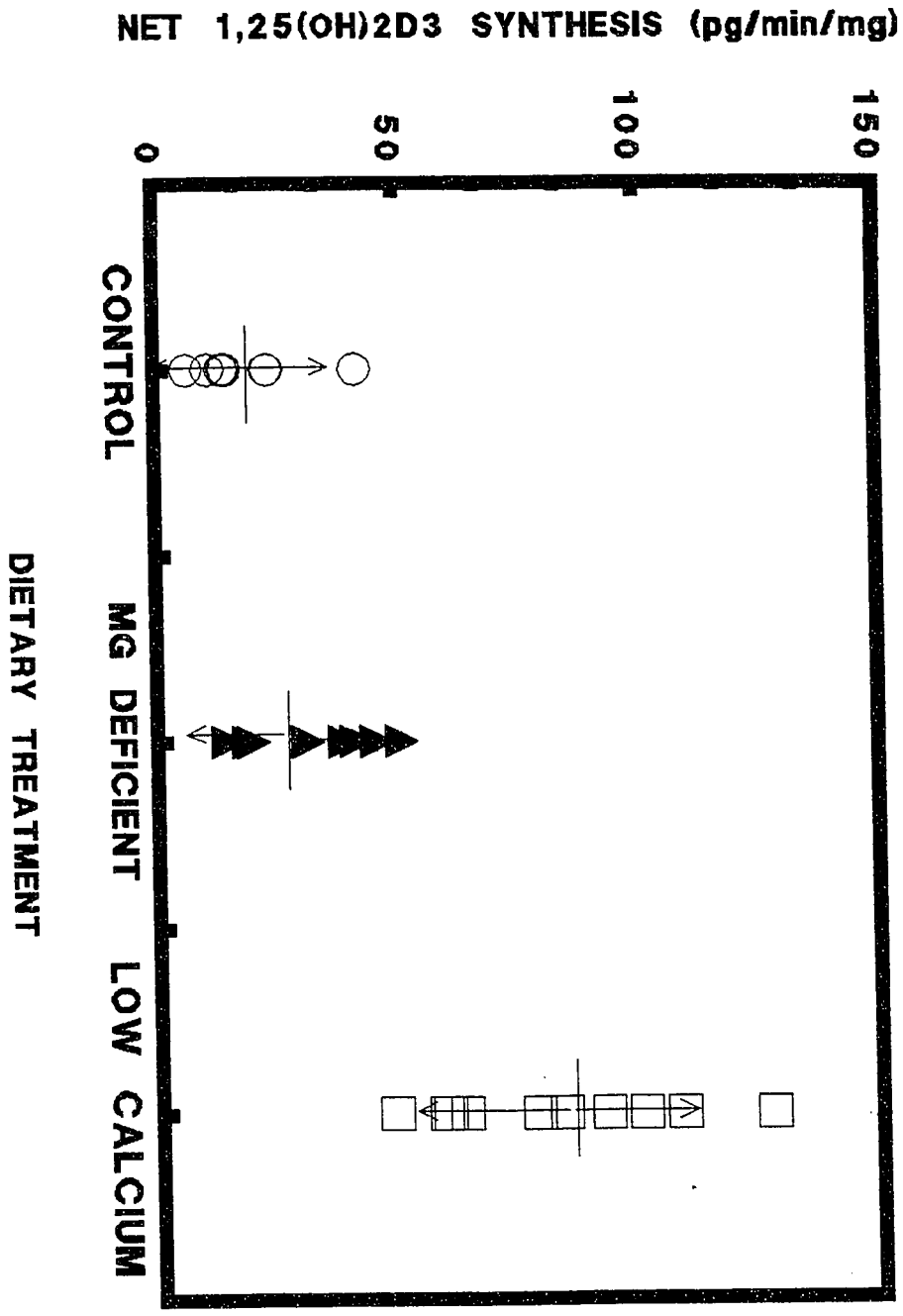

NET 1,25(OH)2D3 SYNTHESIS (pg/min/mg)
0
50
100
150
CONTROL
MG DEFICIENT
LOW CALCIUM
DIETARY TREATMENT

FIGURE 3.4.2.7 Correlation of 1αhydroxylase activity and plasma $1,25(OH)_2D_3$ in chicks pair-fed Control, Mg deficient or Low Ca diets for 4, 12 or 15 days.

Significant correlation (r = 0.77) between 1αhydroxylase activity and plasma $1,25(OH)_2D_3$ was observed for all dietary groups. DATA points are means of duplicate determinations.

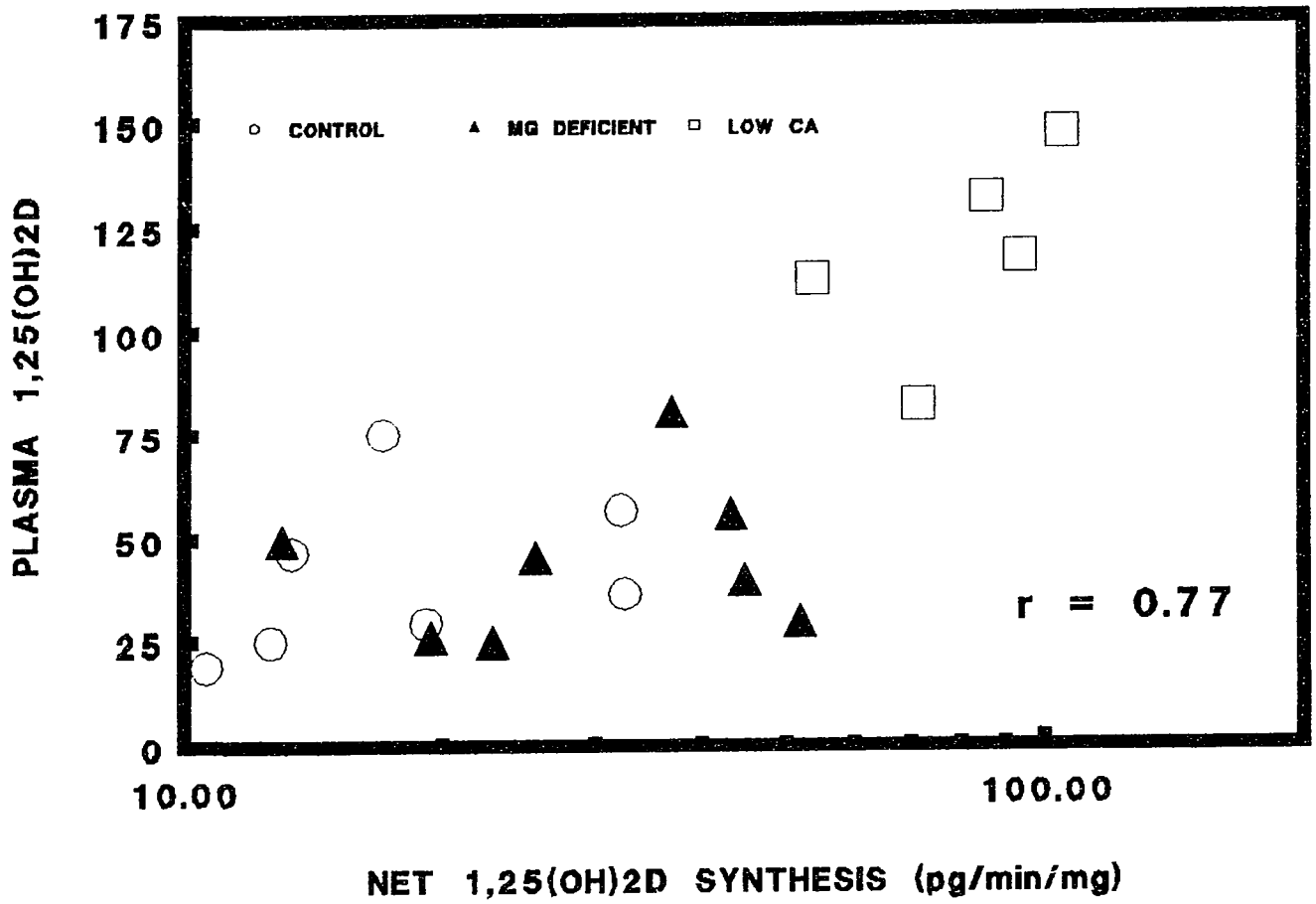

175
150
125
100
75
50
25
0
CONTROL
MG DEFICIENT
LOW CA
r = 0.77
10.00
100.00
PLASMA 1,25(OH)2D
NET 1,25(OH)2D SYNTHESIS (pg/min/mg)

FIGURE 3.4.2.8 Percentage basal PTH stimulation of 1αhydroxylase activity measured in renal tubules isolated from individual chicks fed Control or Mg deficient diets for 12 days.
Data is mean ± SEM for duplicate determinations of 1αhydroxylase activity in 3 individual chick tubule preparations per dietary group. Tubules were incubated with bovine n1-34 $1X10^{-7}$ M PTH in the presence of phosphodiesterase inhibitor for 15 minutes prior to addition of 25(OH)D and assay of 1αhydroxylase activity for 4-5 minutes as described in "Materials and Methods". No significant difference in PTH stimulation was observed between control and Mg deficient chicks.

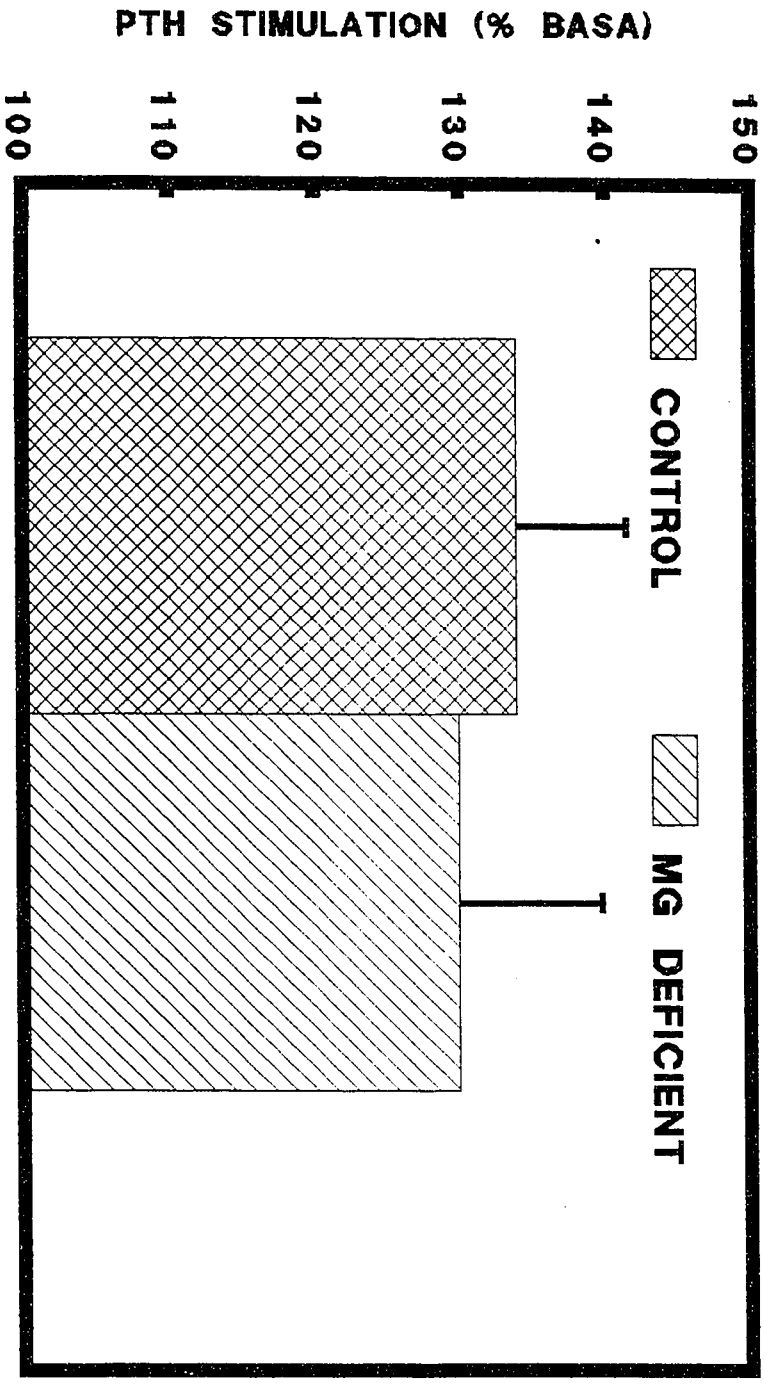

PTH STIMULATION (% BASA)
100
110
120
130
140
150
CONTROL
MG DEFICIENT

TABLE 3.4.2.2

Renal membrane adenylate cyclase activity in chicks pair-fed Control, Mg deficient or Low Ca diets for 15 days. Data are means ± SEM, n = 6-10.

	BASAL ACTIVITY[1]	PTH[2] INCREASE ABOVE BASAL	FORSKOLIN[3] INCREASE ABOVE BASAL
CONTROL	2.0 ± 0.3	2.8 ± 0.3	32.8 ± 3.3
MG DEFICIENT	1.5 ± 0.2	2.3 ± 0.2	37.6 ± 5.9
LOW CA	1.2 ± 0.2	2.1 ± 0.1	32.9 ± 4.1

[1]pmoles/min/mg membrane protein
[2]n1-34 bovine PTH 0.5 ug/ml (approximately $1X10^{-7}M$)
[3]10^{-4}

FIGURE 3.4.2.9 Dose response of PTH stimulation of renal membrane adenylate cyclase activity of chicks pair-fed Control, Mg deficient or Low Ca diets for 15 days.
Data are mean ± SEM, n = 4-10 chick preparations. No significant differences were observed in PTH stimulation between Mg deficient, low Ca and control chicks for the doses of PTH tested.

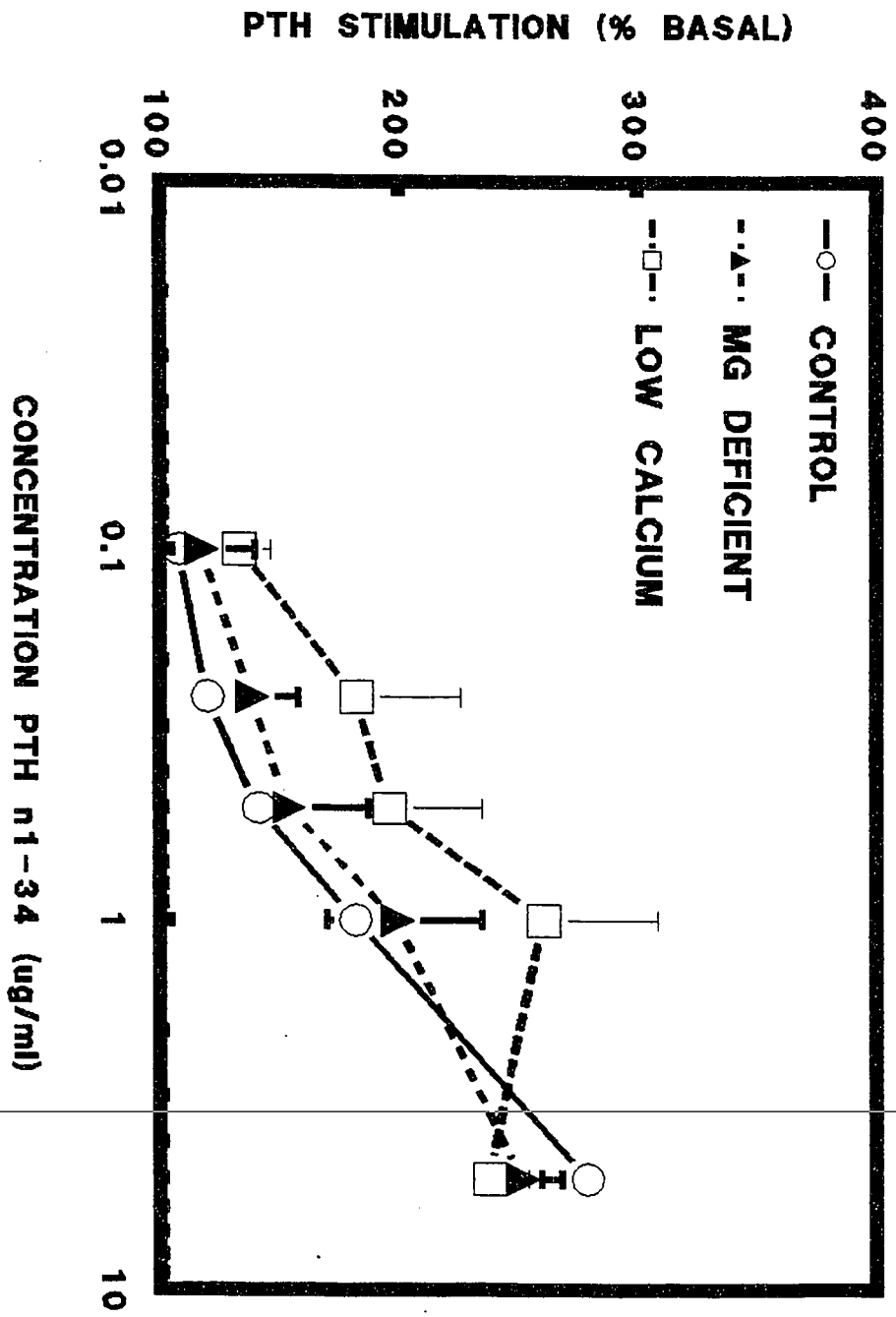

PTH STIMULATION (% BASAL)
100
200
300
400
CONTROL
MG DEFICIENT
LOW CALCIUM
0.01
0.1
1
10
CONCENTRATION PTH n1-34 (ug/ml)

TABLE 3.4.2.3

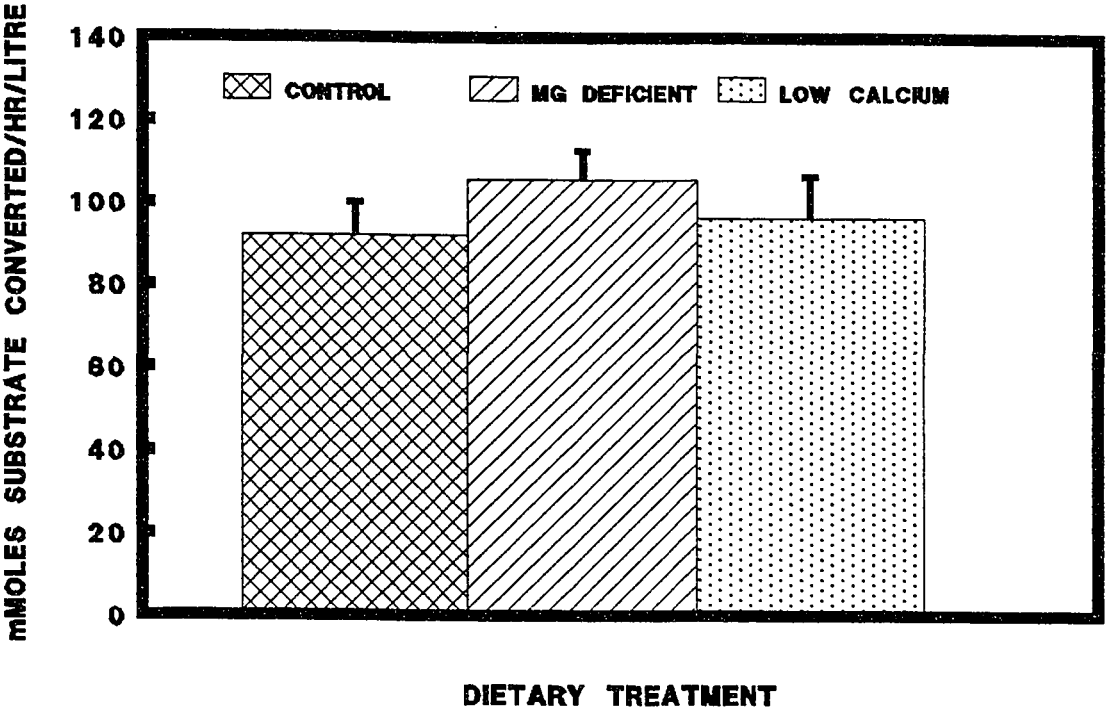
Bone mineral content in chicks pair-fed Control, Mg deficient or Low Ca diets for 15 days. Data are means ± SEM, n = 7-12.

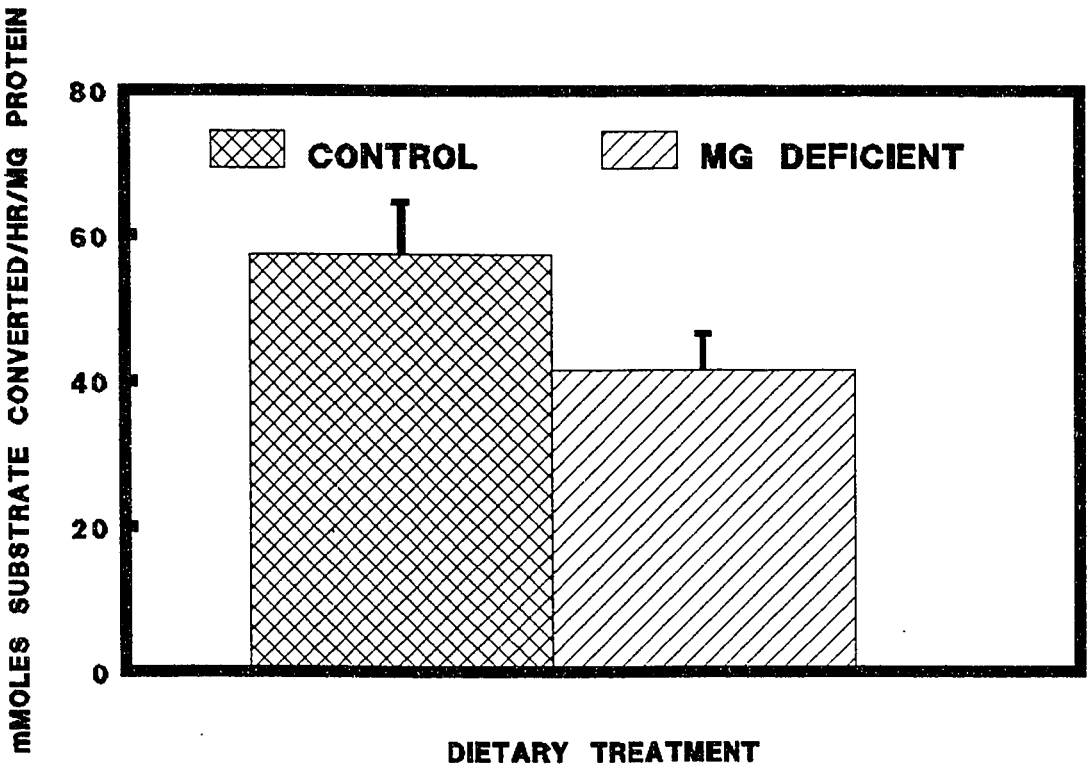
	Total Ash[1]	Ca[1]	P[1]	Mg[1]
Control	199.0 ± 3.5[a]	63.2 ± 0.7[a]	37.2 ± 0.6[a]	1.26 ± 0.02[a]
Low Ca	161.5 ± 9.5[b]	48.9 ± 1.3[b]	26.6 ± 0.9[b]	1.21 ± 0.04[a]
Mg Def	241.9 ± 6.7[c]	77.6 ± 2.3[c]	46.0 ± 1.5[c]	0.50 ± 0.02[b]

[1]mg/g dry weight of bone
[a,b,c]Means with different letters in their superscripts are significantly ($p < 0.05$) different by one way ANOVA, followed by Newman Keuls.

FIGURE 3.4.2.10 (top) Plasma alkaline phosphatase activity in chicks pair-fed Control, Mg deficient or Low Ca diets for 15 days.
Data are mean ± SEM, n = 6-10 chicks. No significant differences were observed in plasma alkaline phosphatase activity in control, Mg deficient or low Ca chicks.

FIGURE 3.4.2.11 (bottom) Bone epiphyseal alkaline phosphatase activity measured in tibiae isolated from chicks pair-fed Control or Mg deficient diets for 15 days.
Data are means ± SEM, n = 6-24 chicks. No significant differences were observed between alkaline phosphatase activity measured in bone epiphysis obtained from Mg deficient and control chicks.


mMOLES SUBSTRATE CONVERTED/HR/LITRE
140
120
100
80
60
40
20
0
CONTROL
MG DEFICIENT
LOW CALCIUM
DIETARY TREATMENT


mMOLES SUBSTRATE CONVERTED/HR/MG PROTEIN
80
60
40
20
0
CONTROL
MG DEFICIENT
DIETARY TREATMENT

3.4.3. DISCUSSION

The development of hypocalcaemia during dietary Mg deficiency was not associated with elevated circulating $1,25(OH)_2D_3$ or increased 1αhydroxylase activity. This is an inappropriate response as compared to the positive control group fed low dietary Ca, which exhibited a three fold increase in plasma $1,25(OH)_2D_3$, a two fold increase in 1αhydroxylase activity and maintained plasma Ca just below the normal range. These results support previous evidence of impaired vitamin D metabolism in Mg deficiency (Rude et al., 1985, Reddy and Sivakumer, 1976, Traba et al., 1985). For example, it has been reported that Mg deficient rats fed low dietary Ca developed hypocalcaemia, whereas control rats maintained normocalcaemia during low Ca stress (Welsh and Weaver, 1988). In this previous study PTH was comparably elevated in Mg deficient and control rats during low dietary Ca, but $1,25(OH)_2D_3$ was significantly lower in the Mg deficient rats. Rude et al. (1985) reported persistently low serum $1,25(OH)_2D_3$ in Mg deficient subjects responding to Mg therapy despite normalization of PTH levels. While the results described in this thesis demonstrate refractoriness of 1αhydroxylase activity to hypocalcaemia in Mg deficiency, neither synthesis or circulating $1,25(OH)_2D_3$ were decreased at any time examined. Thus $1,25(OH)_2D_3$ synthesis was not impaired by Mg deficiency, but rather was non-responsive to hypocalcaemia. Taken in unison with data from previous studies, these observations suggest that in vivo stimulation of 1αhydroxylase in response to hypocalcaemia is impaired by Mg deficiency.

There are several stages in which Mg deficiency could conceivably alter vitamin D metabolism. The data from the current studies rule out insufficient substrate ($25(OH)D_3$) concentration as a cause of insufficient $1,25(OH)_2D_3$ production in response to hypocalcaemia, since circulating $25(OH)D_3$ was higher in Mg deficient than in Ca depleted chicks. No significant elevation in circulating $24,25(OH)_2D_3$ was observed in Mg deficient chicks, as compared to control or low Ca birds, suggesting that $25(OH)D_3$ was not shunted through

this alternate pathway. However, direct measurement of renal 24 hydroxylase activity in the three dietary groups would be necessary before reaching a definitive conclusion. The correlation between 1αhydroxylase activity and plasma $1,25(OH)_2D_3$ suggests that metabolic clearance of $1,25(OH)_2D_3$ was not increased in Mg deficiency. Measurement of metabolic clearance, utilizing radiolabeled $1,25(OH)_2D_3$, would be necessary to directly examine this suggestion.

Mg is a cofactor for the 1αhydroxylase (Gray et al., 1972) thus, Mg deficiency may limit the enzyme's activity directly if cellular availability of Mg is altered, resulting in decreased basal activity. However, neither 1αhydroxylase activity or circulating $1,25(OH)_2D_3$ were decreased in these studies, even when plasma Mg was as low as 0.6 mg% (day 15). Carpenter et al. (1987) also observed no effect of media Mg concentration on $1,25(OH)_2D_3$ production by isolated rat mitochondria. $[Mg]_i$ has not been measured in proximal tubules or mitochondria of Mg deficicient animals, however, it is known that $[Mg]_i$ is generally not affected by changes in extracellular Mg even in severe Mg depletion (Gunther and Vormann, 1985). Mg is a cofactor for several enzymatic reactions involved in energy metabolism (Gunther, 1981). Thus, renal tubule viability and energy metabolism might be compromised by Mg deficiency, thereby indirectly altering synthesis of $1,25(OH)_2D_3$. Mg participates in the regulation of glycolysis through its actions on the regulatory enzyme, phosphofructokinase, whose activity is sensitive to intracellular $[Mg^{2+}]_i$ (Gunther, 1981). However, the metabolism of ^{14}C glucose to $^{14}CO_2$ was not impaired in renal tubules from Mg deficient compared to control chicks. Tubule viability, assessed by trypan blue exclusion, was also comparable between tubules isolated from Mg deficient and control chicks. Although cellular metabolism and viability did not appear to be severely compromised by acute Mg deficiency, further studies should examine more specific indices of mitochondrial integrity such as the activity of specific mitochondrial marker enzymes including succinate dehydrogenase and cytochrome oxidase. Taken in

unison, these observations suggest that $[Mg]_i$ is not limiting in acute Mg deficiency, and likely does not explain the unresponsiveness of the 1αhydroxylase to the prevailing hypocalcaemia.

One well documented effect of decreased extracellular Mg is increased cell Ca concentration (George and Heaton, 1975). Kidney calcification associated with Mg deficiency begins in the proximal tubular mitochondria (Sarkar, 1988), the site of 1αhydroxylase activity. It is possible that accumulation of intramitochondrial Ca, in the Mg deficient chick renal tubules, affected 1αhydroxylase responsiveness, as Ca has been shown to exert independent regulatory effects on $1,25(OH)_2D_3$ production (Carpenter et al., 1990). Since studies presented in this thesis suggest that PTH stimulation of 1αhydroxylase was intact in the Mg deficient chicks, it is possible that elevated $[Ca]_i$ or factors influenced by Ca which inhibit the 1αhydroxylase compromised 1αhydroxylase responsiveness. This might account for the refractoriness of the 1αhydroxylase to the hypocalcaemia of Mg deficiency. Elevations in $[Ca]_i$ have been associated with activation of PKC (Leach and Blumberg, 1989) which has recently been implicated in the inhibition of the 1αhydroxylase (Henry and Luntao, 1989) and stimulation of the 24 hydroxylase (Mandla, Boneh and Tenenhouse, 1990). Future experiments to examine cytosolic Ca and PKC activity in renal tubules from Mg deficient animals should address this question.

Although hyperphosphatemia inhibits 1αhydroxylation of $25(OH)D_3$ (Carpenter, 1990) there was no significant correlation between the degree of hyperphosphatemia and plasma $1,25(OH)_2D_3$ in the Mg deficient chicks. It remains possible that intracellular P concentration was altered by Mg deficiency, however further studies are necessary to test this possibility.

It has been suggested that Mg deficiency impairs PTG function (Chase and Slatopolsky, 1974). This might account for the inappropriately normal $1,25(OH)_2D_3$ production observed in these studies. Due to the lack of a sensitive and specific antibody to chick PTH, it was not possible to measure

immunoreactive PTH in Mg deficient chicks. Histological evidence of PTG hyperactivity in response to hypocalcaemia has been previously demonstrated in chicks fed the same Mg deficient diet used in the present study (Welsh et al., 1981). As well, normal PTG responsiveness to changes in extracellular Ca during Mg deficiency has been documented in several studies (Anast and Forte, 1983, Welsh, Schwartz and Krook, 1981), and PTH secretion in vitro is enhanced by moderate decreases in extracellular Mg (Habener and Potts, 1976, Takatsuki, Hanley and Sherwood, 1980). Thus, in the early stages of Mg deficiency, it is likely that PTH secretion is increased, as has been documented in the rat (Welsh and Weaver, 1988), whereas chronic, severe Mg depletion may lead to functional failure of the PTG (Breitenbach et al., 1973). Based on these previous observations, it was expected that 1αhydroxylase activity might increase initially, in Mg deficient chicks, followed by a gradual decline which correlated with the degree of hypomagnesaemia. The time course experiments presented in this thesis instead suggest a complete lack of responsiveness of the 1αhydroxylase to the prevailing hypocalcaemia. However, future studies will be necessary to accurately assess PTG activity and circulating PTH in acute Mg deficiency.

Target tissue resistance to PTH might account for the inappropriately normal renal 1αhydroxylase activity during Mg deficiency. PTH interacts with a membrane receptor (kidney, bone) linked via G proteins to adenylate cyclase. Many of PTH's actions have been associated with elevated intracellular cAMP (Slatopolsky et al., 1990). Since Mg is required for adenylate cyclase (Rude and Oldham, 1987), PTH stimulated cAMP generation might be impaired by Mg deficiency. However, PTH stimulation of renal membrane adenylate cyclase activity, in vitro, was not reduced in the hypocalcaemic Mg deficient chicks in the present studies, indicating intact PTH membrane receptor integrity and adenylate cyclase responsiveness. This is in agreement with studies conducted by Forbes and Parker (1980) who demonstrated comparable in vitro PTH stimulated cAMP synthesis in renal membranes isolated from control and Mg

deficient rats. In contrast, in vivo PTH stimulated (renal) cAMP generation was compromised in Mg deficient rats compared to controls (Forbes and Parker, 1980) suggesting that in vitro PTH studies conducted in isolated membrane fractions, in the presence of Mg, may not reflect the in vivo milieu of Mg deficiency. However, PTH stimulation of 1αhydroxylase activity in isolated renal tubules, described in this thesis, was also not impaired by Mg deficiency. These results suggest normal renal responsiveness to PTH despite Mg deficieny. In support of this observation, Mg deficient rats on low Ca diets responded with comparable urinary cAMP generation when compared to control rats (Forbes and Parker, 1980). In addition, Breitenbach et al. (1973) examined the efficacy of in vivo PTH infusions to elevate extracellular Ca and concluded that PTH resistance exists only in chronic Mg deficiency. Further in vivo PTH studies, examining indices of vitamin D metabolism, will be required to clarify PTH target tissue responsiveness in acute Mg deficiency.

Since $1,25(OH)_2D_3$ functions to stimulate intestinal Ca absorption and (with PTH) release of Ca from bone and renal Ca reabsorption, alterations in vitamin D metabolism may be pivotal in the development of hypomagnesaemic hypocalcaemia. This may occur through changes in circulating $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$ target tissue responsiveness. The inappropriately normal circulating $1,25(OH)_2D_3$ observed in these studies may have contributed to the genesis of the hypocalcaemia. Skeletal resistance to $1,25(OH)_2D_3$ was suggested by Carpenter et al. (1987, 1988) who reported reduced circulating osteocalcin in Mg deficient rats while Mira et al. (1982) demonstrated reduced serum alkaline phosphatase activity in Mg deficient rats. However in the studies presented in this thesis, plasma and epiphyseal alkaline phosphatase activity were not compromised in Mg deficient chicks suggesting skeletal $1,25(OH)_2D_3$ resistance was not present. Chou et al. (1979) demonstrated normal active intestinal Ca absorption in Mg deficient chicks and both Chou et al. (1979) and Rayssiguier et al. (1982) demonstrated similar expression of intestinal calbindin D 28k in intestinal mucosa from Mg deficient and control animals.

Since calbindin D 28k is induced by $1,25(OH)_2D_3$ in vivo (Christakos, et al., 1989) and $1,25(OH)_2D_3$ enhances active intestinal Ca transport (Haussler, 1986), the observation that $1,25(OH)_2D_3$ did not increase in Mg deficiency offers a physiological explanation for these observations. In addition these reports substantiate the argument that target tissue responsiveness to $1,25(OH)_2D_3$ is not compromised in Mg deficient chicks. However in the current study, bone Ca, P and total ash were increased in Mg deficient chicks, but decreased in low Ca chicks, compared to control animals. This data suggests retention of Ca in bone during Mg deficiency despite hypocalcaemia. Decreased bone resorption in acutely Mg deficient chicks has been well documented and is associated with the onset of hypocalcaemia (Welsh et al., 1981). An examination of other specific vitamin D target tissue proteins and functions would be necessary to clarify the existence of vitamin D target tissue resistance and its relevance to hypocalcaemia in Mg deficiency.

3.4.4. CONCLUSIONS

The progression of hypocalcaemia in Mg deficient chicks was not associated with increases in either plasma $1,25(OH)_2D_3$ or 1αhydroxylase activity. The lack of responsiveness of the 1αhydroxylase to hypocalcaemia in the Mg deficient chicks was not associated with inadequate $25(OH)D_3$ or increased circulating $24,25(OH)_2D_3$. Preliminary evidence suggested that renal adenylate cyclase and 1αhydroxylase responsiveness to PTH stimulation in vitro was intact in Mg deficient animals.

3.5 VITAMIN D METABOLISM AND PTH IN VIVO RESPONSIVENESS DURING MAGNESIUM DEFICIENCY IN CHICKS

3.5.1. EXPERIMENTAL PROTOCOL

Two week old white leghorn chicks were fed and housed as described under "Materials and Methods". They received deionized water ad libitum and were fed one of four soy-based diets as follows: Control (0.1% Mg; 1.5% Ca), Mg deficient (0.013% Mg; 1.5% Ca), LowCa (0.1% Mg; 0.2% Ca) or Mg deficient/ LowCa (0.013% Mg; 0.2% Ca). Two studies were performed: in Study I, four

groups of chicks (six to eight per group) were fed the diets for 12 days and then sacrificed. Food intake in the control, Low Ca and Mg deficient groups was matched to that consumed by the chicks fed the Mg deficient/Low Ca diet, which had the lowest intake. In Study II, two groups of chicks (12 per group) were fed control or Mg deficient diets for 11 days, at which time six Mg deficient and six control chicks were killed. The remaining chicks were fed diets with initial Mg intakes but of lower dietary Ca (0.2% Ca) for seven days before they were sacrificed. Food intake of the chicks on the control diets was matched to that of the Mg deficient chicks for the duration of the study. For both studies, blood was collected into heparinized syringes by cardiac puncture under halothane anaesthesia; plasma was stored at -70°C for subsequent mineral and vitamin D metabolite analysis. Left tibiae were removed and analyzed for dry weight and mineral content.

Due to differences in experimental design, the duration of Low Ca challenge was 12 days in Study I and only seven days in Study II. Chicks sacrificed after Mg deficiency in Study II were comparable to those from Study I, but chicks sacrificed after Low Ca challenge in Study II were older and the controls had been pair-fed longer than control animals in Study I. Due to these differences in duration of treatments, age, and growth rate of animals, it was inappropriate to compare results of the two studies directly. Therefore, statistical analyses for each study were done independently.

3.5.2. RESULTS

STUDY I

Growth rates of the four groups were not significantly different due to the pair-feeding regime (Table 3.5.2.1). Significant hypomagnesaemia was observed in both groups of chicks fed Mg deficient diets regardless of the Ca content (Table 3.5.2.1). However, chicks receiving the Mg deficient/low Ca diet had plasma Mg levels which were significantly higher than those of the Mg deficient chicks fed adequate Ca. In addition, plasma Mg increased in chicks fed low Ca with adequate Mg compared to chicks fed adequate levels of both

Ca and Mg. Mg deficient chicks fed adequate Ca had significantly lower plasma Ca (7.8 mg%) than chicks fed either control (9.6 mg%), low Ca (9.1 mg%), or Mg deficient/low Ca (9.4 mg%) diets. Thus, hypocalcaemia did not develop in either of the groups fed low dietary Ca. Circulating $1,25(OH)_2D_3$ was not raised in the hypocalcaemic Mg deficient chicks compared to controls, but was significantly raised in both groups that consumed diets low in Ca (ie, Mg deficient/low Ca and control/low Ca) (Table 3.5.2.1). The magnitude of the increase in plasma $1,25(OH)_2D_3$ was higher (4.5-fold increase) in the low Ca animals fed adequate Mg than in the Mg deficient/low Ca chicks (three fold increase). Plasma P was significantly higher in the Mg deficient chicks fed adequate Ca than in control chicks. In both groups fed low dietary Ca, plasma P was significantly lower than in either of the groups fed adequate dietary Ca, and the lowest levels were observed in Mg deficient/low Ca chicks (Table 3.5.2.1).

Bone Mg levels (mg/g dry wt) were significantly lower in Mg deficient and Mg deficient/low Ca chicks as compared to control and low Ca chicks, although Mg deficient/low Ca chicks showed significantly higher bone Mg than Mg deficient chicks (Table 3.5.2.2). Mg deficient chicks had increased bone Ca (mg/g dry wt) as compared to controls, although the difference was not significant. Bone Ca was not significantly different in low Ca and Mg deficient/low Ca chicks, yet both groups fed low Ca had significantly lower bone Ca than their respective controls. While bone P was similar in control and Mg deficient chicks and significantly reduced in low Ca and Mg deficient/low Ca chicks, the lowest levels were observed in the Mg deficient/low Ca chicks (Table 3.5.2.2). Similar results were obtained for mineral ash density (data not shown).

STUDY II

As observed in Study I, body weights of the Mg deficient chicks were comparable to those of the control animals due to the pair-feeding regime (Table 3.5.2.3). Significant hypomagnesaemia and hypocalcaemia developed in

Mg deficient chicks, as compared to controls, within 11 days (Table 3.5.2.3). Although the absolute values for plasma Ca differed somewhat between studies, the % change between control and Mg deficient birds was comparable (82% in Study I vs 80% in Study II). Plasma $1,25(OH)_2D_3$ was not significantly different in the control and Mg deficient animals, again similar to the data obtained from Study I (Table 3.5.2.3). In contrast to Study I, plasma P was not significantly altered by Mg deficiency in Study II (Table 3.5.2.3).

After exposure to low dietary Ca for seven days, body weight was significantly lower in the Mg deficient/low Ca animals than the low Ca controls despite continuation of the pair-feeding regime (Table 3.5.2.3). Plasma Mg remained significantly lower in Mg deficient animals than in low Ca fed chicks, but was increased relative to the Mg deficient animals fed adequate dietary Ca sacrificed on day 11. Low Ca challenge caused a significant increase in plasma Ca in the Mg deficient chicks, whereas in control chicks plasma Ca decreased significantly after seven days of low dietary Ca (Table 3.5.2.3). Thus there was no difference in plasma Ca between control and Mg deficient chicks after exposure to low dietary Ca. Plasma P was not different between Mg deficient and control animals after exposure to low dietary Ca, but was significantly lower than that of the corresponding diet group during adequate dietary Ca (Table 3.5.2.3). A significant increase in circulating $1,25(OH)_2D_3$ occurred in both control (220% increase) and Mg deficient animals (160% increase) during low Ca stress. The increase in $1,25(OH)_2D_3$ in Study II was less than that observed in Study I, possibly due to the shorter duration of low Ca stress and the larger size of the chicks at the time of low Ca challenge in Study II (Table 3.5.2.3).

In both control and Mg deficient chicks, exposure to low dietary Ca elicited a comparable decrease in bone Ca and P concentrations (mg/g dry bone and mg/g ash), relative to data from chicks killed on day 11 (3.5.2.4). There was no effect of low dietary Ca on bone Mg concentration; thus bone Mg remained lower in Mg deficient chicks than in control chicks (3.5.2.4). Similar results were obtained for mineral ash concentration (data not shown).

TABLE 3.5.2.1

Body weights, plasma minerals and plasma $1,25(OH)_2D_3$ in chicks pair-fed two levels of Ca and Mg for 12 days. Data are mean ± SEM, n = 6-8.

	Control	MgDef	Low Ca	MgDef/ Low Ca
Body wt (g)	171 ± 12	179 ± 16	185 ± 8	202 ± 4
Plasma:				
Mg (mg%)	2.3 ± 0.1[a]	0.7 ± 0.1[b]	2.8 ± 0.1[c]	1.5 ± 0.7[d]
Ca (mg%)	9.6 ± 0.2[a]	7.8 ± 0.8[b]	9.1 ± 0.1[a]	9.4 ± 0.3[a]
P (mg%)	5.8 ± 0.3[a]	7.1 ± 0.3[b]	4.6 ± 0.2[c]	3.8 ± 0.1[d]
1,25DHCC[1] (pg/ml)	29 ± 2[a]	37 ± 4[a]	134 ± 7[b]	111 ± 18[b]

[a,b,c,d]Means with different letters in their superscripts are significantly ($P<0.05$) different by one way ANOVA, followed by Newman Keuls.
[1]$1,25(OH)_2D_3$

TABLE 3.5.2.2

Weight, ash and mineral content of tibiae from chicks pair-fed two levels of Ca and Mg for 12 days. Data are mean ± SEM, n = 6-8.

	Control	MgDef	Low Ca	MgDef/low Ca
Dry wt(g)	0.79 ± 0.06	0.95 ± 0.09	0.79 ± 0.05	0.76 ± 0.03
Ash wt(g)	0.28 ± 0.02^{a}	0.35 ± 0.04^{b}	0.20 ± 0.01^{c}	0.24 ± 0.01^{a,c}
Mg[1]	2.78 ± 0.05^{a}	1.04 ± 0.05^{b}	2.72 ± 0.08^{a}	1.42 ± 0.04^{c}
Ca[1]	138.5 ± 10.9^{a,b}	162.3 ± 12.0^{a}	88.6 ± 3.9^{c}	121.0 ± 10.6^{b,c}
P[1]	68.7 ± 1.2^{a}	65.0 ± 4.4^{a}	51.9 ± 1.0^{b}	37.7 ± 1.8^{c}

[1]Expressed as mg/g dry weight of bone.
^{a,b,c}Means with different letters in their superscripts are significantly ($P<0.05$) different by one way ANOVA, followed by Newman Keuls.

TABLE 3.5.2.3

Body Weights, plasma minerals, and plasma $1,25(OH)_2D_3$ in control and Mg deficient chicks before and after exposure to low dietary Ca. Data are means ± SEM, n = 6.

	Adequate Dietary Ca		Low Dietary Ca	
	Control	MgDef	Control/Low Ca	MgDef/Low Ca
Body wt (g)	196 ± 5[a]	189 ± 10[a]	272 ± 11[b]	242 ± 8[c]
Plasma:				
Mg (mg %)	1.90 ± 0.09[a]	0.65 ± 0.08[b]	1.98 ± 0.09[a]	1.07 ± 0.10[c]
Ca (mg%)	12.8 ± 0.3[a]	10.2 ± 0.7[b]	11.7 ± 0.3[c]	11.4 ± 0.3[c]
P (mg%)	5.92 ± 0.20[a]	6.00 ± 0.31[a]	4.21 ± 0.31[b]	4.02 ± 0.16[b]
$1,25(OH)_2D_3$ (pg/ml)	32.4 ± 3.0[a]	49.6 ± 5.4[a]	71.1 ± 5.3[b]	76.9 ± 8.9[b]

[a,b,c]Means with different letters in their superscript are significantly ($P<0.05$) different by one way ANOVA, followed by Newman Keuls.

TABLE 3.5.2.4

Bone weight, ash and mineral content of tibiae from control and Mg deficient chicks before and after exposure to low dietary Ca. Data are mean ± SEM, n = 6.

	Adequate Dietary Ca		Low Dietary Ca	
	Control	MgDef	Control/Low Ca	MgDef/Low Ca
Dry wt (g)	0.90 ± 0.04[a]	0.96 ± 0.04[a]	1.18 ± 0.05[b]	1.18 ± 0.03[b]
Ash wt (g)	0.35 ± 0.01	0.38 ± 0.01	0.38 ± 0.01	0.40 ± 0.01
Mg[1]	2.40 ± 0.05[a]	0.92 ± 0.05[b]	2.35 ± 0.05[a]	1.05 ± 0.04[b]
Ca[1]	133.7 ± 3.9[a]	145.1 ± 3.3[a]	109.6 ± 5.3[b]	120.0 ± 3.2[b]
P[1]	80.7 ± 0.7[a]	81.4 ± 0.6[a]	70.6 ± 0.7[b]	72.6 ± 2.1[b]

[1]Expressed as mg/g dry weight of bone.
[a,b]Means with different letters in their superscripts are significantly ($P<0.05$) different by one way ANOVA, followed by Newman Keuls.

3.5.3. DISCUSSION

These studies demonstrate that Mg deficient chicks responded to low dietary Ca with increased circulating $1,25(OH)_2D_3$, decreased bone Ca content and maintenance of plasma Ca levels. Since Mg deficient chicks responded regardless of whether low Ca challenge was given subsequent to or concomittent with Mg depletion, Mg deprivation did not induce irreversible tissue changes that prevented restoration of normocalcaemia. The association of changes in plasma $1,25(OH)_2D_3$ with maintainance of normocalcaemia during Mg deficiency indicates a pivotal role for altered vitamin D metabolism in the genesis of hypomagnesaemic hypocalcaemia.

Interpretation of these results must be made in the light of the degree of Mg deficiency induced in each group. The data from study I suggested that simultaneous induction of Mg and Ca deficiencies resulted in a less severe Mg deficiency, as indicated by significantly higher plasma and bone Mg in Mg deficient/low Ca chicks than in Mg deficient chicks (Table 3.5.2.1). Thus, although the Mg deficient/low Ca chicks responded with an increase in plasma $1,25(OH)_2D_3$ and maintained normocalcaemia, the lesser degree of hypomagnesaemia induced in these animals precluded a clear assessment of the effect of hypomagnesaemia on Ca homeostasis in response to low dietary Ca. Therefore in Study II, chicks were fed the Mg deficient diet to induce hypomagnesaemia (34% of control) before exposure to low dietary Ca. Again in this study, low dietary Ca induced increases in plasma $1,25(OH)_2D_3$ (Table 3.5.2.3) in Mg deficient and control animals, and both groups had comparable plasma Ca after seven days of low Ca challenge. To study further the mechanism of this response, it would be necessary to characterize the temporal changes in plasma Ca, Mg, and $1,25(OH)_2D_3$ in association with the induction of low Ca challenge.

Low dietary Ca leads to PTG stimulation and elevated PTH. Elevated PTH stimulates synthesis of $1,25(OH)_2D_3$ and increases bone resorption. Thus, the increase in circulating $1,25(OH)_2D_3$, decrease in bone Ca content and

maintenance of normocalcaemia observed in the Mg deficient animals exposed to low Ca stress described in this thesis implied that PTH synthesis, secretion and actions were intact despite plasma Mg levels as low as 0.65 mg% (Study II) (Table 3.5.2.3). Although interpretation of these results should ideally be made in light of the prevailing PTH concentrations in all of the animals; the lack of a specific and sensitive antibody for chick PTH precluded these measurements. In previous studies, serum PTH was not reduced in Mg deficient rats fed adequate Ca, and increased comparably in control and Mg deficient rats during acute exposure to low dietary Ca. These studies indicated that PTH secretion was maintained in rats despite serum Mg as low as 0.6 mg% compared to controls (2.0 mg%) (Welsh and Weaver, 1988). Similarly, no reduction in circulating PTH was reported in hypocalcaemic calves (Rayssiguier et al., 1977) or dogs (Levi et al., 1974) with primary Mg deficiency, or in human Mg deficiency (Fuss et al., 1985, Rude et al., 1985, Yamamoto et al., 1985). These reports suggest that impaired PTH secretion which has been reported in other cases of human Mg deficiency likely resulted from secondary alterations, complicating factors (especially in human studies) or the duration of hypomagnesaemia. The ability of Mg deficient chicks to respond to low Ca challenge, in the present studies, is consistent with the hypothesis that PTG responsiveness is intact in the early stages of acute Mg deficiency. However, confirmation of this hypothesis should be made by direct measurement of circulating PTH when available.

Previous studies have suggested that end organ resistance to PTH and/or $1,25(OH)_2D_3$ exists during Mg deficiency, as indicated by reduced bone resorption and inappropriately normal or low circulating $1,25(OH)_2D_3$ and renal 1αhydroxylase activity despite hypocalcaemia (Takatsuki et al., 1980, Rosler and Rabinowitz, 1973, Forbes and Parker, 1980, Welsh and Weaver, 1988). In Mg deficient chicks fed adequate dietary Ca, increased bone Ca content (DeLuca, 1984, Tables 3.4.2.3 and 3.5.2.2) and decreased bone turnover result in retention of Ca in bone (Welsh and Weaver, 1988) which has been correlated

to the development of hypocalcaemia (Welsh et al., 1981). In Study I retention of Ca in bone, characteristically observed in Mg deficient animals fed adequate dietary Ca, was prevented by lowering dietary Ca. Although this observation suggests that bone responsiveness to PTH and $1,25(OH)_2D_3$ was intact during hypomagnesaemia, the lesser degree of bone and plasma Mg depletion in the Mg deficient/low Ca chicks (as compared to the Mg deficient chicks fed adequate Ca) may have facilitated this response. However, in Study II, where plasma Mg was 0.6 mg% at the time of low Ca challenge, similar increases in plasma $1,25(OH)_2D_3$ in response to low dietary Ca were associated with comparable reductions in bone Ca concentration in Mg deficient (17.3% decrease) and control (18.1% decrease) animals. Thus, raised plasma $1,25(OH)_2D_3$ (and presumably PTH) induced by low dietary Ca allowed appropriate bone resorption to occur despite hypomagnesaemia and bone Mg depletion. The data from both Study I (simultaneous Ca and Mg deprivation) and Study II (Ca deprivation subsequent to Mg deprivation) demonstrated that renal responsiveness to PTH was intact in Mg deprived chicks, since increased circulating $1,25(OH)_2D_3$ during low dietary Ca is mediated by PTH stimulation of renal 1αhydroxylase. These observations are consistent with the earlier studies, presented in this thesis, describing intact in vitro PTH responsiveness of renal membrane adenylate cyclase and renal tubule 1αhydroxylase in Mg deficient and control chicks (Refer to section 3.4.2). These observations are supported by the work of Forbes and Parker (1980) who demonstrated renal refractoriness, by decreased urinary cAMP to PTE infusions in Mg deficient animals consuming adequate dietary Ca compared to controls, which was resolved when animals consumed diets low in Ca. While the second low Ca study verified intact end organ responsiveness to PTH and $1,25(OH)_2D_3$ in Mg deficiency, more importantly it denied the presence of irreversible tissue changes due to Mg deficiency that would prevent normocalcaemia from ensuing.

These studies suggest that $1,25(OH)_2D_3$ synthesis in response to low

dietary Ca remained intact despite Mg deprivation. These data are consistent with previous observations that 1αhydroxylase activity is not impaired in hypomagnesaemic chicks (refer to section 3.4.2 Figures 3.4.2.4 and 3.4.2.6) and studies reporting that media Mg concentration had no effect on $1,25(OH)_2D_3$ production by isolated rat mitochondria (Carpenter et al., 1987). Gunther (1986) reported that $[Mg]_i$ dependent functions appear to be refractory to profound changes in extracellular Mg, thus it is possible that $[Mg]_i$ levels do not change drastically despite significant decreases in extracellular Mg. Assuming that $[Mg^{2+}]_i$ per se does not limit 1αhydroxylase activity, the major question remains as to why plasma $1,25(OH)_2D_3$ does not increase in hypocalcaemic Mg deficient chicks fed adequate Ca, but does increase when Mg deficient chicks are exposed to low dietary Ca. The experiments presented in this section of this thesis support the hypothesis that altered cellular Ca metabolism may play a role in the development of hypocalcaemia observed in Mg deficiency. Mg deficiency increases total and free $[Ca]_i$ (George and Heaton, 1975). Kidney calcification occurs within three days of Mg deprivation, with preferential mitochondrial Ca phosphate deposition in the cells lining the proximal convoluted tubule (Sarkar, 1988), the site of the 1αhydroxylase. Thus increased $[Ca]_i$ could directly or indirectly impair the responsiveness of the 1αhydroxylase to the extracellular hypocalcaemia. Since animals fed low dietary Ca diet are in a state of negative Ca balance, it is possible that the accumulation of $[Ca]_i$ normally associated with Mg deficiency, was reduced by low dietary Ca thereby permitting 1αhydroxylase responsiveness to hypocalcaemia.

Dietary P deprivation and hypophosphataemia stimulate $1,25(OH)_2D_3$ production independently of PTH. Previous time course studies (presented in section 3.4.2) indicated that the increase in 1αhydroxylase activity in Ca deprived chicks followed the development of hypophosphataemia. In the experiments described in this section, Mg deficient animals fed low dietary Ca, which exhibited increases in circulating $1,25(OH)_2D_3$, were significantly

hypophosphataemic relative to Mg deficient animals fed adequate dietary Ca. The studies described in this thesis indicate that plasma P in Mg deficient chicks is either normal or elevated; hypophosphataemia is never present (refer to sections 3.4.2, 3.5.2). Thus, reductions in plasma P may be permissive for the stimulation of 1αhydroxylase activity in response to hypocalcaemia. Future studies to delinate the effects of Mg deficiency, with respect to $[Ca]_i$ and P, on vitamin D metabolism in kidney, will be necessary to examine these possibilities.

These low Ca challenge studies indicated that Ca intake modulated the degree of Mg deficiency. For example, in both studies, reduction of dietary Ca resulted in an increase in both plasma and bone Mg. Since the Mg deficient chicks on low dietary Ca were pair-fed, and thus did not consume more Mg, these observations suggest that low dietary Ca increases Mg retention, either by increasing intestinal Mg absorption or by reducing renal Mg excretion. Mg deficient rats exposed to a low Ca diet exhibit a similar phenomenon (Welsh and Weaver, 1988). Such an effect could be due to a direct interaction between Ca and Mg at either the kidney or the intestine, or could be secondary to increased circulating $1,25(OH)_2D_3$. Further studies would be necessary to clarify the mechanism.

The association of changes in plasma $1,25(OH)_2D_3$ with maintainance of normocalcaemia during Mg deficiency suggests a pivotal role for altered vitamin D metabolism in the genesis of hypomagnesaemic hypocalcaemia. To clarify the role of vitamin D, the effects of $1,25(OH)_2D_3$ supplementation on the development of hypocalcaemia in Mg deficient chicks were next examined.

3.5.4. CONCLUSIONS

These studies illustrate hypomagnesaemia per se does not inhibit adaptation to low dietary Ca. Future studies are required to clarify the roles of PTH, $[Ca]_i$ and P on the regulation of 1αhydroxylase activity during Mg deficiency. The association of normocalcaemia with elevated circulating $1,25(OH)_2D_3$ during Mg deficiency indicates a pivotal role for $1,25(OH)_2D_3$ in

3.6. VITAMIN D AND THE PATHOPHYSIOLOGY OF THE HYPOCALCAEMIA OF MAGNESIUM DEFICIENCY

3.6.1. EXPERIMENTAL PROTOCOL

Two week old white Leghorn chicks were fed and housed as described under "Materials and Methods". They received deionized water ad libitum and were fed one of two soya-based diets as follows: Control (0.1% Mg; 1.5% Ca) or Mg deficient (0.013% Mg; 1.5% Ca).

On day zero (one day before dietary regime commenced) chicks were implanted (under halothane anaesthesia) with Alzet osmotic mini pumps (Model 2001) containing either $1,25(OH)_2D_3$ (dissolved in ethanol, propylene glycol:ethanol, 95:5) or vehicle (propylene glycol:ethanol, 95:5). The day after surgery, half of each group were randomly assigned to control or Mg deficient diets. Days 12 and 14 were chosen as end points due to the consistent development of hypocalcaemia by day 11 in Mg deficient chicks in previous studies. Although two sets of experiments were conducted, the results from both studies were comparable, therefore the data were pooled. In addition, as the data were similar for each time point, only results from day 14 are presented and discussed. Food intake was matched to the Mg deficient non supplemented group, which consistently had the lowest intake. At sacrifice, blood was collected and plasma was stored for mineral and vitamin D metabolite analysis. The duodenal loop was excised for calbindin D-28 analysis. Left tibiae were removed for mineral analysis while right tibiae were removed for histological analysis.

3.6.2. RESULTS

DETERMINATION OF OPTIMAL INFUSION RATE OF $1,25(OH)_2D_3$

The positive relationship between $1,25(OH)_2D_3$ infusion rate and circulating $1,25(OH)_2D_3$ in normal chicks implanted with osmotic mini pumps for 14 days is shown in Figure 3.6.2.1. An infusion rate of 7ng/hour was chosen for the experiments described below on $1,25(OH)_2D_3$ supplementation in Mg deficiency, since this rate resulted in an 80% increase in circulating

$1,25(OH)_2D_3$ above non-supplemented levels, an increase comparable to that induced by low dietary Ca in control and Mg deficient chicks.

GROWTH

14 days of Mg deficiency did not significantly affect growth or final body weights due to the pair feeding regime. $1,25(OH)_2D_3$ supplementation did not significantly affect growth of chicks on either control or Mg deficient diets; thus, final body weights did not differ among the four treatment groups (Figure 3.6.2.2).

PLASMA MINERALS AND $1,25(OH)_2D_3$

Chicks fed the Mg deficient diet for 14 days had significantly lower plasma Mg (33% of control) and Ca (80% of control) than chicks fed the control diet. There was no significant increase in plasma $1,25(OH)_2D_3$ in the Mg deficient chicks despite the reduction in plasma Ca. Mg deficiency did not affect plasma P (Table 3.6.2.1). These results are comparable to those observed previously (refer to section 2.1: Figures 2.1.1, 2.1.2, 2.1.3 and 2.1.4) and indicate that the osmotic pumps containing vehicle did not affect the response to Mg deficiency.

$1,25(OH)_2D_3$ supplementation significantly increased circulating $1,25(OH)_2D_3$ in both control and Mg deficient chicks, with no effect of Mg deficiency on the level of circulating $1,25(OH)_2D_3$ achieved (Table 3.6.2.1). $1,25(OH)_2D_3$ supplementation significantly increased plasma Ca in Mg deficient, but not control chicks (compare Mg deficient, vehicle treated with Mg deficient, $1,25(OH)_2D_3$ supplemented). There was no difference in plasma Ca in Mg deficient birds supplemented with $1,25(OH)_2D_3$ and either control group (vehicle or $1,25(OH)_2D_3$ supplemented). Thus, of all four groups, only the Mg deficient, vehicle treated chicks exhibited significantly lower plasma Ca when analyzed by one way ANOVA. In contrast, $1,25(OH)_2D_3$ supplementation did not significantly alter plasma Mg or P in either control or Mg deficient birds (compare vehicle treated to supplemented chicks in Table 3.6.2.1).

INTESTINAL CALBINDIN D-28k

INTESTINAL CALBINDIN D-28k

There was no difference in the amount of calbindin D-28k detected by immunoblotting of intestinal samples from vehicle infused control and Mg deficient chicks (Figure 3.6.2.3). In both groups of chicks supplemented with $1,25(OH)_2D_3$, a visible increase in the amount of immunoreactive calbindin D-28K was observed, with no detectable difference between control and Mg deficient animals.

BONE COMPOSITION AND MORPHOLOGY

Chicks fed Mg deficient diets for 14 days had heavier bones, significantly lower bone Mg and significantly higher bone Ca compared to chicks receiving the control diet. Supplementation with $1,25(OH)_2D_3$ increased total bone Ca content in both control and Mg deficient chicks, but did not influence bone Mg in either dietary group (Table 3.6.2.2). Histological examination of mid diaphysis cross sections suggested an increase in the amount of unmineralized osteoid present in Mg deficient hypocalcaemic non supplemented chicks compared to non supplemented controls. Excess unmineralized osteoid was not present in bone sections from Mg deficient $1,25(OH)_2D_3$ supplemented chicks in comparison to supplemented controls. Bone sections from $1,25(OH)_2D_3$ supplemented controls appeared denser when compared to bone sections from non supplemented controls (Figure 3.6.2.4).

TABLE 3.6.2.1

Plasma minerals and circulating $1,25(OH)_2D_3$ in control and Mg deficient chicks 14 days after implantation with osmotic mini-pumps containing 1,25DHCC or vehicle. Data are mean ± SEM, n = 8-10 chicks per group.

	Vehicle		1,25DHCC Supplementation	
	Control	MgDef	Control	MgDef
Mg[1]	1.4 ± 0.1^b	0.4 ± 0.1^a	1.3 ± 0.1^b	0.5 ± 0.1^a
Ca[1]	11.3 ± 0.2^b	8.2 ± 0.5^a	11.3 ± 0.4^b	10.1 ± 0.8^b
P[1]	6.4 ± 0.3	6.8 ± 0.3	6.3 ± 0.3	6.2 ± 0.4
$1,25(OH)_2D_3$[2]	22.0 ± 3.0^a	39.3 ± 3.0^a	78.6 ± 12^b	78.7 ± 11^b

[1]mg/dl
[2]pg/ml
[a,b,c]Means with different letters in their superscripts are statistically significant ($P<0.05$) by one way ANOVA, followed by Newman Keuls.

TABLE 3.6.2.2

Bone mineral composition in control and Mg deficient chicks 14 days after implantation with mini osmotic pumps containing $1,25(OH)_2D_3$ or vehicle. Data are mean ± SEM, n = 10 for bone weight and somatic index, n = 6 for Mg and Ca content.

	Vehicle		$1,25(OH)_2D_3$ Supplementation	
	Control	MgDef	Control	MgDef
Dry wt Bone (g)	1.3 ± 0.1^a	1.4 ± 0.1^a	1.1 ± 0.1^c	1.2 ± 0.1^a
Bone Somatic Index[1]	0.46 ± 0.01^b	0.54 ± 0.01^a	0.44 ± 0.01^b	0.50 ± 0.01^a
Mg[2]	2.3 ± 0.03^b	0.75 ± 0.02^a	2.2 ± 0.03^b	0.85 ± 0.03^a
Ca[2]	125 ± 11^b	145 ± 5^a	148 ± 17^a	168 ± 5^c

[1]Mg dry weight bone/100 g body wt
[2]Expressed as mg mineral/g bone
[a,b,c]Means with different letters in their superscripts are significantly ($P<0.05$) different by one way ANOVA, followed by Newman Keuls.

FIGURE 3.6.2.1 Determination of an optimal $1,25(OH)_2D_3$ infusion rate by 14 days implantation with osmotic mini pumps.
Data are mean, n = 2-3 animals per dosage. Data is expressed as percentage vehicle implanted controls. Chicks were implanted, under anaesthesia, with Alzet osmotic mini pumps containing vehicle (propylene glycol:ethanol, 95:5) or $1,25(OH)_2D_3$ (propylene glycol:$1,25(OH)_2D_3$ dissolved in ethanol, 95:5). Chicks were sacrificed after 14 days and circulating $1,25(OH)_2D_3$, Ca and body weights were assessed. A linear elevation in circulating $1,25(OH)_2D_3$ was observed at infusion rates greater than 1.3 ng/hr. An infusion rate of 7 ng/hr increased circulating $1,25(OH)_2D_3$ to 180% of vehicle implanted controls, did not induce hypercalcaemia or decrease body weight in the implanted chicks and was chosen for subsequent experimentation.

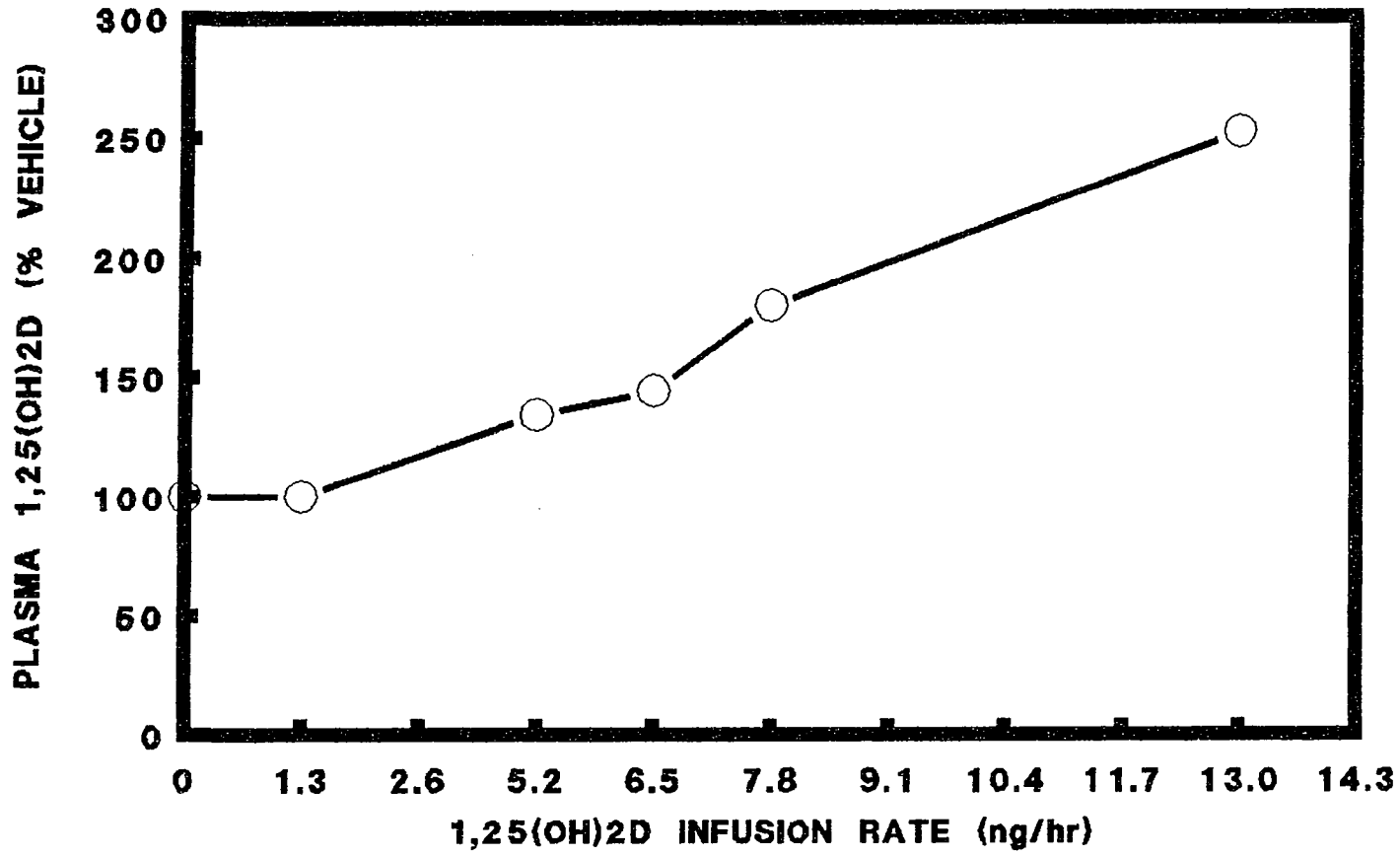

PLASMA 1,25(OH)2D (% VEHICLE)
300
250
200
150
100
50
0
0
1.3
2.6
5.2
6.5
7.8
9.1
10.4
11.7
13.0
14.3
1,25(OH)2D INFUSION RATE (ng/hr)

FIGURE 3.6.2.2 Body weights of chicks pair fed Control or Mg deficient diets for up to 16 days with and without $1,25(OH)_2D_3$ supplementation. Data are mean ± SEM, n = 6 chicks per group. No significant differences in body weights were observed between the four experimental groups at any time examined.

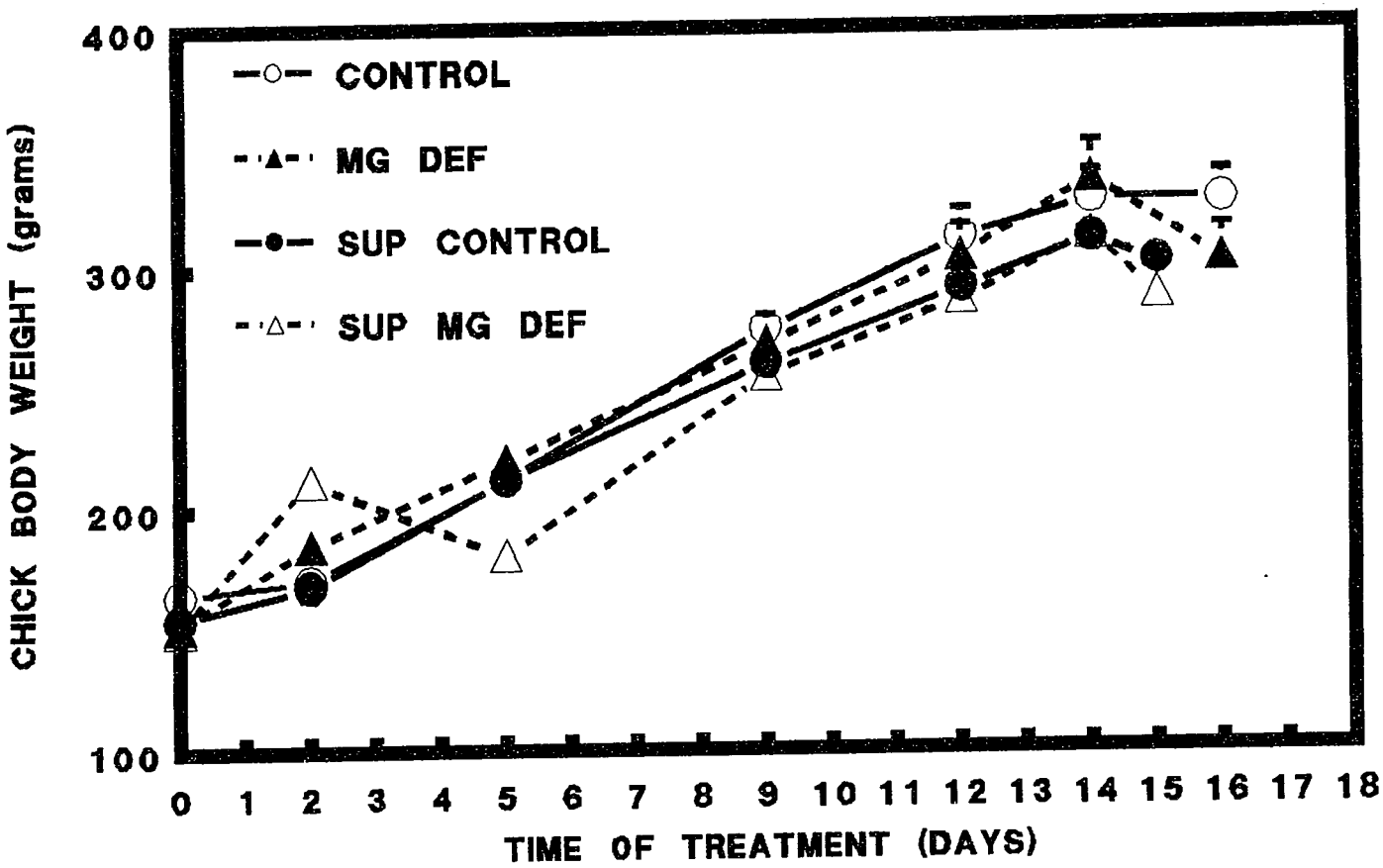
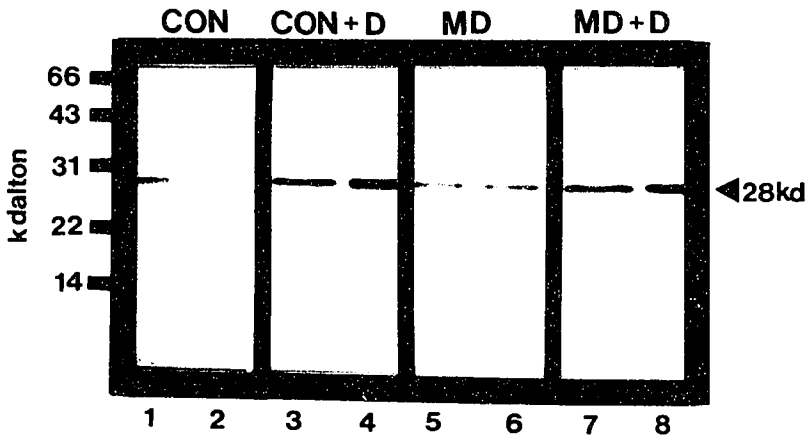

400
300
200
100
CHICK BODY WEIGHT (grams)
CONTROL
MG DEF
SUP CONTROL
SUP MG DEF
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
TIME OF TREATMENT (DAYS)

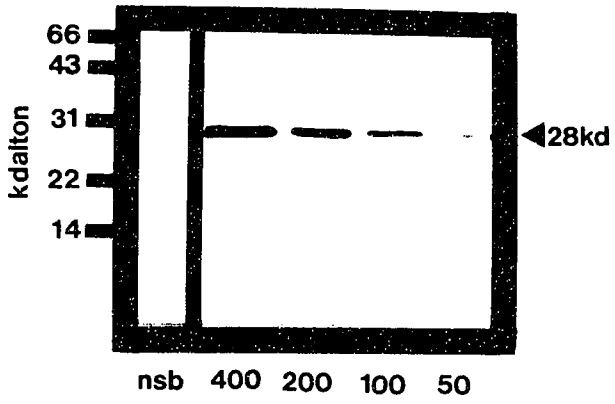
FIGURE 3.6.2.3 Calbindin D 28 immunoblot of intestinal mucosa from Control and Mg deficient chicks supplemented with or without $1,25(OH)_2D_3$ for up to 16 days.

Illustrated are representative intestinal calbindin D 28k immunoblots from 2 Mg deficient and 2 control chicks with or without $1,25(OH)_2D_3$ supplementation for up to 16 days. A dose response of increasing concentrations of heat stable cytosolic intestinal mucosal protein from a control chick is also illustrated. No discernable differences were observed in calbindin D 28k detected by immunoblot in intestinal mucosa from Mg deficient and control chicks irrespective of $1,25(OH)_2D_3$ status. Supplementation with $1,25(OH)_2D_3$ elevated immunologically detectable intestinal mucosal calbindin D 28k comparably in Mg deficient and control chicks.

TOP,
INTESTINAL CALBINDIN D28k IN CONTROL (CON) AND MG DEFICENT (MD) CHICKS WITH (+) AND WITHOUT 1,25(OH)2D3 SUPPLEMENTATION

BOTTOM,
PROTEIN DOSE RESPONSE

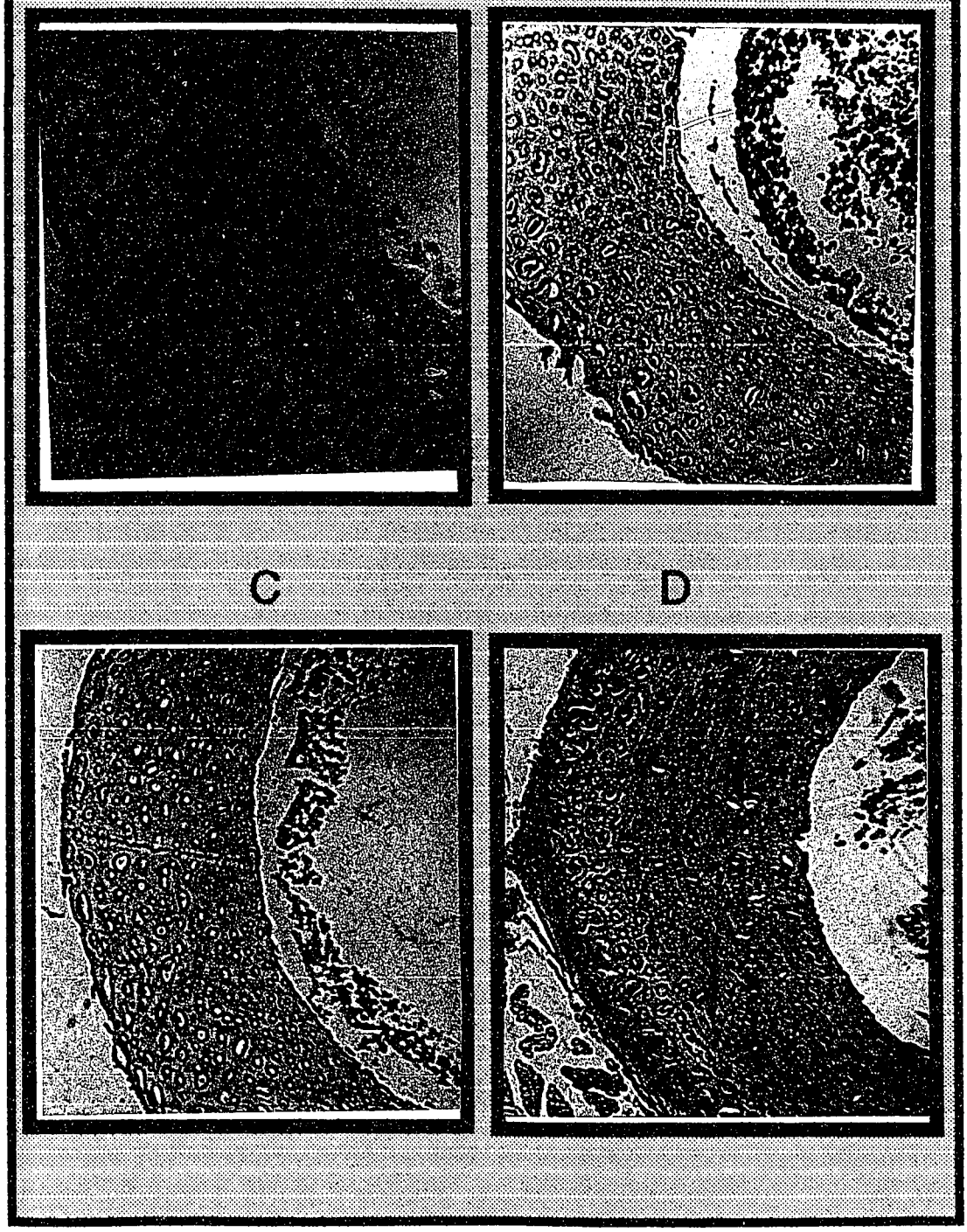




PROTEIN ng/lane

FIGURE 3.6.2.4 Histological examination of mid-diaphysis tibiae cross sections from chicks fed Control or Mg deficient diets for up to 16 days with and without $1,25(OH)_2D_3$ supplementation.
Illustrated is one representative mid diaphysis tibiae cross section from chicks fed control or Mg deficient diets for up to 16 days with and without $1,25(OH)_2D_3$ supplementation. Increased unmineralized osteoid is indicated in non supplemented Mg deficient hypocalcaemic chicks by increased osteoid diameters present in newly formed bone (A panel) in comparison to non supplemented control chicks (B panel). Bone cross sections from Mg deficient chicks supplemented with $1,25(OH)_2D$ do not suggest the presence of increased unmineralized osteoid when compared to sections from non supplemented Mg deficient chicks and sections obtained from non supplemented or supplemented controls (C panel compared to A panel, B panel and D panel). Cross sections of bone prepared from $1,25(OH)_2D_3$ supplemented controls appear denser than cross sections of bone prepared from non supplemented controls (compare D panel with B panel).

BONE HISTOLOGY



3.6.3. DISCUSSION

The first series of studies in this thesis demonstrated that renal 1αhydroxylase activity and circulating $1,25(OH)_2D_3$ did not increase during the development of Mg deficiency, despite the onset and progression of hypocalcaemia. It was postulated that the failure of the 1αhydroxylase to respond to the hypocalcaemia might have perpetuated and exacerbated the hypocalcaemia. The second series of studies demonstrated that exposure to low dietary Ca concomittant with or subsequent to Mg deprivation increased circulating $1,25(OH)_2D_3$ and prevented hypocalcaemia. In those studies, however, it was not clear whether increased plasma Ca was related to increased circulating $1,25(OH)_2D_3$ or other changes induced by low dietary Ca. In these $1,25(OH)_2D_3$ supplementation studies, elevated $1,25(OH)_2D_3$ increased plasma Ca in the Mg deficient chicks without increasing either plasma or bone Mg. Thus, it is clear that elevation of $1,25(OH)_2D_3$ alone was sufficient to maintain normocalcaemia during the induction of Mg deficiency. Since $1,25(OH)_2D_3$ production is stimulated by PTH in vivo, the observation that exogenous administration of $1,25(OH)_2D_3$ prevents hypocalcaemia in Mg deficient chicks is not incompatible with, and does not exclude, a role for PTH.

Taken in unison, these studies suggest a pivotal role for altered vitamin D metabolism in the genesis of hypomagnaesemic hypocalcaemia.

The maintenance of normocalcaemia in the $1,25(OH)_2D_3$ supplemented Mg deficient group may have been due to a general increase in intestinal absorption and/or renal reabsorption of Ca. Although the exact function of calbindin D-28K is not understood, the level of this vitamin D dependent protein is correlated with circulating $1,25(OH)_2D_3$ and active Ca absorption (DeLuca, 1984, Christakos et al., 1989). The increase in calbindin D-28 in $1,25(OH)_2D_3$ supplemented chicks suggests that intestinal Ca absorption may have been increased in response to increased plasma $1,25(OH)_2D_3$. An increase in net Ca absorption could explain the increase in plasma Ca observed in Mg deficient supplemented chicks, while an increase in Ca balance and increased

bone mineralization implied by increased Ca absorption could explain the increase in bone Ca in both control and Mg deficient supplemented groups.

A net increase in bone Ca may be interpreted as either a decrease in resorption, enhancement of formation (with adequate mineralization) or an integration of both processes (Nijweide, Burger and Feyen, 1986). Insufficient extracellular Ca precludes normal mineralization of newly formed bone matrix, therefore once hypocalcaemia develops in Mg deficiency, newly formed bone is poorly mineralized (detected morphologically as unmineralized osteoid). $1,25(OH)_2D_3$ supplementation of Mg deficient chicks was associated with an increase in total bone Ca, compared to Mg deficient, non supplemented chicks. Since Mg deficient $1,25(OH)_2D_3$ supplemented chicks were not hypocalcaemic, it is possible that the mineralization defect classically observed in bone from hypocalcaemic Mg deficient chicks was alleviated by $1,25(OH)_2D_3$ supplementation. Histological examination of tibiae cross sections from each animal group suggested increased osteoid mineralization (Figure 3.6.2.4) in Mg deficient $1,25(OH)_2D_3$ supplemented birds, as compared to the Mg deficient non supplemented chicks. However a definitive assessment of these results awaits methodical histological examination of osteoclast number and quantitation of unmineralized osteoid by image analysis with statistical inferences.

Bone resorption is also impaired by Mg deficiency (Welsh et al. 1981). The defect has been attributed to changes in bone mineral composition as well as alterations in PTH and $1,25(OH)_2D_3$ metabolism (Shils, 1988). $1,25(OH)_2D_3$ supplementation may have altered bone resorption in Mg deficient chicks. Conventionally a decrease in Ca concentration (mg Ca/g ash) may be interpreted as increased net bone resorption, as illustrated by reduced Ca density in low Ca fed control chicks (Stern, 1990). Mg deficiency initially induces reductions in bone resorption and once hypocalcaemia develops; decreased osteoid mineralization, thereby obscuring interpretation of bone resorption by examination of mineralization indices. Therefore, no clear assessment of this issue in these studies can be made without quantitation of

indices of bone formation and resorption. In view of the documented effect of $1,25(OH)_2D_3$ on PTH synthesis (Russell, Lettieri and Sherwood, 1986), $1,25(OH)_2D_3$ supplementation should have resulted in depressed circulating PTH. Considering the critical role for PTH in bone resorption; $1,25(OH)_2D_3$ supplementation would be associated with depressed bone resorption. Indeed increased bone Ca levels were observed in the control $1,25(OH)_2D_3$ supplemented chicks. Future studies will be necessary to characterize skeletal changes in $1,25(OH)_2D_3$ supplemented Mg deficient animals.

It has been suggested that target tissue resistance to $1,25(OH)_2D_3$ occurs in Mg deficiency (Carpenter et al., 1987, ibid, 1988). However, in the present study, intestinal calbindin D-28k was not different between non supplemented control and Mg deficient chicks, reflecting similar circulating $1,25(OH)_2D_3$ and confirming a previous observation (Chou et al., 1979). More importantly, calbindin D-28 was increased comparably in intestine of Mg deficient and control chicks during $1,25(OH)_2D_3$ supplementation. In conjunction with the normalization of plasma Ca upon $1,25(OH)_2D_3$ supplementation, it seems likely that target tissue responsiveness to $1,25(OH)_2D_3$ remains intact in acute Mg deficiency. However, additional target tissue effects of $1,25(OH)_2D_3$ should be examined to substantiate these conclusions.

Exogenous administration of $1,25(OH)_2D_3$ did not affect plasma or bone Mg in Mg deficient chicks. Earlier studies described in this thesis (section 3.5.2) suggested that $1,25(OH)_2D_3$ may enhance overall Mg retention since increases in plasma and bone Mg were observed in those low Ca fed animals with elevated $1,25(OH)_2D_3$. The $1,25(OH)_2D_3$ supplementation studies described in this section suggest that increased $1,25(OH)_2D_3$ status alone does not enhance overall Mg retention. More direct indices of Mg absorption and reabsorption and the influence of $1,25(OH)_2D_3$ would be required to test this suggestion further.

3.6.4. CONCLUSIONS

Supplementation with $1,25(OH)_2D_3$ during induction of Mg deficiency was associated with normocalcaemia. Thus alterations in vitamin D metabolism appear to play a pivotal role in the pathogenesis of hypomagnesaemic hypocalcaemia. Intestinal target tissue responsiveness to $1,25(OH)_2D_3$, indicated by comparable elevations in calbindin D-28 in supplemented Mg deficient and control chicks, compared to non supplemented animals, appeared intact during Mg deficiency. Therefore it is probable that acute Mg deficiency is not associated with target tissue resistance to vitamin D. Finally, $1,25(OH)_2D_3$ does not appear to have a major role in the regulation of Mg homeostasis.

CHAPTER 4 - VITAMIN D METABOLISM AND INSULIN DEPENDENT DIABETES

4.1. RATIONALE AND SPECIFIC OBJECTIVES

Insulin dependent diabetes has consistently been associated with low circulating $1,25(OH)_2D_3$ which may be due to defective synthesis or increased catabolism of $1,25(OH)_2D_3$. Decreased renal net synthesis of $1,25(OH)_2D_3$ during diabetes may be due to direct or indirect effects of insulin. Direct consequences of insulin deficiency could include alterations in renal metabolic integrity, decreased steady state levels of one or more components of the 1αhydroxylase enzyme or altered transduction of signals which modulate $1,25(OH)_2D_3$ synthesis. The signal transduction pathways known to be involved in renal vitamin D hydroxylation include both adenylate cyclase and PI systems. Indirect effects of insulin deficiency could include alterations in circulating PTH, $25(OH)D_3$ concentration, Ca or Mg homeostasis, growth factor secretion or actions or tissue specific changes such as renal hypertrophy.

A systematic examination of synthesis and catabolism of $1,25(OH)_2D_3$ during diabetes is required to ascertain the aetiology of aberrant vitamin D metabolism in insulin dependent diabetes and conversely, the role of insulin in regulation of renal vitamin D hydroxylation. Previous studies have utilized dietary and/or surgical manipulations to elevate 1αhydroxylase activity, however while these strategies afford ease of measurement they obscure and confuse the vitamin D metabolic profile. Thus it would be desirable to conduct studies on vitamin D metabolism in diabetes without prior dietary or surgical alterations to clarify the situation.

The specific objectives of the studies on vitamin D metabolism in insulin dependent diabetes were:

1. To set up a mammalian assay system which would reflect in vivo $1,25(OH)_2D_3$ production and in which signal transduction pathways involved in regulation of 1αhydroxylase could be studied.

2. To systematically study the effects of insulin deficiency on vitamin D metabolism.
3. To assess the effects of insulin deficiency on indices of signal transduction and the relevance of these changes to vitamin D metabolism.
4. To determine the aetiology of altered vitamin D metabolism in insulin deficiency by studying the temporal association of insulin deficiency and aberrant vitamin D metabolism.

4.2. ANIMAL MODEL

The animal model for the insulin dependent diabetic studies was chosen based on the following criteria:

1. Demonstration and characterization of insulin dependent diabetes, reversible by insulin therapy, in the animal model.
2. Demonstration of perturbations in vitamin D and Ca metabolism in the diabetic animal model.
3. Control of the precise time of diabetes onset and degree of insulin deficiency in the animal model.

Fulfillment of these criteria presupposed the existence of a literature base which would provide a necessary reference for experimental work. They would minimize the metabolic derangements observed in severe and chronic diabetes and facilitate the conduction of temporal insulin deficiency studies.

4. Ability to measure 1αhydroxylase activity in vitro in kidney preparations from the animal.
5. Availability of assays for determination of metabolites such as insulin and PTH in the animal model.
6. Sufficient size of experimental animal to obtain serum and tissue samples large enough for individual measurements.

Fulfillment of these criteria would facilitate the measurement and analysis of Ca homeostasis and vitamin D metabolism in the animal model and allow for the analysis of metabolic and physiological correlations.

7. The last criteria was for sexually immature growing animals. Fulfillment of this criteria would avoid complication of the experimental results with effects from sex steroids, facilitate mineral deficiency studies and avoid age related changes in insulin and vitamin D metabolism.

Based on these criteria, STZ induced diabetes in young male Sprague Dawley rats was chosen as the experimental model for the following reasons:

1. The time and severity of diabetes onset could be controlled by age, mode and dose of STZ injection.
2. A considerable body of experimental data already existed on vitamin D metabolism, Ca homeostasis and insulin dependent diabetes in this animal model.
3. Many of these alterations are reversed by strict insulin therapy.
4. The size, age and immaturity of the animal facilitated the measurement of indices of diabetes, vitamin D metabolism and Ca homeostasis in individual animals without complicating factors.

It is recognized that the drug STZ may be generally toxic; necrotic alterations and metabolic changes have been described in several tissues in addition to the pancreatic islet beta cells (Bestetti et al., 1987). These effects, which may be related to the dose and mode of drug injection, are, in many cases, temporary (Like and Rossini, 1976). However, to avoid possible misinterpretations of the experimental data, studies were conducted to select a STZ dosage and injection protocol which would minimize these effects. The dosage of STZ chosen significantly reduced, but did not eliminate, circulating insulin yet induced significant hyperglycaemia quickly. This dose was without apparent toxicity, as determined by monitoring urinary and blood glucose, blood insulin, urinary ketones and protein and body weight.

To verify the STZ induced diabetic model, several initial studies were conducted in spontaneously BB diabetic rats (Marliss et al., 1982). It was anticipated that data obtained from this model could be compared to data from the STZ model to demonstrate the universality of changes in vitamin D

metabolism associated with insulin deficiency. BB rats were not chosen for the principle experimental model since it is not possible to control the time of diabetes onset or its severity in the BB rat. Further, these animals do not exhibit diabetes until the 4th or 5th month of age, when they are older and sexually mature with associated consequent complications. At the time of study BB rats were derived from a colony maintained at Health and Welfare Canada, thus it was not possible to control their dietary intakes. Finally, the BB rat model has a high prevalence of hypertension, with its associated alterations in Ca and vitamin D homeostasis (Marliss et al., 1982).

4.3 EXPERIMENTAL PROTOCOLS

4.3.1. VITAMIN D METABOLISM IN INSULIN DEPENDENT DIABETES; EFFECTS OF IN VIVO AND IN VITRO INSULIN

In the first set of experiments vitamin D metabolism and mineral homeostasis were assessed in two models of insulin dependent diabetes (STZ induced diabetes and BB spontaneous diabetes) with and without insulin therapy, and compared to their respective controls. In addition the effect of in vitro insulin therapy on net $1,25(OH)_2D_3$ synthesis in renal proximal tubules from control and STZ diabetic rats was assessed.

Indices of diabetes included decreased growth rates, elevated urinary and plasma glucose and reduced circulating insulin compared to age matched controls. Effective diabetic control was assumed when body weights comparable to control animals were achieved, negligible urinary glucose was detected three days in a row, and circulating glucose levels less than 250mg/dl were obtained. Indices of mineral homeostasis were circulating Ca, Mg, P and PTH levels. Indices of vitamin D metabolism included circulating $25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ and net $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ synthesis, measured in purified renal proximal tubules. Renal tubule viability was judged comparable when trypan blue exclusion criteria were met and comparable glucose metabolism from ^{14}C glucose was obtained. Kidney parameters were renal somatic index (mg kidney/g body weight), total protein and DNA

concentration per mg tissue protein. Animals exhibiting compromised renal function (ie. proteinuria) or compromised metabolic function (urinary ketoacidosis and/or weight loss) were excluded from the experiments.

4.3.2. VITAMIN D METABOLISM, SIGNAL TRANSDUCTION AND INSULIN DEPENDENT DIABETES

In the second set of experiments, the role of the adenylate cyclase and PI signal transduction pathways in regulation of renal vitamin D hydroxylation in normal and diabetic animals was addressed.

Signal transduction experiments were conducted in freshly isolated proximal or mixed renal tubules obtained from rats fed semi purified control or low Ca diets. Where indicated, primary cultures of renal tubules were used. Synthesis of cAMP was assessed in response to PTH and forskolin. Stimulation of in vitro $1,25(OH)_2D_3$ production by PTH (adenylate cyclase signal transduction mediated stimulation of $1,25(OH)_2D_3$ production) was expressed as absolute 1αhydroxylase activity or % basal activity as indicated.

PKC activity was assessed in membrane and cytosolic preparations isolated from purified proximal or mixed renal tubules in response to phorbol ester stimulation and expressed as absolute activity or % basal activity. Acute stimulation of PKC activity was achieved with co-incubation of renal tubules with phorbol esters for up to 60 minutes. Chronic stimulation of PKC activity was achieved with co-incubation or renal tubules with phorbol esters for up to 18 hours. Specificity of PKC mediated effects was demonstrated after PKC downregulation (chronic PKC stimulation) and in parallel incubations with the PKC inhibitor staurosporine. The effect of dose and incubation time of phorbol esters on in vitro $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ production were examined (PKC stimulation).

The effect of diabetes on cAMP synthesis was assessed by comparing PTH and forskolin stimulation of cAMP generation induced in diabetic versus control tubules. The effect of diabetes on PTH stimulation of net $1,25(OH)_2D_3$ synthesis was assessed in proximal renal tubules purified from diabetic and

control rats.

The effect of diabetes on the adenylate cyclase signal transduction system in vivo was assessed by feeding STZ diabetic rats a low Ca diet and monitoring parameters known to be influenced by PTH. The response of diabetic rats to low dietary Ca was compared to the response of diabetic rats to low dietary P in which changes in vitamin D metabolism are independent of the PTH adenylate cyclase system. The effect of diabetes on renal tubule PKC activity was assessed in acutely STZ diabetic rats and compared to vehicle treated controls.

4.3.3. TEMPORAL CHANGES IN VITAMIN D METABOLISM ASSOCIATED WITH INSULIN DEFICIENCY

In the third set of experiments, temporal changes in renal biochemistry, Ca homeostasis and vitamin D metabolism were assessed following diabetes induction and insulin withdrawal.

4.4. VITAMIN D METABOLISM IN INSULIN DEPENDENT DIABETES; EFFECTS OF IN VIVO AND IN VITRO INSULIN

4.4.1. EXPERIMENTAL PROTOCOL

Steptozotocin induced diabetes

Male weanling Sprague Dawley rats were fed and housed as described under "Materials and Methods". They received AIN semi-purified normal rat diet and tap water ad libitum. To induce diabetes, rats weighing 120-130g were injected intraperitoneally with 85mg/kg STZ in citrate buffer (pH 4.5); control rats received citrate buffer injection. Animals which developed diabetes within 48 hours were used for study. Nine acute seven to ten day studies with six to twelve animals per group were conducted. In two studies, diabetic animals were treated every morning with NPH insulin (Lilly); the amount given was adjusted as necessary to produce early morning trace glucosuria (<2.8 mmol). In two studies; rats were fasted for 12 hours before sacrifice for measurement of circulating Ca, Mg and P. At sacrifice, blood was collected from the neck into heparinized tubes for assays of Ca, Mg, P, glucose and vitamin D metabolites.

In three experiments, serum was collected instead of plasma for measurement of PTH and insulin. In one experiment kidneys were removed, weighed and analyzed for protein and DNA content. Basal net synthesis of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ were assessed in renal proximal tubules from individual control, untreated diabetic and insulin treated diabetic animals. Tissue yield limitation necessitated the measurement of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ synthesis in preparations from different animals. The effect of in vitro insulin on $1,25(OH)_2D_3$ synthesis was studied in four experiments by pre-incubating tubules in K1 media supplemented with 5 ug/ml insulin (gift of Eli Lilly, Indianapolis, IN) for up to 18 hours, after which $25(OH)D_3$ was added and $1,25(OH)_2D_3$ production was measured after one hour. In some cases, smaller yields from control kidneys necessitated pooling of proximal tubules. Assays conducted in pooled tubules were analyzed as an n of one.

Spontaneous Diabetes

Spontaneously diabetic male BB Wistar rats were fed and housed as described under "Materials and Methods". Animals were obtained in groups of five-six one day after detection of diabetes by glucosuria. The mean age of the animals on receipt varied from 45-60 days. Control animals were age-matched male Wistar rats bred from the same Wistar colony from which the diabetic rats were originally bred in 1967. All animals received free access to Purina lab chow and tap water. Three acute studies with six-eight animals per group were conducted in which the animals were sacrificed within seven days of receipt. In one experiment, diabetic rats were treated with NPH insulin. In another experiment, animals were fasted for 12 hours prior to sacrifice for measurement of circulating Ca, Mg and P. At sacrifice, blood was collected from the neck into heparinized tubes for assays of Ca, Mg, P, glucose and vitamin D metabolites. In two experiments, serum was collected instead for PTH and insulin assay. In one experiment kidneys were removed, weighed and analyzed for protein and DNA. In another experiment, proximal tubules were isolated from untreated diabetic and control rat kidneys for measurement of

$1,25(OH)_2D_3$ production.

4.4.2. RESULTS

STZ Induced Diabetic Model

Effect of insulin deficiency

Untreated STZ diabetic animals did not lose weight over the experimental period, but grew at a slower rate than did control rats. The final body weights, therefore, of untreated diabetic rats were significantly lower than that of control animals (Table 4.4.2.1). As expected, serum insulin was significantly reduced and serum glucose was significantly elevated in the diabetic untreated animals.

Serum $1,25(OH)_2D_3$ was significantly reduced in the untreated diabetic rats compared to controls, and net $1,25(OH)_2D_3$ production in proximal tubules isolated from these diabetic animals was significantly lower than that in tubules isolated from control animals (Table 4.4.2.4). Serum $24,25(OH)_2D_3$ and net $24,25(OH)_2D_3$ production were also significantly lower in STZ untreated rats than in control animals (Table 4.4.2.5). Serum $25(OH)D_3$ and PTH were not altered by insulin deficiency (Table 4.4.2.5, Figure 4.4.2.3). Plasma Ca and P were not affected by diabetes, however plasma Mg levels were significantly lower in STZ diabetic rats than in controls (Table 4.4.2.3).

Despite the reduced body weight in the diabetic versus control animals both the wet weight and somatic index (mg kidney/g body weight) of the kidney were significantly higher in diabetic untreated rats than in control rats. Total renal protein was significantly higher in diabetic rats than in control rats. The increase in protein in the diabetic kidney was not associated with an increase in total DNA, resulting in a significantly lower DNA:protein ratio in diabetic untreated rats than in controls (Table 4.4.2.9).

Effect of in vivo insulin therapy

The efficacy of insulin therapy in normalizing glucose homeostasis of STZ injected rats is indicated by the lack of significant differences between final body weights and serum glucose of control and diabetic treated rats

(Table 4.4.2.6). There was no significant difference in serum $1,25(OH)_2D_3$ or net $1,25(OH)_2D_3$ production between control and insulin treated STZ diabetic rats (Table 4.4.2.7). Serum $24,25(OH)_2D_3$ and net $24,25(OH)_2D_3$ production remained lower in treated STZ diabetic rats as compared to controls, however the difference was no longer significant (Table 4.4.2.7).

Correlation between circulating $1,25(OH)_2D_3$ and in vitro $1,25(OH)_2D_3$ production

When the data for all animals (control, STZ diabetic untreated and STZ diabetic insulin treated) were combined, there was a significant correlation ($r=0.72$; $p<0.05$) between in vitro $1,25(OH)_2D_3$ production and circulating $1,25(OH)_2D_3$ (Figure 4.4.2.1).

Effect of in vitro insulin on $1,25(OH)_2D_3$ production

$1,25(OH)_2D_3$ production in tubules isolated from diabetic animals which were pre-incubated for 18 hours in K1 media was approximately 40% of that in pre-incubated control tubules. This response was comparable to that observed in freshly isolated tubules (Table 4.4.2.4) indicating no change in the impairment in $1,25(OH)_2D_3$ production in diabetic tubules kept in K1 media overnight. Although there was a trend toward increased $1,25(OH)_2D_3$ production with time when tubules from STZ diabetic rats were incubated with insulin, this increase was not statistically significant (Figure 4.4.2.4). $1,25(OH)_2D_3$ production in diabetic tubules after 18 hours exposure to insulin remained approximately 50% (Figure 4.4.2.4 [no insulin]) that of control tubules incubated for 18 hours with vehicle or insulin (data not shown).

Spontaneously Diabetic BB Model

Effect of insulin deficiency

Although initial body weights of control and BB diabetic rats were comparable, the average body weight of BB diabetic rats dropped slightly during the first seven days after diagnosis, while Wistar control rats continued to gain weight. Thus, final body weights (Table 4.4.2.2) were significantly lower in BB diabetic rats than in controls. As expected, circulating insulin was lower

and blood glucose was higher in BB diabetic rats as compared to controls. Like the STZ diabetic model, circulating $1,25(OH)_2D_3$ and in vitro $1,25(OH)_2D_3$ production were significantly lower in BB diabetic rats than in controls (Table 4.4.2.4). In contrast to STZ diabetes, however, circulating $25(OH)D_3$ was significantly higher, and PTH was significantly lower, in BB diabetic rats than in control animals (Table 4.4.2.5, Figure 4.4.2.3). BB diabetes did not affect circulating $24,25(OH)_2D_3$. Plasma Ca was significantly lower in BB rats than in controls. Although plasma Mg was lower in BB diabetic rats than in controls, unlike the STZ model, the difference in plasma Mg was not significant. Plasma P was not affected by insulin deficiency in BB rats (Table 4.4.2.3).

Kidney weight, expressed per body weight, and total kidney protein were significantly elevated in BB compared to control rats. The increase in total protein of diabetic kidneys was not associated with a comparable increase in DNA, resulting in a significantly lower DNA:protein ratio in BB diabetic compared to control animals (Table 4.4.2.9).

Effect of in vivo insulin therapy

Insulin therapy normalized body weights, blood glucose and circulating $1,25(OH)_2D_3$ in spontaneously diabetic BB rats (Table 4.4.2.8).

Correlation between circulating $1,25(OH)_2D_3$ and in vitro $1,25(OH)_2D_3$ production

As in STZ diabetes and controls, there was a significant correlation ($r=0.68$; $p<0.05$) between circulating $1,25(OH)_2D_3$ and in vitro $1,25(OH)_2D_3$ production in the BB diabetic and control rats (Figure 4.4.2.2).

TABLE 4.4.2.1

Indices of diabetes in rats injected with STZ[2] compared to vehicle injected controls

	CONTROL[1] (vehicle)	DIABETIC[1] (STZ)[2]
BODY WEIGHT (g)	189 ± 4	140 ± 3*
INSULIN (ng/ml)	26 ± 3	9 ± 1*
GLUCOSE (mg/dl)	128 ± 4	467 ± 40*

[1]Data are mean ± SEM, n ≥ 9
[2]Streptozotocin
*Significantly ($p<0.05$) different compared to control

TABLE 4.4.2.2

Indices of diabetes in spontaneously diabetic BB rats compared to age matched Wistar controls

	CONTROL[1]	SPONTANEOUSLY DIABETIC[1]
BODY WEIGHT (g)	406 ± 26	306 ± 20*
INSULIN (ng/ml)	32 ± 4	13 ± 2*
GLUCOSE (mg/dl)	125 ± 2	633 ± 69*

[1]Data are mean ± SEM, n ≥ 4
*Significantly ($p<0.05$) different compared to control

TABLE 4.4.2.3

Plasma minerals in STZ[2] induced and spontaneously diabetic rats compared to control rats

	PLASMA Ca[1]	PLASMA Mg[1]	PLASMA P[1]
SPRAGUE DAWLEY CONTROL	11.2 ± 0.4	1.82 ± 0.06	7.56 ± 0.44
STZ[2] DIABETIC	10.1 ± 0.4	1.48 ± 0.06*	6.69 ± 0.32
WISTAR CONTROL	12.71 ± 0.25	1.79 ± 0.24	4.45 ± 0.11
BB[3] DIABETIC	10.40 ± 0.57*	1.63 ± 0.04	4.42 ± 0.07

Data are expressed as mean ± SEM, n ≥ 7
[1]mg/dl
[2]Streptozotocin
[3]Spontaneously diabetic rat model
*Significantly ($p<0.05$) different compared to control

TABLE 4.4.2.4

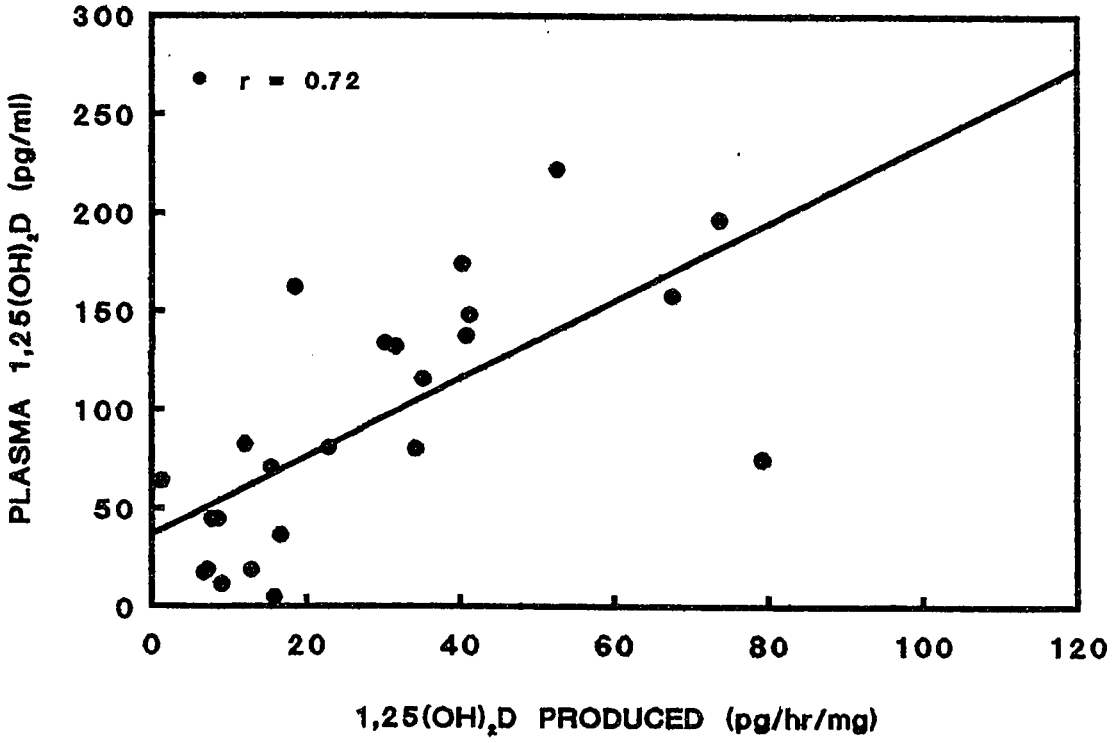
Circulating $1,25(OH)_2D_3$ and in vitro $1,25(OH)_2D_3$ synthesis in STZ[2] induced and spontaneously diabetic rats compared to respective control rats

	CIRCULATING $1,25(OH)_2D_3$[1] (pg/ml)	NET SYNTHESIS $1,25(OH)_2D_3$[1] (fmoles/min/mg)
SPRAGUE DAWLEY CONTROL	124 ± 8	1.3 ± 0.3
STZ[2] DIABETIC	29 ± 4*	0.4 ± 0.1*
WISTAR CONTROL	44 ± 10	1.6 ± 0.3
BB[3] CONTROL	20 ± 6*	0.7 ± 0.1*

[1]Data are mean ± SEM, n ≥ 4
[2]Streptozotocin
[3]Spontaneously diabetic rat model
*Significantly ($p<0.05$) different compared to control

FIGURE 4.4.2.1 (top) Correlation between net synthesis of $1,25(OH)_2D_3$ measured in renal proximal tubules and circulating $1,25(OH)_2D_3$ in STZ diabetic and Sprague Dawley controls.
Data are mean of duplicate determinations of circulating $1,25(OH)_2D_3$ and in vitro net synthesis of $1,25(OH)_2D_3$ measured in isolated proximal tubules from individual diabetic and control rats. A significant correlation between net synthesis rates and circulating $1,25(OH)_2D_3$ was observed $r = 0.72$ ($p<0.05$).

FIGURE 4.4.2.2 (bottom) Correlation between net synthesis of $1,25(OH)_2D_3$ measured in renal proximal tubules and circulating $1,25(OH)_2D_3$ in BB diabetic and Wistar controls.
Data are mean of duplicate determinations of circulating $1,25(OH)_2D_3$ and in vitro net synthesis of $1,25(OH)_2D_3$ measured in isolated proximal tubules from individual diabetic and control rats. A significant correlation between net synthesis rates and circulating $1,25(OH)_2D_3$ was observed $r = 0.69$ ($p<0.05$).


r = 0.72
PLASMA 1,25(OH)2D (pg/ml)
1,25(OH)2D PRODUCED (pg/hr/mg)
0
50
100
150
200
250
300
0
20
40
60
80
100
120

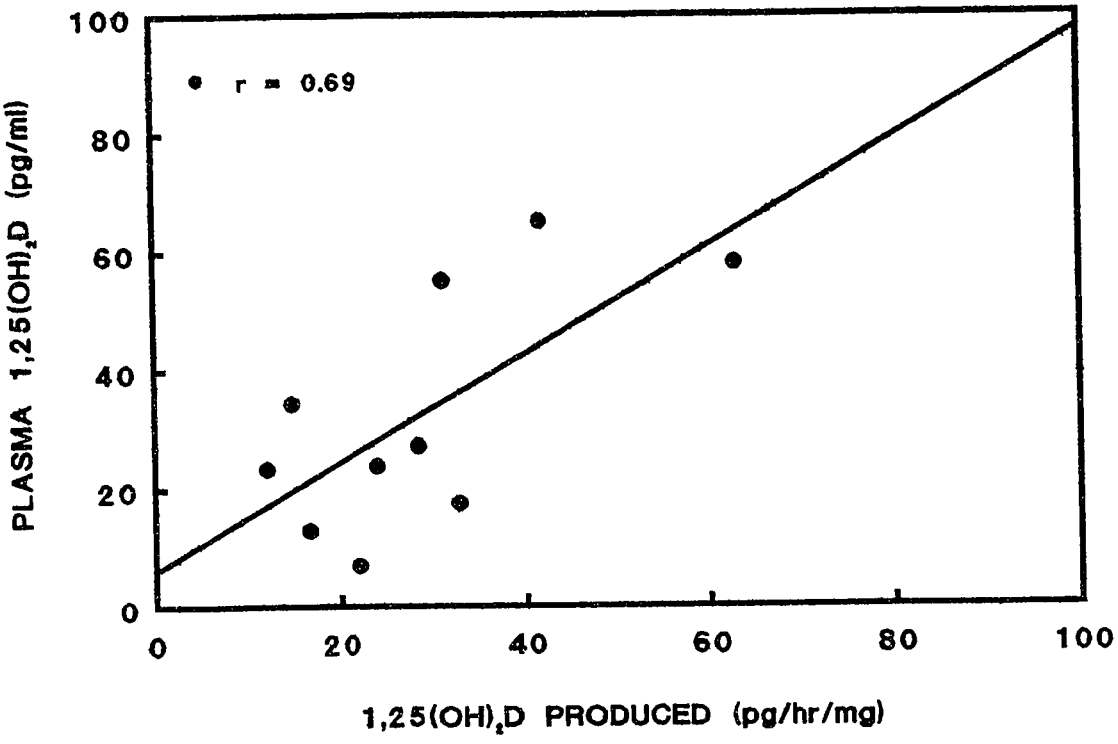

r = 0.69
PLASMA 1,25(OH)2D (pg/ml)
1,25(OH)2D PRODUCED (pg/hr/mg)
0
20
40
60
80
100
0
20
40
60
80
100

TABLE 4.4.2.5

Indices of vitamin D metabolism in STZ and BB diabetic rats compared to respective control rats

	CIRCULATING $25(OH)D_3$[1] (ng/ml)	CIRCULATING $24,25(OH)_2D_3$[1] (ng/ml)	NET $24,25(OH)_2D_3$ SYNTHESIS[1] (pmoles/min/mg)
SPRAGUE DAWLEY CONTROL	15 ± 1	10.0 ± 0.9	0.85 ± 0.1
STZ[2] DIABETIC	19 ± 2	6.7 ± 0.8*	0.64 ± 0.2*
WISTAR CONTROL	13 ± 1	22.5 ± 1.7	N/A
BB[3] CONTROL	21 ± 2*	25.8 ± 2.1	N/A

[1]Data are means ± SEM, n ≥ 6
[2]Streptozotocin
[3]Spontaneously diabetic rat model
*Significantly ($p>0.05$) different compared to control
N/A Data not available

FIGURE 4.4.2.3 Circulating PTH in STZ and BB diabetic rats compared to respective control rats.
Data are mean ± SEM, n = 12-20 determinations of duplicates from individual control and diabetic rats. No significant differences between circulating PTH were observed between Sprague Dawley (SD) controls and Streptozotocin (STZ) diabetic rats. BB diabetic rats had significantly (*) ($p<0.05$) lower circulating PTH in comparison to Wistar (W) controls.

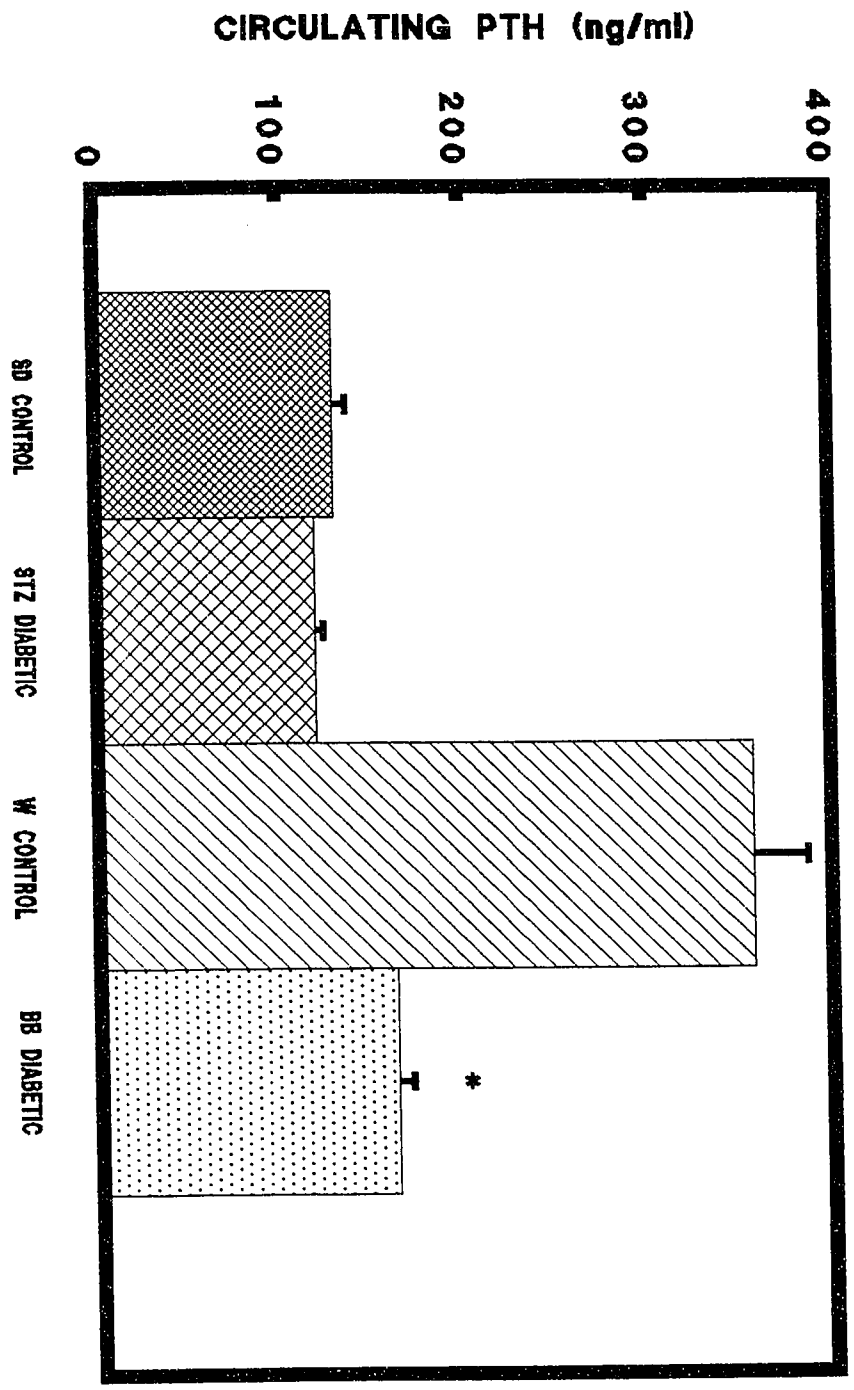

CIRCULATING PTH (ng/ml)
0
100
200
300
400
SD CONTROL
STZ DIABETIC
W CONTROL
BB DIABETIC
*

TABLE 4.4.2.6

Indices of diabetes in STZ diabetic rats treated with insulin for 3-5 days and control rats

	CONTROL[1]	STZ[2] DIABETIC PLUS INSULIN[1]
BODY WEIGHT (g)	228 ± 15	217 ± 7
INSULIN (ng/ml)	26 ± 3	79 ± 33
GLUCOSE (mg/dl)	128 ± 4	124 ± 44

[1]Data are mean ± SEM, n ≥ 6
[2]Streptozotocin

TABLE 4.4.2.7

Indices of vitamin D metabolism in STZ diabetic rats treated with insulin for 3-5 days and control rats

	CONTROL[1]	STZ[2] DIABETIC PLUS INSULIN[1]
CIRCULATING $1,25(OH)_2D_3$ (pg/ml)	150 ± 13	152 ± 23
NET SYNTHESIS $1,25(OH)_2D_3$ (fmoles/min/mg)	2.1 ± 0.4	2.1 ± 0.3
CIRCULATING $24,25(OH)_2D_3$ (ng/ml)	10.0 ± 0.9	5.2 ± 2.1
NET SYNTHESIS $24,25(OH)_2D_3$ (pmoles/min/mg)	0.9 ± 0.1	1.2 ± 0.5

[1]Data are mean and SEM, n ≥ 4-6
[2]Streptozotocin
*Significantly ($p>0.05$) different compared to respective controls

TABLE 4.4.2.8

Indices of diabetes and plasma $1,25(OH)_2D_3$ in BB diabetic rats treated with insulin and control rats

	CONTROL[1]	BB[2] DIABETIC PLUS INSULIN[1]
BODY WEIGHT (g)	323 ± 8	276 ± 13*
GLUCOSE (ng/ml)	109 ± 8	221 ± 73
CIRCULATING $1,25(OH)_2D_3$ (pg/ml)	102 ± 19	88 ± 15

[1]Data are mean ± SEM, n ≥ 5
[2]Spontaneously diabetic rat model
*Significantly ($p<0.05$) different compared to control

FIGURE 4.4.2.4 Effect of in vitro insulin on net $1,25(OH)_2D_3$ synthesis in renal proximal tubules isolated from STZ diabetic rats.
Data are mean ± SEM, n = 7-20 determinations of net $1,25(OH)_2D_3$ synthesis rates from individual proximal tubules isolated from STZ diabetics and treated in vitro with or without insulin for 1, 4, or 18 hours as compared to rates observed in control tubules. Control tubules were treated and incubated in parallel with diabetic tubules, however no significant differences were observed between control groups therefore data was pooled and averaged. Data is expressed as a % of control tubules net $1,25(OH)_2D_3$ synthesis rate. Net $1,25(OH)_2D_3$ synthesis rates in diabetic tubules remained significantly lower than rates observed in control tubules irregardless of time of incubation with in vitro insulin.

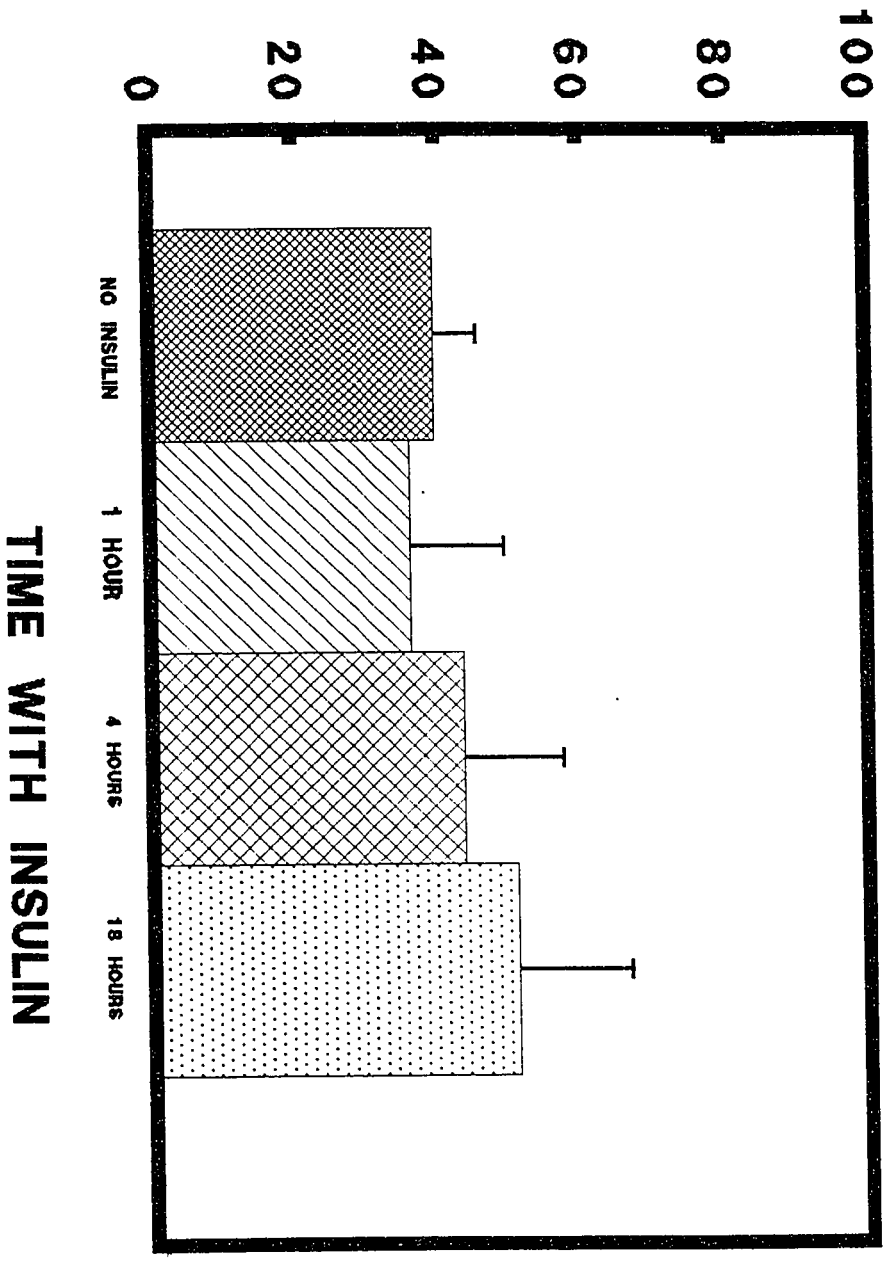

NET 1,25(OH)2D SYNTHESIS (% CONTROL)
0
20
40
60
80
100
NO INSULIN
1 HOUR
4 HOURS
18 HOURS
TIME WITH INSULIN

TABLE 4.4.2.9

Indices of renal growth in STZ and BB diabetic rats compared to respective controls

	SOMATIC INDEX[1]	TOTAL PROTEIN (mg)	ug DNA/mg PROTEIN
SPRAGUE DAWLEY CONTROL[2]	10.1 ± 0.3	96.0 ± 3.4	9.5 ± 0.5
STZ[3] DIABETIC[2]	16.1 ± 0.6*	118.7 ± 5.1*	7.4 ± 0.3*
WISTAR CONTROL[2]	8.5 ± 0.2	97.8 ± 3.0	18.6 ± 0.7
BB[4] DIABETIC[2]	10.1 ± 0.3*	114.0 ± 7.7*	15.7 ± 0.7

[1]mg kidney/g body weight
[2]Data are means ± SEM, n = 6
[3]Streptozotocin
[4]Spontaneously diabetic rat model
*Significantly ($p>0.05$) different compared to control

4.4.3. DISCUSSION

The results from these studies demonstrate reduced circulating $1,25(OH)_2D_3$ and decreased net synthesis of $1,25(OH)_2D_3$ in two models of insulin dependent diabetes. Therefore these studies complement earlier reports of impaired 1αhydroxylase activity measured in homogenates (Ikeda et al., 1985), mitochondria (Rao and Kung, 1988) or renal slices (Wongsurawat et al., 1983) from STZ diabetic rats and reduced circulating $1,25(OH)_2D_3$ levels in STZ and BB diabetic rats (Schneider et al., 1977, Nyomba et al., 1989). In contrast to previous studies (Wongsurawat et al., 1983, Ikeda et al., 1985), diabetic rats were not subjected to prior dietary or surgical manipulation to facilitate measurement of 1αhydroxylase activity. Instead measurement of basal 1αhydroxylase activity was achieved by integrating improved isolation strategies for vitamin D metabolites (SepPak affinity and adsorption chromatography, HPLC adsorption and affinity chromatography) with detection by radioreceptor assay (pg sensitivity) with a purified proximal tubule system, which minimized interference from vitamin D binding proteins. In addition, these studies were conducted in tubule preparations from individual animals, thus correlations to circulating vitamin D metabolites, observed in each animal could be made. The similarities in vitamin D metabolism observed in both the BB spontaneously diabetic and the STZ induced diabetic model supports the existence of perturbations in vitamin D metabolism which have previously been predominantly documented in STZ diabetic rats.

The reversibility of the altered vitamin D metabolism in both animal models upon insulin therapy implicates a role for insulin directly or indirectly in the maintenance of normal vitamin D metabolism. Wilson et al. (1982) reported that exogenous insulin increased circulating $1,25(OH)_2D_3$ in STZ diabetic rats within 12 hours. The studies described in this thesis confirm normalization of circulating $1,25(OH)_2D_3$ with in vivo insulin therapy in both models of diabetes (Table 4.4.2.7, 4.4.2.8) as well as normalization of 1αhydroxylase (Table 4.4.2.7) activity in STZ diabetes. To determine whether

insulin directly influences 1αhydroxylation, the effect of pre-incubation with insulin on $1,25(OH)_2D_3$ net synthesis was assessed in proximal renal tubules isolated from both control and STZ diabetic rats. The data indicates that insulin did not alter $1,25(OH)_2D_3$ net synthesis in control renal tubules, nor did insulin significantly increase $1,25(OH)_2D_3$ net synthesis in tubules from STZ diabetic rats (Figure 4.4.2.4). This observation is consistent with that of Henry (1981) who reported that insulin did not affect basal 1αhydroxylase activity in primary cultures of renal tubules from vitamin D deficient (non diabetic) chicks. These results suggest that insulin does not directly modulate net synthesis of $1,25(OH)_2D_3$. However while in vitro insulin did not significantly elevate net synthesis of $1,25(OH)_2D_3$ in STZ diabetic tubules there was a small increase in activity apparent by four hours and again at 18 hours; this small (10%) increase after 18 hours was abolished by co-incubation of tubules with cycloheximide (data not shown). Thus it is possible that the incubation time was insufficient for the effects of insulin to become apparent or that additional factors act synergistically with insulin to modulate $1,25(OH)_2D_3$ production. Alternately insulin may not affect basal 1αhydroxylase activity, but may be permissive for PTH stimulation of $1,25(OH)_2D_3$ net synthesis (Henry, 1981, Korkor et al., 1987). Further studies to examine the interactions between insulin and other modulators of 1αhydroxylase activity will be required to ascertain whether insulin directly influences renal $1,25(OH)_2D_3$ production.

Several factors influence net synthesis of $1,25(OH)_2D_3$ and modulate circulating $1,25(OH)_2D_3$ levels. Insulin absence may alter one or more of these factors, thereby indirectly altering vitamin D metabolism. By comparing the two diabetic models with and without insulin therapy, several factors, which could theoretically relate to impaired $1,25(OH)_2D_3$ production can be excluded. Neither diabetic model was associated with low circulating $25(OH)D_3$, the substrate for the renal 1αhydroxylase (Wilson et al., 1982, Table 4.4.2.5), thus it is unlikely that decreased substrate availability would explain the impaired $1,25(OH)_2D_3$ production associated with insulin absence. However, the

plausibility of a role for transcalciferin (McLeod and Cooke, 1989) in substrate ($25(OH)D_3$) transport for the renal 25 hydroxy vitamin D hydroxylases has been suggested, and circulating transcalciferin is significantly lower in diabetic rats as compared to controls (Nyomba et al., 1989), thus alterations in intracellular substrate in diabetes cannot be excluded. The concept of enhanced conversion of $25(OH)D_3$ to $24,25(OH)_2D_3$, during diabetes, has been proposed by Wongsurawat et al. (1983). They documented increased net synthesis of $24,25(OH)_2D_3$ from $25(OH)D_3$ in renal slices isolated from STZ diabetic rats fed low Ca diets compared to low Ca fed non diabetic rats. Enhanced 24 hydroxylase activity would imply increased metabolic clearance of $1,25(OH)_2D_3$ to $1,24,25(OH)_3D$, since $1,25(OH)_2D_3$ can also act as a substrate for the 24 hydroxylase (Tomon, Tenenhouse and Jones, 1990). However our data (Stone et al., 1991a&b) as well as other studies (Imura, Seino and Ishida, 1985) have described normal or reduced circulating $24,25(OH)_2D_3$ in diabetes. In addition, Spencer et al. (1980) reported no difference in metabolic clearance of $^3H1,25(OH)_2D_3$ in STZ diabetic and control rats fed adequate Ca. The results from the studies described in this thesis indicate that circulating $24,25(OH)_2D_3$ levels and 24 hydroxylase activity were significantly reduced in STZ diabetic rats fed adequate Ca diets. In addition, circulating $24,25(OH)_2D_3$ was normal in BB spontaneously diabetic rats fed normal rat chow which also exhibited low circulating $1,25(OH)_2D_3$. Upon insulin therapy 24 hydroxylase activity increased and circulating $24,25(OH)_2D_3$ levels increased slightly but not significantly; while 1αhydroxylase activity and circulating $1,25(OH)_2D_3$ rose significantly and were normalized in STZ diabetics. In addition significant positive correlations were obtained between net synthesis of $1,25(OH)_2D_3$ and circulating $1,25(OH)_2D_3$ in both diabetic animal models while no correlation was obtained between 24 hydroxylase activity and circulating $1,25(OH)_2D_3$or 1αhydroxylase activity in STZ diabetic animals; suggesting enhanced metabolic clearance was not present during insulin absence. Taken in unison these results suggest that increased metabolic clearance of $1,25(OH)_2D_3$ or enhanced

conversion of $25(OH)D_3$ to $24,25(OH)_2D_3$ did not exist in our acutely diabetic rats with normal Ca intakes and was not responsible for the lower circulating $1,25(OH)_2D_3$ or 1αhydroxylase activity observed in with these diabetic animals. However direct measurement of 24 hydroxylase activity and/or $1,25(OH)_2D_3$ catabolism in other vitamin D target tissues such as the enterocyte during diabetes would be necessary to eliminate this possibility. The discrepancy between the observations reported in this thesis of reduced renal 24 hydroxylase activity and that of enhanced 24 hydroxylase made by Wongsurawat et al. (1983) may be explained by the differences in circulating $1,25(OH)_2D_3$ in experimental animals due to differences in Ca intakes. $1,25(OH)_2D_3$ induces 24 hydroxylase activity in a VDR dependent mechanism (Tanaka and DeLuca, 1974). Although VDR numbers are elevated in some target tissues during diabetes, target tissue effects of $1,25(OH)_2D_3$ are usually normal or decreased in diabetes (Nyomba et al., 1989, Stone et al., 1991a&b). Thus while intestinal VDR number were increased in previous studies in our lab, immunoassayable intestinal calbindin D9 and alkaline phosphatase activity were lower in diabetic animals (Stone at al., 1991a&b). Moreover, renal calbindin D28 was not increased in diabetic kidney (Stone et al., 1991a&b). Therefore 24 hydroxylase activity in our diabetic animals might have been reduced due to the low circulating $1,25(OH)_2D_3$ levels present. In the studies reported by Wongsurawat et al. (1983), diabetic animals were fed low dietary Ca and had increased synthesis of $1,25(OH)_2D_3$, which might explain the elevated 24 hydroxylase activity. Finally, 24 hydroxylase activity in the studies described in this thesis was measured in (purified) proximal tubules whereas Wongsurawat et al. (1983) measured 24 hydroxylase activity in renal slices which are both distal and proximal tubules. Thus the different 24 hydroxylase rates obtained may have reflected differences in tissue composition. Future studies are required to examine these issues more thoroughly.

The aberrant vitamin D metabolism in diabetes must be interpreted with respect to circulating PTH, a major regulator of both 1αhydroxylase and

24 hydroxylase activities. Although circulating PTH was low in BB diabetes, STZ diabetic rats had decreased circulating $1,25(OH)_2D_3$ and decreased 1αhydroxylase activity despite normal circulating PTH (Figure 4.4.2.3). Thus, although PTH may contribute to impaired $1,25(OH)_2D_3$ production in BB diabetes, insulin deficiency is not universally associated with low circulating PTH and several studies have reported elevated circulating PTH in diabetes (Schedl, Heath and Wanger, 1978, Colette et al., 1989, Schneider et al., 1974). The differences in circulating PTH between the two diabetic models may be related to age (BB rats were older and exhibited slower growth rates than STZ animals), disease severity (higher blood glucose and weight loss was observed in BB diabetics) and/or diet (BB rats were fed chow, with 1.2% Ca, whereas STZ rats were fed AIN semi-purified diet with 0.6% Ca). The dietary and age related factors may have precipitated Ca overload in BB diabetics, due to decreased growth rates and increased Ca intake (resulting from hyperphagia). In support of this hypothesis Nyomba et al. (1989) have reported higher net Ca absorption in BB diabetic rats (secondary to hyperphagia) compared to control rats. The decreased circulating PTH observed in BB rats is consistent with this suggestion, since PTH secretion would be reduced in response to Ca absorption in excess of need. However circulating Ca was significantly lower in BB diabetic rats compared to controls. In addition the autoimmune processes associated with diabetes onset in BB rats may have affected PTG function independently of effects on glucose homeostasis (Marliss et al., 1982). Further studies will be required to clarify these differences and the relevance to the altered vitamin D metabolism associated with insulin dependent diabetes.

Target tissue resistance to PTH may contribute to the decreased 1αhydroxylase activity observed in diabetes since synthesis of $1,25(OH)_2D_3$ was decreased despite adequate circulating PTH in STZ diabetics (Aksnes et al., 1988). As previously discussed, insulin is permissive for PTH stimulation of $1,25(OH)_2D_3$ production in vitro and insulin is necessary in vivo for achievement of comparable $1,25(OH)_2D_3$ synthesis in response to low dietary

Ca (Henry, 1981, Korkor et al., 1987, Wongsurawat and Armbrecht, 1985), a response mediated by PTH. Although stimulation of $1,25(OH)_2D_3$ production was blunted by diabetes, PTH elevated serum Ca and inhibited tubular reabsorption of phosphate equally in diabetic and control rats, suggesting not all renal PTH target tissue effects are impaired by insulin deficiency (Wongsurawat et al., 1985). Studies described in the next section (4.5) will assess the effect of diabetes on aspects of the PTH signal transduction pathway in kidney.

Although it has been suggested that Mg deficiency may precipitate aberrant vitamin D metabolism during diabetes (Saggese et al., 1988), reductions in plasma Mg were not observed in both STZ and BB diabetes. In addition, the severity of hypomagnesaemia in STZ diabetes was small in comparison to that observed in the primary Mg deficiency studies (Section 3) in which alterations in vitamin D and Ca homeostasis were observed. Thus while hypomagnesaemia may complicate the disturbed vitamin D metabolism in diabetes, it is unlikely that it is a primary factor.

Increased renal mass despite decreased growth rate was observed in both animal models of diabetes and was reversed by in vivo insulin therapy as has been previously reported and characterized (Mogenson and Anderson, 1973, SeyerHansen, 1976). The increase in renal mass was secondary to hypertrophy, as indicated by increased protein content without a compensatory increase in DNA content. A direct relationship between hyperglycaemia and renal hypertrophy (Flyvbjerg and Orskov, 1990) has previously been demonstrated in diabetes. These observations may partly explain the decreased $1,25(OH)_2D_3$ net synthesis rates (expressed per mg protein) observed in diabetes (by changing the specific activity). However the 22% increase in renal protein/DNA ratio does not explain the 50-70% decrease in 1αhydroxylase activity nor does it explain the decreased circulating $1,25(OH)_2D_3$ observed in diabetics. Instead, it is possible that the renal hypertrophy reflects other alterations in physiology or biochemistry that indirectly or directly alter

vitamin D metabolism in diabetes.

4.4.4. CONCLUSIONS

In summary, decreased circulating $1,25(OH)_2D_3$ was associated with decreased net synthesis of $1,25(OH)_2D_3$ in vitro in two models of insulin dependent diabetes. The low circulating $1,25(OH)_2D_3$ and impaired 1αhydroxylase activity were normalized by in vivo insulin therapy, but 1αhydroxylase activity in vitro was not increased by insulin exposure. The altered vitamin D metabolism was not associated with decreased circulating $25(OH)D_3$, increased renal 24 hydroxylase activity or increased circulating $24,25(OH)_2D_3$. Increased metabolic clearance of $1,25(OH)_2D_3$ was not indicated during diabetes. Reductions in circulating PTH were not consistently observed. Neither Mg deficiency nor Ca overload were major contributing factors to the aberrant vitamin D metabolism during diabetes. Renal hypertrophy was evident in both diabetic models and was reversed by in vivo insulin therapy, but the change in renal protein did not fully explain decreased $1,25(OH)_2D_3$ synthesis. In conclusion these data validate the STZ diabetic rat model and the purified renal proximal tubule system for studying the effects of insulin deficiency on vitamin D metabolism. They suggest that the alteration in vitamin D metabolism observed in untreated diabetes is likely at the level of $1,25(OH)_2D_3$ net synthesis and probably not a direct effect of insulin absence on the 1αhydroxylase enzyme system.

4.5 VITAMIN D METABOLISM, SIGNAL TRANSDUCTION AND INSULIN DEPENDENT DIABETES

4.5.1. EXPERIMENTAL PROTOCOL

Male weanling Sprague Dawley rats were fed and housed as described under "Materials and Methods". They received either AIN semi-purified normal, low Ca or low P diets and deionized or tap water ad libitum. Diabetes was induced as described previously. Several studies were conducted in control and low Ca fed non diabetic animals to optimize assay conditions and characterize the role of the adenylate cyclase and PKC in vitamin D

metabolism prior to measurements in diabetic animals.

4.5.1.a. Adenylate Cyclase Signal Transduction Pathway

The effect of PTH on net $1,25(OH)_2D_3$ production was optimized for dosage and incubation time in freshly purified and primary cultures of renal proximal tubules respectively. Using the dosage of PTH which maximally stimulated $1,25(OH)_2D_3$ synthesis, a time course of PTH stimulated cAMP generation was conducted. The time course of stimulation of adenylate cyclase with forskolin was optimized in primary cultures of mixed renal tubules.

4.5.1.a.1. In Vitro PTH

Two studies were conducted to examine the effect of seven days of STZ diabetes on in vitro PTH stimulation of adenylate cyclase and vitamin D metabolism. In both studies, serum was assayed for glucose, insulin, $1,25(OH)_2D_3$ and PTH. In one study basal, PTH and forskolin stimulated cAMP synthesis was assayed in mixed renal tubules. In a second experiment basal and PTH stimulation of net $1,25(OH)_2D_3$ synthesis was measured in proximal tubules isolated from untreated STZ diabetic and control rats. For these studies renal tubules were incubated in K1 medium with 5 ug/ml insulin and a phosphodiesterase inhibitor for four hours.

4.5.1.a.2. In Vivo PTH

One 14 day study was conducted to examine the effect of mineral restriction on vitamin D metabolism in diabetes. One group of animals was fed low Ca diets, which elevates circulating PTH and increases net synthesis of $1,25(OH)_2D_3$. A second group of animals was fed low P diets, which results in increased net synthesis of $1,25(OH)_2D_3$ independent of increased circulating PTH. The rationale was to determine if $1,25(OH)_2D_3$ (synthesis and circulating levels) would be increased in diabetic rats in response to low Ca and low P. Thus the specificity of alterations in 1αhydroxylase stimulation could be monitored. Rats were injected with STZ or citrate buffer and seven days after diabetes onset six control and six diabetic animals were sacrificed for baseline values. The remaining animals were fed either low Ca or low P diets with six

diabetic and six control animals per dietary group. After seven days on the experimental diets, all remaining animals were sacrificed. Rats were fasted for 12 hours prior to sacrifice, and serum was frozen for assay of Ca, P, glucose, insulin, $1,25(OH)_2D_3$ and PTH. Renal proximal tubules were purified from STZ diabetic and control rats and assayed for net $1,25(OH)_2D_3$ synthesis. Femora were removed for bone analysis and minerals.

4.5.1.b. Phosphoinositide Signal Transduction Pathway

4.5.1.b.1. In Vitro Protein Kinase C Activation

The effect of in vitro PKC activation on $25(OH)D_3$ metabolism was assessed in mixed or proximal tubules freshly isolated from control and low Ca fed rats. Both 12-O-tetradecanoyl phorbol 13-acetate (TPA), a known modulator of PKC activity and 4α-phorbol 12,13 didecanoate (4αPDD), a structurally similar but inactive phorbol ester, were tested. Time course and dose response experiments were conducted to determine if the effects of PKC stimulation on $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ production could be dissociated. The role of PKC in mediating the effects of phorbol esters on vitamin D hydroxylation was assessed by down regulation and PKC inhibition experiments. The temporal relationship between PKC activation and translocation and renal vitamin D hydroxylations was characterized.

4.5.1.b.2. In vivo Protein Kinase C activity

One seven day STZ diabetic study was conducted to determine if renal tubule membrane or cytosolic PKC activity was altered in diabetic animals. At sacrifice, serum was frozen for the determination of glucose, insulin and $1,25(OH)_2D_3$. Membrane and cytosolic PKC activity was assayed in renal tubules freshly isolated from STZ diabetic and control animals.

4.5.2. RESULTS

4.5.2.a. Adenylate Cyclase Signal Transduction and Vitamin D Metabolism

4.5.2.a.1. In vitro PTH and Vitamin D Metabolism in Insulin Dependent Diabetes

Net synthesis of $1,25(OH)_2D_3$ was significantly increased in proximal tubules after four hours incubation with $1x10^{-7}$ PTH (Figure 4.5.1.a.1.1). Stimulation was observed with $1x10^{-7}$ M PTH within two hours and was significant within three hours (Figure 4.5.1.a.1.2). The efficacy of in vitro PTH to stimulate $1,25(OH)_2D_3$ net synthesis in renal proximal tubules in STZ diabetic versus control rats was tested at $1x10^{-7}$ M PTH for four hours.

PTH $1x10^{-7}$ M significantly enhanced renal tubule synthesis of cAMP to 180 and 215% of basal values after one and two minutes exposure, respectively. cAMP synthesis declined thereafter but remained elevated above basal values for up to 20 minutes (Figure 4.5.1.a.1.3). 10μM forskolin significantly elevated synthesis of cAMP above basal within 20 minutes; cAMP synthesis remained elevated above basal values for up to 60 minutes (Figure 4.5.1.a.1.4).

Basal cAMP synthesis was significantly greater in tubules isolated from STZ diabetic rats as compared to control rats. Maximal adenylate cyclase activity (induced by direct stimulation of the catalytic subunit by 10μM forskolin) was comparable between renal tubules isolated from STZ diabetic and control rats (Figure 4.5.1.a.1.5). % stimulation of cAMP synthesis by $1x10^{-7}$ M PTH was comparable in renal tubules isolated from STZ diabetic and control rats (Figure 4.5.1.a.1.6). However % stimulation of cAMP synthesis by 10μM forskolin was reduced due to the enhanced basal enzyme rates in diabetic rats (data not shown).

Basal 1αhydroxylase activity was significantly lower in tubules isolated from diabetic rats in comparison to controls (Data not shown). The percent stimulation of net basal $1,25(OH)_2D_3$ synthesis, by $1x10^{-7}$ M PTH was not significantly different in proximal tubules isolated from control and STZ diabetic rats (Figure 4.5.1.a.1.7).

FIGURE 4.5.1.a.1.1 Dose response effect of in vitro PTH on net $1,25(OH)_2D_3$ synthesis in renal proximal tubules.
Data are mean ± SEM of duplicate or triplicate determinations from 3 separate (fresh) preparations of proximal tubules isolated from low Ca fed rats. Rats were fed low Ca diets to facilitate measurement of 1αhydroxylase activity. Tubules were pre-incubated with phosphodiesterase inhibitor or vehicle for 3 hours at 37 C after which 5 μM $25(OH)D_3$ and vehicle or rat n1-34 PTH was added and tubules were incubated for a further hour. Reactions were terminated by addition of equivolume acetonitrile and vitamin D metabolites were isolated and quantitated as described under "Materials and Methods". Data is expressed as % basal (vehicle) net $1,25(OH)_2D_3$ synthesis rate. Significant (*) ($p<0.05$) stimulation of basal net $1,25(OH)_2D_3$ synthesis was observed at a PTH concentration of $1x10^{-7}$ M.

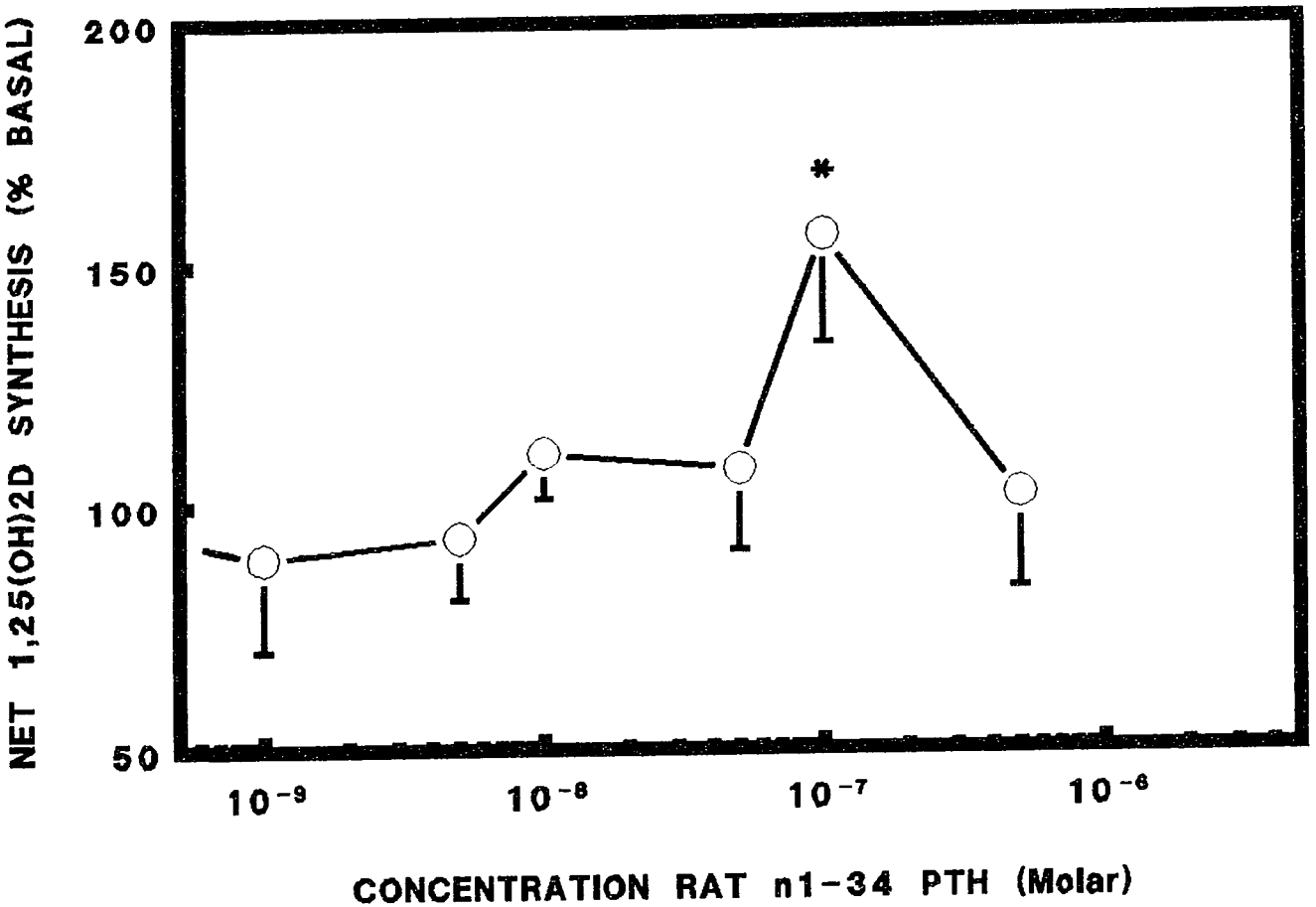

NET 1,25(OH)2D SYNTHESIS (% BASAL)
200
150
100
50
*
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
CONCENTRATION RAT n1-34 PTH (Molar)

FIGURE 4.5.1.a.1.2 Time course of in vitro PTH stimulation of net $1,25(OH)_2D_3$ synthesis in renal proximal tubules.
Data are mean ± SEM of duplicate or triplicate determinations of synthesis rates observed in individual primary cultures of proximal tubules (90% confluent). 5 µM $25(OH)D_3$ was added as substrate for quantitation of $1,25(OH)_2D_3$ synthesis and dishes were treated with vehicle (Basal) or phosphodiesterase inhibitor and rat n1-34 $1x10^{-7}$ M PTH and incubated for varying lengths of time at 37 C in an incubator. Reactions were terminated by addition of equivolume acetonitrile and vitamin D metabolites were isolated and quantitated as described under "Materials and Methods". Data is expressed as % basal (vehicle) net $1,25(OH)_2D_3$ synthesis rate. Stimulation of $1,25(OH)_2D_3$ synthesis by PTH was observed after 2 hours of incubation and was significant (*) ($p<0.05$) after 3 hours.

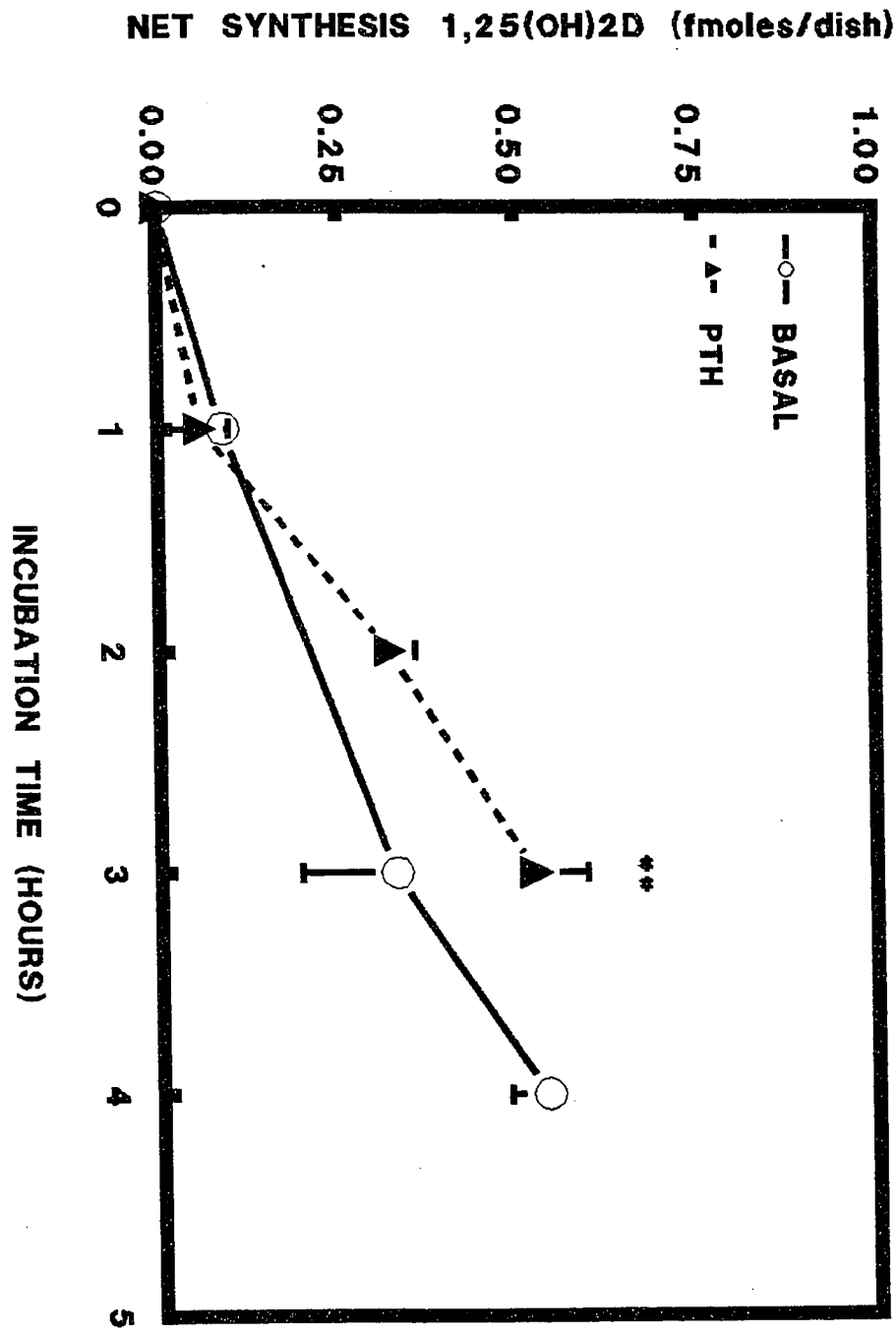

NET SYNTHESIS 1,25(OH)2D (fmoles/dish)
0.00
0.25
0.50
0.75
1.00
0
1
2
3
4
5
INCUBATION TIME (HOURS)
—○— BASAL
- -▲- - PTH
**

FIGURE 4.5.1.a.1.3 Time course of PTH stimulation of cAMP synthesis in renal tubules.
Data are mean ± SEM of triplicate determinations from 2-3 separate (fresh) preparations of renal tubules. Prelabelled tubules were incubated with phosphodiesterase inhibitor and either vehicle (Basal) or rat n1-34 $1x10^{-7}$ M PTH for the incubation times indicated. Reactions were terminated by removal of supernatant, washing of cellular pellet and cell lysis. Newly synthesized cAMP was isolated and quantitated as described under "Materials and Methods". Data is expressed as % basal (vehicle plus phosphodiesterase inhibitor) of newly synthesized cAMP. Significant (*) ($p<0.05$) stimulation by PTH was observed after 1 and 2 minutes and remained elevated relative to basal levels for up to 20 minutes.

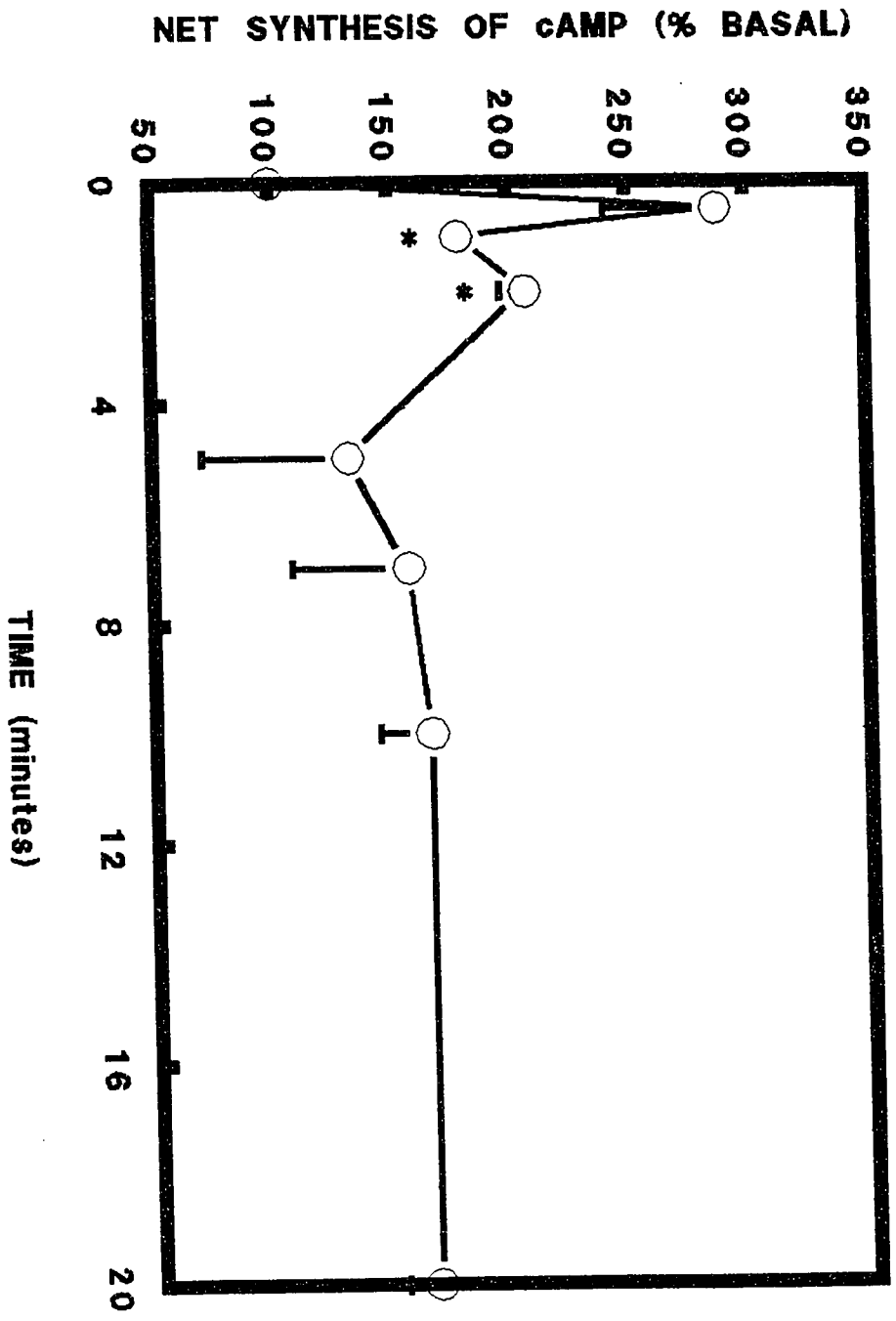

NET SYNTHESIS OF cAMP (% BASAL)
50
100
150
200
250
300
350
0
4
8
12
16
20
TIME (minutes)
*
*

FIGURE 4.5.1.a.1.4 Time course of forskolin stimulation of cAMP synthesis in renal tubules.
Data are mean ± SEM of triplicate determinations of synthesis rates observed in individual primary cultures of renal tubules. Prelabelled cultures were incubated with phosphodiesterase inhibitor and either vehicle (Basal) or 10 μM forskolin for the incubation times indicated. Reactions were terminated by removal of supernatant, washing of cellular pellet and cell lysis. Newly synthesized cAMP was isolated and quantitated as described under "Materials and Methods". Data is expressed as % basal (vehicle plus phosphodiesterase inhibitor) of newly synthesized cAMP. Significant (*) ($p<0.05$) stimulation by forskolin was observed after 10 and 20 minutes and remained significantly elevated for at least 60 minutes relative to basal levels.

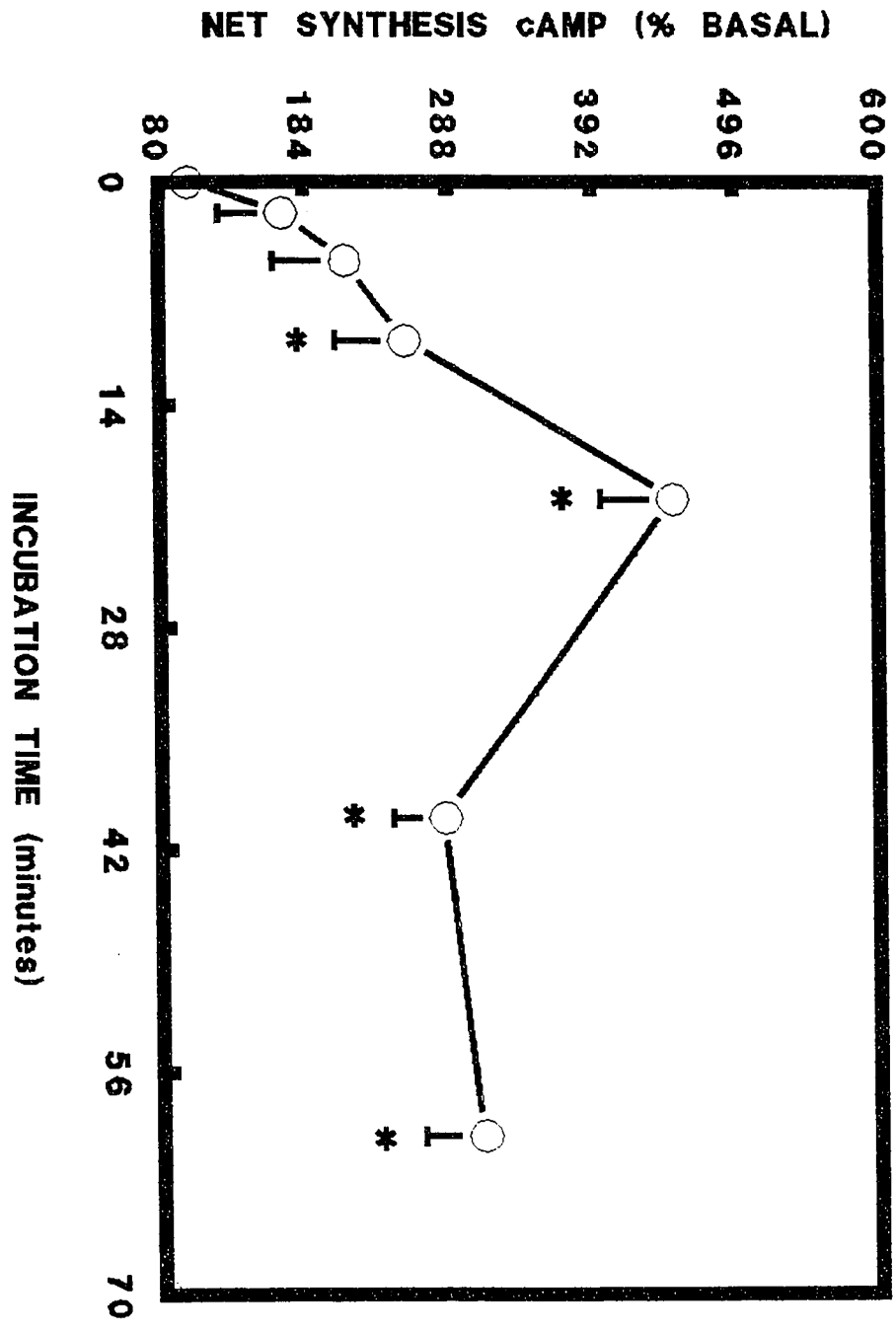

NET SYNTHESIS cAMP (% BASAL)
80
184
288
392
496
600
0
14
28
42
56
70
INCUBATION TIME (minutes)
*
*
*
*

FIGURE 4.5.1.a.1.5 Basal and forskolin stimulated cAMP synthesis measured in renal tubules isolated from STZ diabetic and control rats. Data are mean ± SEM, n = 6-8 determinations of duplicates or triplicates of tubules isolated from individual control and STZ diabetic rats. Prelabelled freshly isolated renal tubules were incubated with vehicle plus phosphodiesterase inhibitor (Basal) for 2 minutes or 10 μM forskolin and phosphodiesterase inhibitor (Forskolin) for 20 minutes. Reactions were terminated by removal of supernatant, washing of cellular pellet and cell lysis. Newly synthesized cAMP was isolated and quantitated as described under "Materials and Methods". Data is expressed as rate of cAMP synthesis (newly synthesized) (pmoles/min/mg tubule protein). Basal cAMP synthesis rates in tubules isolated from STZ diabetic rats were significantly (*) ($p<0.05$) elevated in comparison to tubules isolated from controls, however, maximal cAMP synthesis rates, as determined by forskolin stimulation, were comparable.

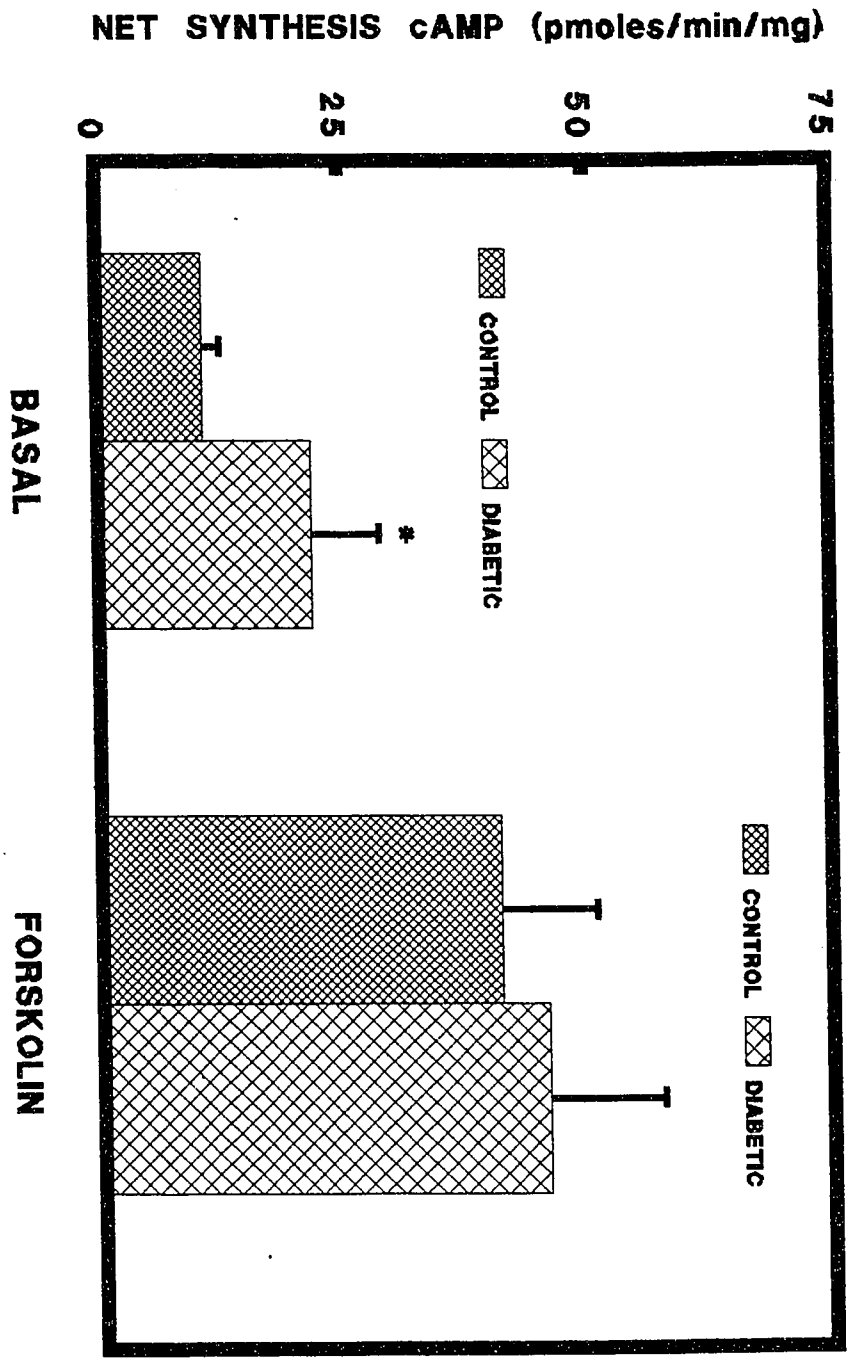
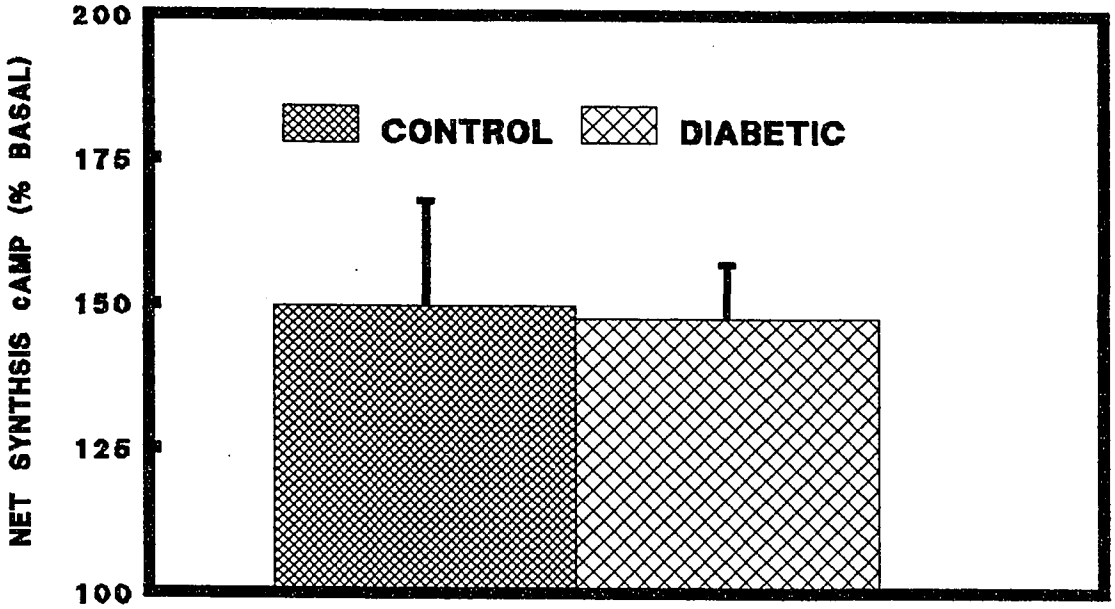
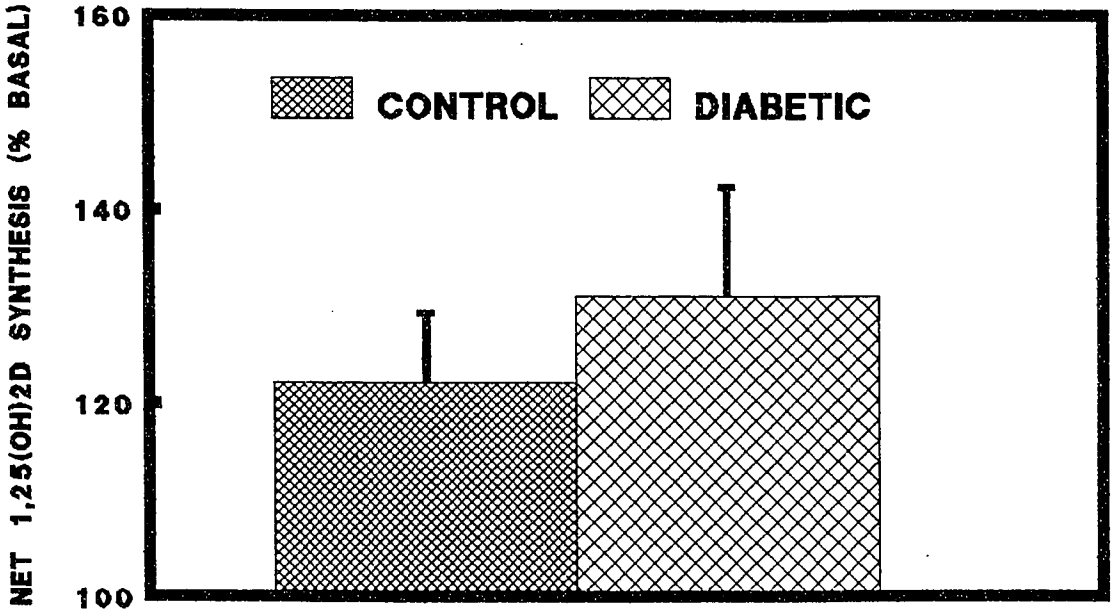

NET SYNTHESIS cAMP (pmoles/min/mg)
0
25
50
75
CONTROL
DIABETIC
*
BASAL
FORSKOLIN

FIGURE 4.5.1.a.1.6 (top) Stimulation of cAMP synthesis by PTH in renal tubules isolated from STZ diabetic and control rats.
Data are mean ± SEM, n = 6-8 determinations of duplicates or triplicates from individual animals. Freshly isolated tubules were incubated with vehicle plus phosphodiesterase inhibitor (Basal) or rat n1-34 $1x10^{-7}$ M PTH and phosphodiesterase inhibitor for 2 minutes. Reactions were terminated by removal of supernatant, washing of cellular pellet and cell lysis. Newly synthesized cAMP was isolated and quantitated as described under "Materials and Methods". Data is expressed as % basal control or STZ diabetic synthesized cAMP. No significant differences were observed between % PTH stimulation of cAMP synthesis in control and STZ diabetic rat tubules although absolute synthesis rates remained depressed in both basal and PTH stimulated STZ diabetic rat tubules (data not shown).

FIGURE 4.5.1.a.1.7 (bottom) Percent stimulation of net $1,25(OH)_2D_3$ synthesis by PTH in proximal tubules isolated from STZ diabetic and control rats.
Data are mean ± SEM, n = 3-4 determinations of tubules isolated from individual control and diabetic animals. Freshly isolated proximal tubules were incubated in the presence of 5 ug/ml insulin with vehicle (Basal) or rat n1-34 $1x10^{-7}$ M PTH and phosphodiesterase inhibitor (PTH stimulated) for 3 hours after which 5 µM $25(OH)D_3$, as a substrate for quantitation of $1,25(OH)_2D_3$ synthesis, was added and the tubules were further incubated for an additional hour. Reactions were terminated by addition of equivolume acetonitrile and vitamin D metabolites were isolated and quantitated as described under "Materials and Methods". Data are expressed as % basal control or STZ diabetic net $1,25(OH)_2D_3$ synthesis rate. No significant differences were observed between efficacy of PTH to stimulate net $1,25(OH)_2D_3$ synthesis in control and STZ diabetic renal proximal tubules. However absolute synthesis rates remained lower in STZ diabetic rats despite PTH stimulation (data not shown).



RAT n1-34 PTH 1x10-7 Molar



RAT n1-34 PTH 1x10-7 Molar

4.5.2.a.2. In vivo PTH, mineral restriction and Vitamin D Metabolism in Insulin Dependent Diabetes

Indices of Diabetes

The low Ca and low P diets did not significantly alter growth rates or indices of diabetes which were similar to that observed in previous studies (Table 4.5.1.a.2.1, refer to section 4.4.2).

Circulating Mineral Levels

STZ diabetic rats fed the AIN control diet were significantly hypocalcaemic compared to control rats fed the same diet, in contrast, diabetic rats fed low Ca or low P diets were normocalcaemic. STZ diabetic rats fed low dietary Ca were significantly hyperphosphatemic compared to low Ca fed control rats and diabetic rats fed AIN control diet. Control rats fed low P diet were significantly hypophosphatemic and hypercalcaemic compared to rats fed the AIN normal diet (Table 4.5.1.a.2.2).

Vitamin D metabolism

Net synthesis of $1,25(OH)_2D_3$ in proximal tubules from STZ diabetic rats fed AIN control diets was 40% that of control rats fed the same diet. Control rats fed low dietary Ca exhibited increased net synthesis of $1,25(OH)_2D_3$ (approximately 260% that of control rats fed control diet). STZ diabetic rats fed low Ca also exhibited increased net synthesis of $1,25(OH)_2D_3$ (350% that of STZ rats fed control diet). Despite the increase in response to low dietary Ca net synthesis of $1,25(OH)_2D_3$ in low Ca fed STZ rats remained significantly lower than that of control rats (Figure 4.5.1.a.2.1). Circulating $1,25(OH)_2D_3$ in STZ diabetic rats fed the AIN control diet was 25% that of control rats fed the same diet. Surprisingly, no change in circulating $1,25(OH)_2D_3$ was observed in response to low dietary Ca or P. However control rats fed either low Ca or low P diets exhibited significant elevations in circulating $1,25(OH)_2D_3$ (190-210% of control levels, Figure 4.5.1.a.2.2).

No significant difference in circulating PTH was observed in control and STZ diabetic animals fed AIN control diets. Circulating PTH was increased in

response to low dietary Ca in control rats, but not in STZ diabetic rats. Control rats fed low dietary P exhibited significant reductions in circulating PTH compared to their AIN fed controls, whereas STZ diabetic fed low P diets exhibited no change in PTH (Figure 4.5.1.a.2.3)

Bone Parameters

Bone mass and bone mineral density were significantly lower in STZ diabetics than in control rats when fed AIN control diets. In contrast, bone parameters were not different between STZ diabetics and controls fed either low Ca or low P diets (Table 4.5.1.a.2.3).

Ca per g bone was significantly reduced in STZ diabetics compared to control rats when fed the AIN control diet. However, bone Ca was not different in low Ca or low P fed STZ diabetics and controls when fed low Ca or low P. Ca per g bone was significantly reduced in response to low Ca or low P diets in control and diabetic rats (Table 4.5.1.a.2.5). Similar results were obtained for Ca per g ash (data not shown).

The low P diet decreased bone P per g bone in control rats but not in, STZ diabetic rat (Table 4.5.1.a.2.4). Similar results were obtained for P per g ash (data not shown).

TABLE 4.5.1.a.2.1

Indices of diabetes in STZ diabetic and control rats fed control, Low Ca and Low P diets

	BODY WEIGHT[1] (g)	GLUCOSE[1] (mg/dl)	INSULIN[1] (microUnits/ml)
AIN CONTROL DIET			
CONTROL	218.3 ± 0.5[a,c]	125.3 ± 9.6[a,c]	15.0 ± 1.1[a,c]
STZ[2] DIABETIC	122.3 ± 4.2[b]	316.7 ± 42.1[b,e]	9.4 ± 1.1[b]
LOW Ca DIET			
CONTROL	232.9 ± 6.2[a,d]	115.5 ± 6.8[a]	22.7 ± 3.9[a,d]
STZ DIABETIC	124.8 ± 6.0[b]	505.9 ± 18.0[b]	10.3 ± 0.9[b]
LOW P DIET			
CONTROL	230.9 ± 5.0[a,d]	105.0 ± 4.0[a,d]	DATA NOT
STZ DIABETIC	123.1 ± 4.4[b]	671.4 ± 84.7[b,f]	AVAILABLE

[1]Data are mean ± SEM, n ≥ 6
[2]Streptozotocin
[a,b]Comparison of control and diabetics within each dietary regime, Means with different letters in their superscripts are significantly ($p<0.05$) different by one tailed STUDENTS T-test
[c,d]Comparison of control groups, effect of dietary regime, Means with different letters in their superscripts are significantly ($p<0.05$) different by one tailed STUDENTS T-test
[e,f]Comparison of diabetic groups, effect of dietary regime, Means with different letters in their superscripts are significantly ($p<0.05$) different by one tailed STUDENTS T-test

TABLE 4.5.1.a.2.2

Plasma Ca and P in STZ diabetic and control rats fed control, Low Ca and Low P diets

	Ca[1] (mg/dl)	P[1] (mg/dl)
AIN CONTROL DIET		
CONTROL	$10.0 \pm 0.2^{a,c}$	5.2 ± 0.1^{c}
STZ[2] DIABETIC	$9.2 \pm 0.2^{b,e}$	5.5 ± 0.2^{e}
LOW Ca DIET		
CONTROL	10.4 ± 0.4^{a}	5.1 ± 0.1^{a}
STZ DIABETIC	9.7 ± 0.2^{a}	6.1 ± 0.4^{b}
LOW P DIET		
CONTROL	$11.1 \pm 0.2^{a,d}$	$4.7 \pm 0.1^{a,d}$
STZ DIABETIC	$10.0 \pm 0.2^{b,f}$	$5.0 \pm 0.2^{b,f}$

[1]Data are mean ± SEM, n ≥ 6

[2]Streptozotocin

[a,b]Comparison of control and diabetics within each dietary regime, Means with different letters in their superscripts are significantly ($p<0.05$) different by one tailed STUDENTS T-test

[c,d]Comparison of dietary regimes on control groups, Means with different letters in their superscripts are significantly ($p<0.05$) different by one tailed STUDENTS T-test

[e,f]Comparison of dietary regimes on diabetic groups, Means with different letters in their superscripts are significantly ($p<0.05$) different by one tailed STUDENTS T-test

FIGURE 4.5.1.a.2.1 Net synthesis of $1,25(OH)_2D_3$ in renal proximal tubules isolated from STZ diabetic and control rats fed control and low Ca diets.

Data are presented as individual synthesis rates of $1,25(OH)_2D_3$ ± SEM, n = 4-6 determinations of duplicate determinations made in proximal tubules freshly isolated from STZ diabetic and control rats fed Control (0.06% Ca) and low Ca (0.013% Ca) diets. Data are expressed as net $1,25(OH)_2D_3$ synthesis (fmoles/min/mg tubule protein). Diabetics fed control diets had significantly lower $1,25(OH)_2D_3$ synthesis rates as compared to controls fed control diets ($p<0.05$). Controls fed low Ca diets exhibited significantly elevated $1,25(OH)_2D_3$ synthesis rates as compared to controls fed control diets ($p<0.05$). However, while STZ diabetics fed low Ca diets exhibited significantly elevated $1,25(OH)_2D_3$ synthesis rates as compared to STZ diabetic rats fed control diets ($p<0.05$). $1,25(OH)_2D_3$ synthesis in STZ diabetics fed low Ca remained significantly lower than controls fed low Ca diets ($p<0.05$).

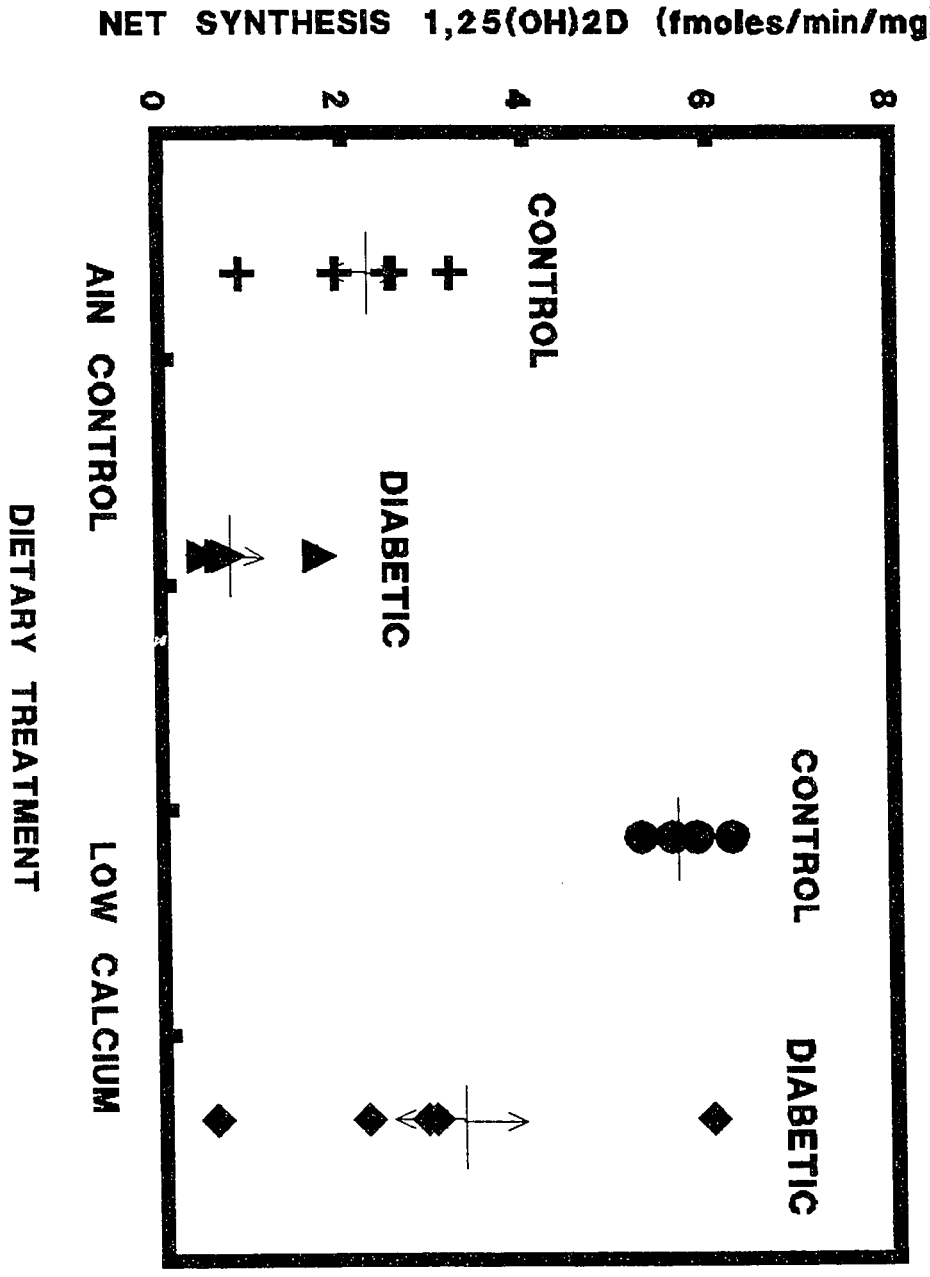

NET SYNTHESIS 1,25(OH)2D (fmoles/min/mg)
0
2
4
6
8
CONTROL
DIABETIC
CONTROL
DIABETIC
AIN CONTROL
LOW CALCIUM
DIETARY TREATMENT

FIGURE 4.5.1.a.2.2 Circulating $1,25(OH)_2D_3$ measured in STZ diabetic and control rats fed control, low Ca or low P diets.
Data are mean ± SEM, n = 6-7 determinations of duplicate measurements of circulating $1,25(OH)_2D_3$ in serum from individual control and STZ diabetic rats fed control, low Ca or low P diets. STZ diabetic rats had significantly (*) ($p<0.05$) lower circulating $1,25(OH)_2D_3$ as compared to controls, irrespective of dietary regime. No significant differences between circulating $1,25(OH)_2D_3$ were observed between STZ diabetics fed control, low Ca or low P diets. Control rats fed low Ca diets had significantly (**) ($p<0.05$) elevated circulating $1,25(OH)_2D_3$ in comparison to controls fed control diets. Control rats fed low P diets had significantly (**) ($p<0.05$) elevated circulating $1,25(OH)_2D_3$ in comparison to controls fed control diets. No significant differences in circulating $1,25(OH)_2D_3$ were observed between controls fed low dietary Ca or low dietary P.

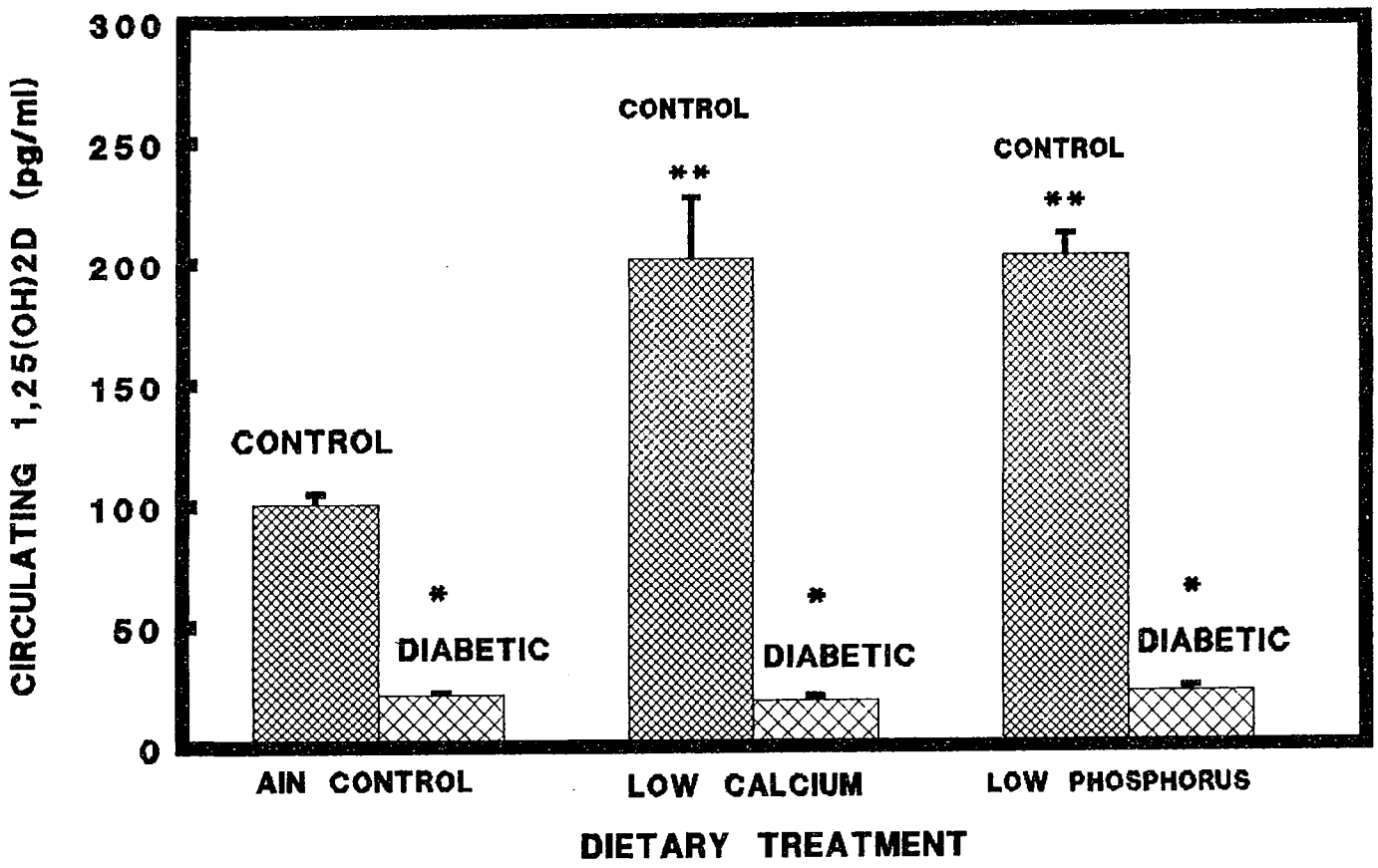

CIRCULATING 1,25(OH)2D (pg/ml)
300
250
200
150
100
50
0
CONTROL
**
CONTROL
**
CONTROL
*
*
*
DIABETIC
DIABETIC
DIABETIC
AIN CONTROL
LOW CALCIUM
LOW PHOSPHORUS
DIETARY TREATMENT

FIGURE 4.5.1.a.2.3 Circulating PTH in STZ diabetic and control rats fed control, low Ca or low P diets.
Data are mean ± SEM, n = 6-7 determinations of duplicate measurements of circulating PTH in serum from individual control and STZ diabetic rats fed control, low Ca or low P diets. No significant differences between circulating PTH were observed between control and STZ diabetics fed control diets. Controls fed low Ca diets had significantly (*) ($p<0.05$) elevated circulating PTH while controls fed low P diets had significantly (**) ($p<0.05$) lower circulating PTH in comparison to controls fed control diets. No significant differences in circulating PTH were observed between STZ diabetic rats fed control, low Ca or low P diets.

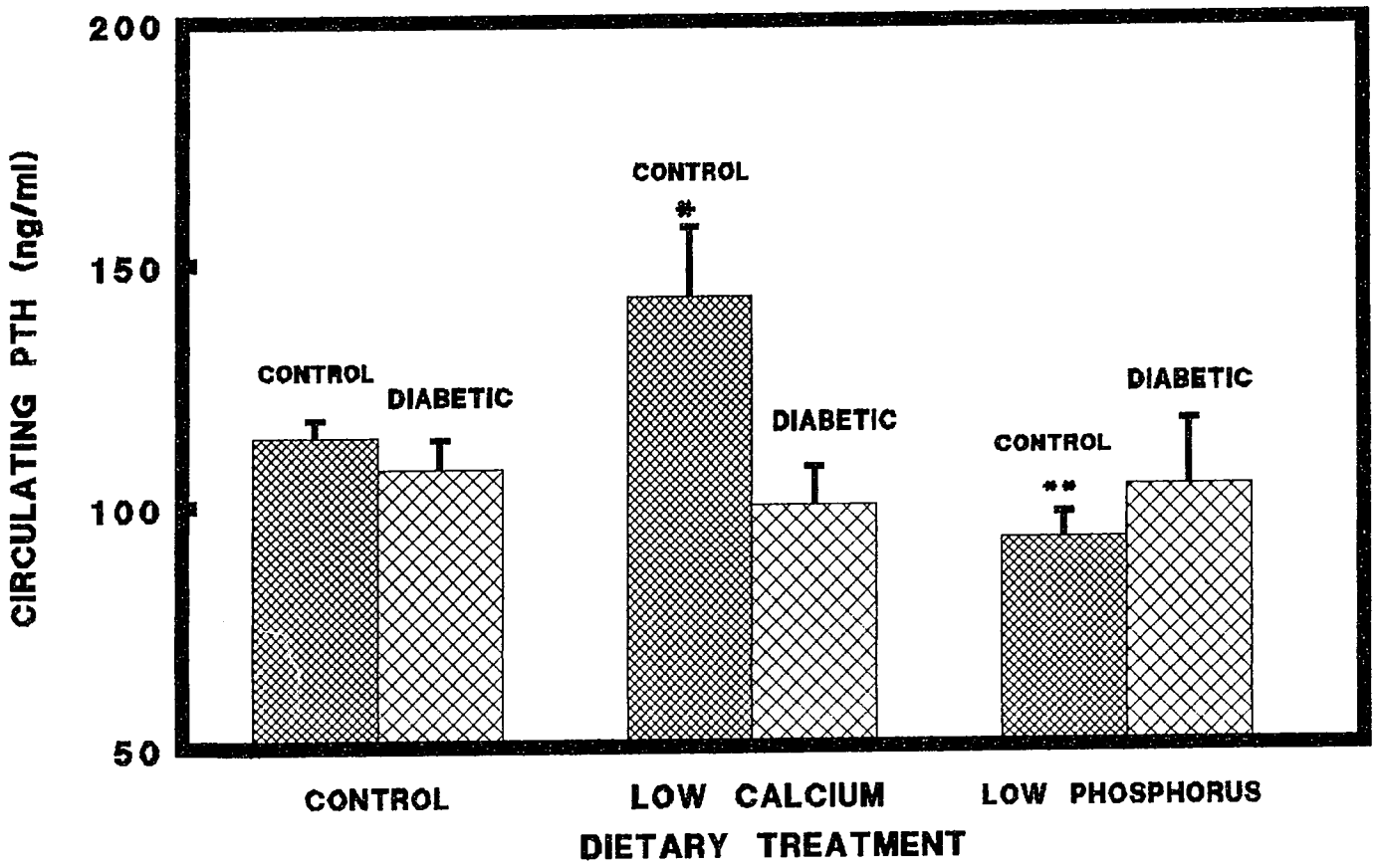

200
150
100
50
CIRCULATING PTH (ng/ml)
CONTROL
DIABETIC
CONTROL
*
DIABETIC
CONTROL
**
DIABETIC
CONTROL
LOW CALCIUM
LOW PHOSPHORUS
DIETARY TREATMENT

TABLE 4.5.1.a.2.3

Bone parameters in STZ diabetic and control rats fed control, Low Ca and Low P diets

	BONE MASS[1] (mg dry weight)	MINERAL DENSITY[1] (mg ASH/g BONE)
AIN CONTROL DIET		
CONTROL	0.361 ± 0.004	0.419 ± 0.006
STZ[2] DIABETIC	0.283 ± 0.005*	0.380 ± 0.011*
LOW Ca DIET		
CONTROL	0.382 ± 0.005	0.407 ± 0.004
STZ DIABETIC	0.302 ± 0.008	0.393 ± 0.008
LOW P DIET		
CONTROL	0.393 ± 0.007	0.425 ± 0.009
STZ DIABETIC	0.318 ± 0.004	0.410 ± 0.010

[1]Data are means ± SEM, n ≥ 6
[2]Streptozotocin
*Significantly ($p<0.05$) different compared to control

TABLE 4.5.1.a.2.4

Bone P in STZ diabetic and control rats fed control, Low Ca and Low P diets

	mg PHOSPHORUS/ g BONE[1]	mg PHOSPHORUS/ g ASH[1]
AIN BASAL DIET		
CONTROL	106.8 ± 1.3[c]	255.2 ± 3.2[a,c]
STZ[2] DIABETIC	106.7 ± 1.6	281.1 ± 5.3[b,e]
LOW CALCIUM DIET		
CONTROL	105.1 ± 1.7	258.5 ± 4.1[a]
STZ DIABETIC	107.6 ± 1.4	271.3 ± 5.5[b]
LOW PHOSPHORUS DIET		
CONTROL	103.3 ± 0.9[a,d]	243.1 ± 3.9[a,d]
STZ DIABETIC	108.3 ± 1.5[b]	264.8 ± 4.7[b,f]

[1]Data are mean ± SEM, n > = 6

[2]Streptozotocin

[a,b]Comparison of control with diabetics within each dietary regime, Means with different letters in their superscripts are significantly (p>0.05) different by one tailed STUDENTS T-test

[c,d]Comparison of control groups, effect of dietary regime, Means with different letters in their superscripts are significantly (p>0.05) different by one tailed STUDENTS T-test

[e,f]Comparison of diabetic groups, effect of dietary regime, Means with different letters in their superscripts are significantly (p>0.05) different by one tailed STUDENTS T-test

TABLE 4.5.1.a.2.5

Bone Ca in STZ diabetic and control rats fed control, Low Ca and Low P diets

	mg Ca/ g BONE[1]	mg Ca/ g ASH[1]
AIN CONTROL DIET		
CONTROL	226.6 ± 4.0[a,c]	541.3 ± 9.8[c]
STZ[2] DIABETIC	197.0 ± 3.5[b]	552.4 ± 14.8[e]
LOW Ca DIET		
CONTROL	198.7 ± 14.8[d]	488.3 ± 9.0[a,d]
STZ DIABETIC	203.1 ± 4.5	514.4 ± 8.5[b,f]
LOW P DIET		
CONTROL	197.6 ± 6.6[d]	465.5 ± 18.4[d]
STZ DIABETIC	201.8 ± 8.1	492.7 ± 23.5[f]

[1]Data are mean ± SEM, n ≥ 6
[2]Streptozotocin
[a,b]Comparison of control and diabetics within each dietary regime, Means with different letters in their superscripts are significantly ($p<0.05$) different by one tailed STUDENTS T-test
[c,d]Comparison of controls, effect of dietary regime, Means with different letters in their superscripts are significantly ($p<0.05$) different by one tailed STUDENTS T-test
[e,f]Comparison of diabetics, effect of dietary regime, Means with different letters in their superscripts are significantly ($p<0.05$) different by one tailed STUDENTS T-test

4.5.2.b.1. In vitro Protein Kinase C and Vitamin D Metabolism

Effect of TPA and 4α-PDD on $1,25(OH)_2D_3$ production

In rat renal proximal tubules, one hour incubation with 160nM TPA reduced $1,25(OH)_2D_3$ synthesis to approximately 55% of control ($p<0.05$). As reported in Table 4.5.1.b.1.1, the phorbol ester 4α-PDD at the same dose (160nM) had no effect on $1,25(OH)_2D_3$ production. Time course data revealed that the inhibitory effect of TPA was extremely rapid, with a 15% decrease in $1,25(OH)_2D_3$ production apparent within ten minutes of incubation (Figure 4.5.1.b.1.1). After 20 minutes of incubation, the amount of $1,25(OH)_2D_3$ accumulated was significantly lower in tubules treated with TPA compared to tubules treated with vehicle ($p<0.05$). The effect persisted for up to 60 minutes, when $1,25(OH)_2D_3$ production in TPA treated tubules was approximately 60% of control. As shown in Figure 4.5.1.b.1.2, 10 nM TPA (the lowest dose tested) significantly inhibited $1,25(OH)_2D_3$ production. The maximal reduction in $1,25(OH)_2D_3$ production occurred with concentrations of TPA above 100nM.

Effect of TPA and 4α-PDD on $24,25(OH)_2D_3$ production

In contrast to the inhibitory effect of TPA on $1,25(OH)_2D_3$ production, 160nM TPA significantly increased $24,25(OH)_2D_3$ production (Figure 4.5.1.b.1.3). 4α-PDD, the phorbol ester which did not affect $1,25(OH)_2D_3$ production, also had no effect on $24,25(OH)_2D_3$ production. The time course for the effect of TPA on $24,25(OH)_2D_3$ synthesis inversely paralleled that for the effect of TPA on $1,25(OH)_2D_3$ synthesis (Figure 4.5.1.b.1.1). However, the effect of TPA on $24,25(OH)_2D_3$ production appeared slightly slower than the effect on $1,25(OH)_2D_3$ production, as no significant effect of TPA on $24,25(OH)_2D_3$ production was observed before 30 minutes incubation. The dose response for the effect of TPA indicated that > 50nM was required for stimulation of $24,25(OH)_2D_3$ (Figure 4.5.1.b.1.2). There was a dose dependent increase in $24,25(OH)_2D_3$ production in the concentration range from 25-200nM TPA.

Effect of PKC down regulation and staurosporine on $1,25(OH)_2D_3$ production

The best characterized cellular effect of TPA (by merit of its structural analogy to DAG) is the activation of PKC by binding directly to the enzyme; thereby raising its affinity for Ca. However other cellular effects of TPA have been demonstrated. Implication of a role for PKC activity in the effect of TPA on $1,25(OH)_2D_3$ production therefore requires demonstration that factors known to modulate PKC activity modulate TPA's effect on $1,25(OH)_2D_3$ production. To test the effect of down regulation of PKC activity on $1,25(OH)_2D_3$ production, tubules were pre-incubated with 160nM TPA for 18 hours, after which the response to another addition of 160nM TPA was assessed. $1,25(OH)_2D_3$ net synthesis in tubules pre-incubated for 18 hours with vehicle and then exposed to 160nM TPA for one hour was approximately 55% of that in tubules pre-incubated for 18 hours with vehicle followed by one hour vehicle (Figure 4.5.1.b.1.4). This response was similar to that observed in freshly isolated tubules (Table 4.5.1.b.1.1) indicating no change in sensitivity to TPA in tubules incubated in K1 media overnight. However, if tubules were pre-incubated with 160nM TPA for 18 hours and then re-exposed to 160nM TPA for one hour, no inhibition of $1,25(OH)_2D_3$ production was observed.

In a similar experiment, tubules were pre-incubated for 18 hours with varying doses of staurosporine (a PKC inhibitor) or vehicle, after which the response to one hour incubation with 160nM TPA was compared to one hour with vehicle. Once again, tubules pre-incubated with vehicle and then exposed to TPA exhibited the expected inhibition of $1,25(OH)_2D_3$ production (Figure 4.5.1.b.1.5). Pre-incubation of tubules with 5nM staurosporine did not affect the inhibition of $1,25(OH)_2D_3$ production mediated by TPA. In contrast, tubules pre-incubated with 10, 25 or 50 nM staurosporine and then exposed to TPA exhibited no inhibition of $1,25(OH)_2D_3$ production compared to tubules pre-incubated with staurosporine and not exposed to TPA.

Effect of TPA on PKC activity

Acute temporal effect of TPA

PKC activity measured in PNF of membrane and cytosol prepared from rat renal tubules was linear up to and including 25ug of membrane protein and 50ug of cytosolic protein (Refer to "Materials and Methods"). The acute effect of TPA on PKC activity was measured in membranes and cytosol derived from proximal tubules exposed to 160nM TPA for up to 60 minutes. As shown in Figure 4.5.1.b.1.6, membrane PKC was increased to approximately 180% of basal activity within one minute of TPA exposure. The increase in PKC activity was transient, peaking at 212% of basal activity after 5 minutes with TPA and declining thereafter. Within 30 minutes, membrane PKC activity was restored to basal levels.

The rapid, transient increase in membrane PKC activity was paralleled by a rapid, transient decrease in cytosolic PKC activity. Cytosolic PKC activity was significantly decreased from basal between one and five minutes after TPA exposure, with a return to baseline by 60 minutes. The maximal decrease in cytosolic PKC activity, observed at 5 minutes, was approximately 60% of control. The parallel decline in cytosolic PKC activity as membrane PKC activity increased is consistent with TPA-induced translocation of cytosolic PKC to membrane.

Effect of chronic exposure to TPA

In contrast to the acute stimulatory effect of TPA on membrane PKC activity, long term (18 hour) exposure of proximal tubules to 160nM TPA resulted in a 54% decrease in membrane PKC activity (Table 4.5.1.b.1.2). Cytosolic PKC activity was also reduced (approximately 44%) after long term exposure to TPA. These changes are indicative of PKC down regulation. The sensitivity of the PKC remaining after down regulation was assessed by re-exposing tubules to TPA for 5 minutes, the time point where peak translocation of PKC was observed in freshly isolated tubules (Figure 4.5.1.b.1.6). Tubules pre-incubated with vehicle (non-down regulated)

responded to 5 minute exposure to TPA with an increase in membrane PKC activity (189% of basal) and a decrease in cytosolic PKC activity (65% of basal) (data not shown). This response is comparable to the acute effect of TPA observed in freshly isolated tubules (Figure 4.5.1.b.1.6) indicating that overnight incubation in K1 media did not significantly alter the sensitivity of tubule PKC to translocation in response to TPA (Data not shown). In contrast, PKC activity in membranes or cytosol from tubules which had been pre-incubated with TPA (down regulated) did not change in response to acute exposure to TPA (Table 4.5.1.b.1.2). These data indicate that chronic exposure of proximal tubules to TPA substantially reduced basal PKC activity, and, further, that the PKC activity remaining after down regulation was insensitive to acute stimulation by TPA.

TABLE 4.5.1.b.1.1

Effect of TPA and 4αPDD on net $1,25(OH)_2D_3$ production in isolated proximal tubules

NET $1,25(OH)_2D$ PRODUCTION[1]	
BASAL[2]	8.0 ± 0.9
TPA[3]	4.7 ± 0.9*
4αPDD[4]	7.0 ± 0.9

Tubules were incubated for 1 hour with 5uM $25(OH)D_3$ and 160nM TPA, 160nM 4αPDD or vehicle. The $1,25(OH)_2D$ produced was extracted and quantitated by radioreceptor assay as described in "Materials and Methods".

[1]Expressed as fmoles $1,25(OH)_2D$ produced per min per mg tubule protein. Data are mean ± sem, n = 3-6 separate tubule isolations with duplicate or triplicate incubations for each isolation.
[2]Tubules exposed to vehicle
[3]Tubules exposed to 160nM TPA
[4]Tubules exposed to 160nM 4αPDD
*Significantly different from basal value

FIGURE 4.5.1.b.1.1 Time course of effect of 160nM TPA on net $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ synthesis in isolated renal tubules. $1,25(OH)_2D_3$ production (measured in proximal renal tubules) and $24,25(OH)_2D_3$ production (measured in renal tubules) was measured with 5 µM $25(OH)D_3$ or 1 µM $25(OH)D_3$ and 100,000 cpm ^3H $25(OH)D_3$ after exposure to 160 nM TPA for up to 60 minutes. Reactions were terminated by addition of equivolume acetonitrile and vitamin D metabolites were isolated and quantitated as described under "Materials and Methods". Data are mean ± SEM, n = 3-4 separate tubule isolations with duplicate or triplicate incubations for each isolation. Data are expressed as % basal (vehicle treated) activity measured in paired incubations exposed to vehicle (4αPDD) for the indicated times. % net synthesis of $1,25(OH)_2D_3$ was decreased after 10 minutes incubation in the presence of TPA and was significantly (*) ($p<0.05$) decreased after 20 minutes, thereafter synthesis rates remained significantly depressed. % net synthesis of $24,25(OH)_2D_3$ was significantly increased (*) ($p<0.05$) after 30 minutes incubation in the presence of TPA, remaining elevated for the duration of the incubation times examined (120 minutes, data not shown).

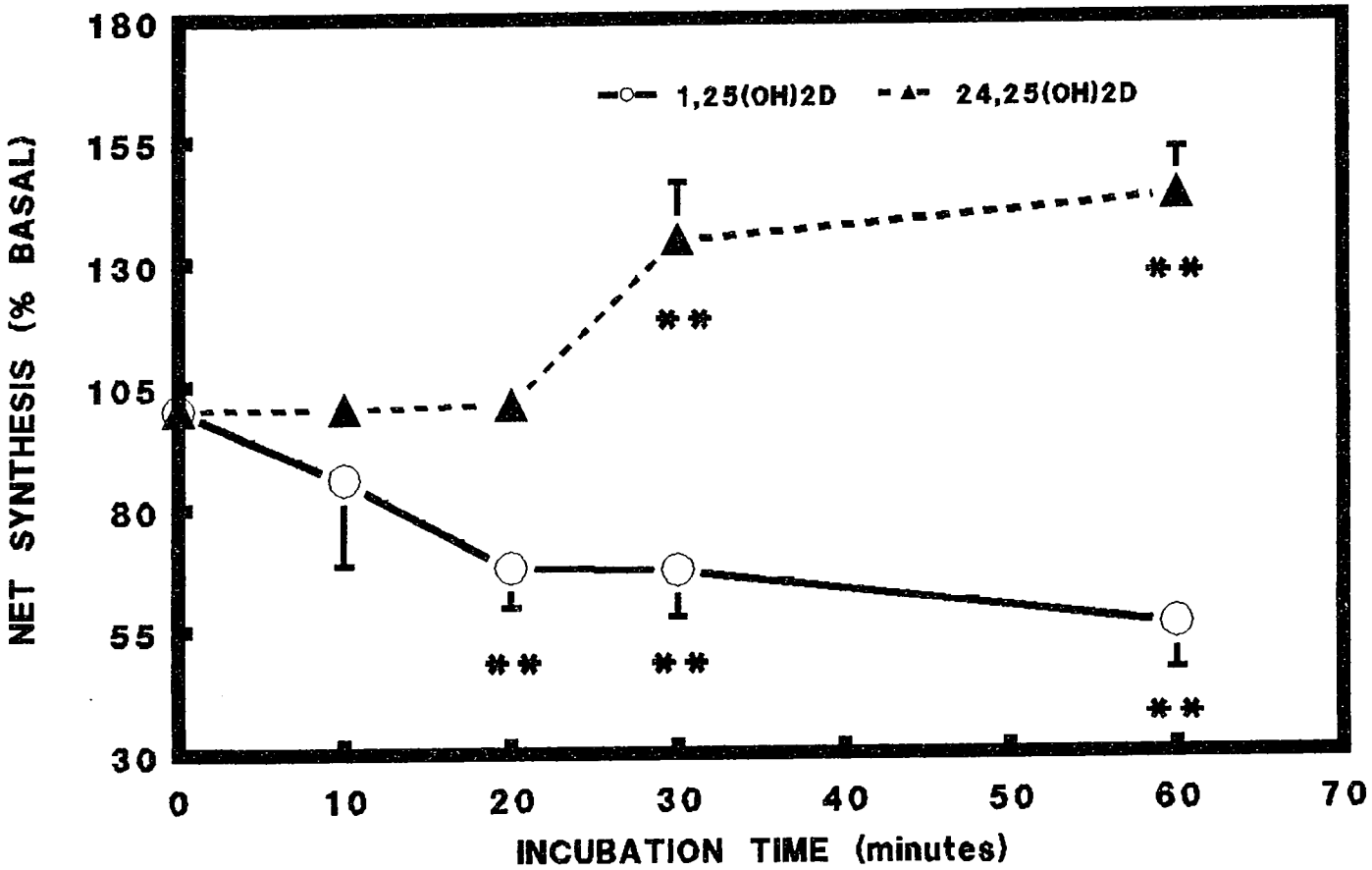

1,25(OH)2D
24,25(OH)2D
NET SYNTHESIS (% BASAL)
180
155
130
105
80
55
30
0
10
20
30
40
50
60
70
INCUBATION TIME (minutes)
**

FIGURE 4.5.1.b.1.2 Net $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ synthesis in isolated renal tubules incubated with increasing doses of TPA.
$1,25(OH)_2D_3$ production (measured in proximal renal tubules) and $24,25(OH)_2D_3$ production (measured in renal tubules) was quantitated after exposure to increasing concentrations of TPA (as indicated) with 5 μM $25(OH)D_3$ or 1 μM $25(OH)D_3$ and 100,000 cpm ^3H $25(OH)D_3$ for 60 minutes. Reactions were terminated by addition of equivolume acetonitrile and vitamin D metabolites were isolated and quantitated as described under "Materials and Methods". Data are mean ± SEM, n = 3 separate tubule isolations with duplicate or triplicate incubations for each isolation. Data are expressed as % basal activity measured in paired incubations exposed to inactive phorbol ester analogue (4αPDD) for 60 minutes. Net synthesis of $1,25(OH)_2D_3$ was significantly (*) ($p<0.05$) decreased in response to 10 nM TPA, and maximal inhibition was observed at TPA concentrations of 100 nM and greater. Significantly (*) ($p<0.05$) increased net synthesis of $24,25(OH)_2D_3$ was observed at concentrations of 100 nM TPA and greater.

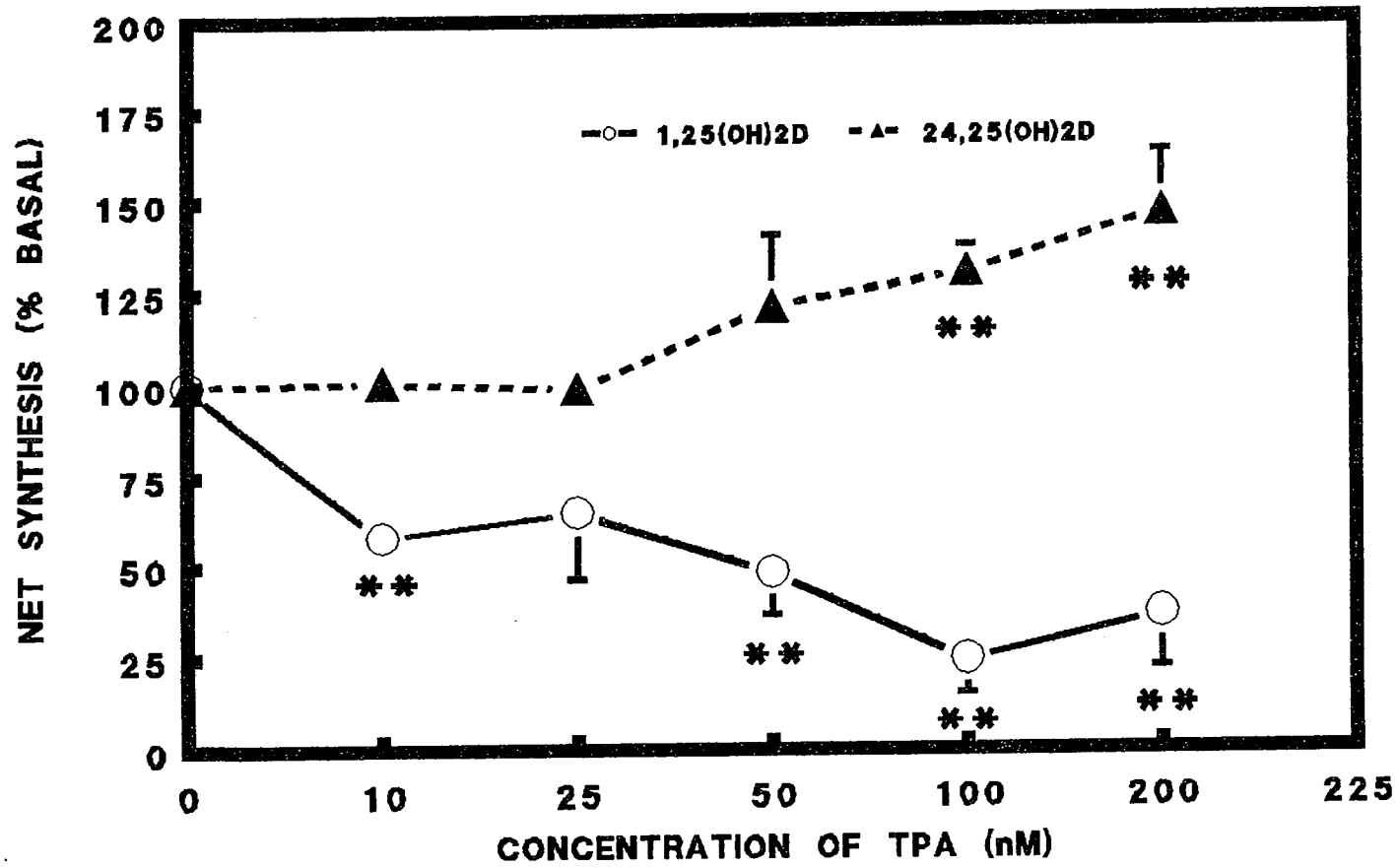

200
175
150
125
100
75
50
25
0
NET SYNTHESIS (% BASAL)
1,25(OH)2D
24,25(OH)2D
**
0
10
25
50
100
200
225
CONCENTRATION OF TPA (nM)

FIGURE 4.5.1.b.1.3 Effect of 160nM TPA or 160nM 4αPDD on net $24,25(OH)_2D_3$ production.

Data are mean ± SEM, n = 3 separate fresh tubule isolations with duplicate or triplicate incubations for each isolation. Tubules were incubated for 1 hour with 1 μM $25(OH)D_3$ and 100,000 cpm ^3H $25(OH)D_3$ and 160 nM TPA, 160 nM 4αPDD or vehicle. Reactions were terminated by addition of equivolume acetonitrile and $24,25(OH)_2D_3$ was isolated and quantitated as described under "Materials and Methods". Data are expressed as % basal $24,25(OH)_2D_3$ produced per min per mg tubule protein. No significant differences between basal and 4αPDD $24,25(OH)_2D_3$ synthesis rates were observed. $24,25(OH)_2D_3$ synthesis was significantly (*) ($p<0.05$) elevated in the TPA treated tubules as compared to the 4αPDD treated tubules.

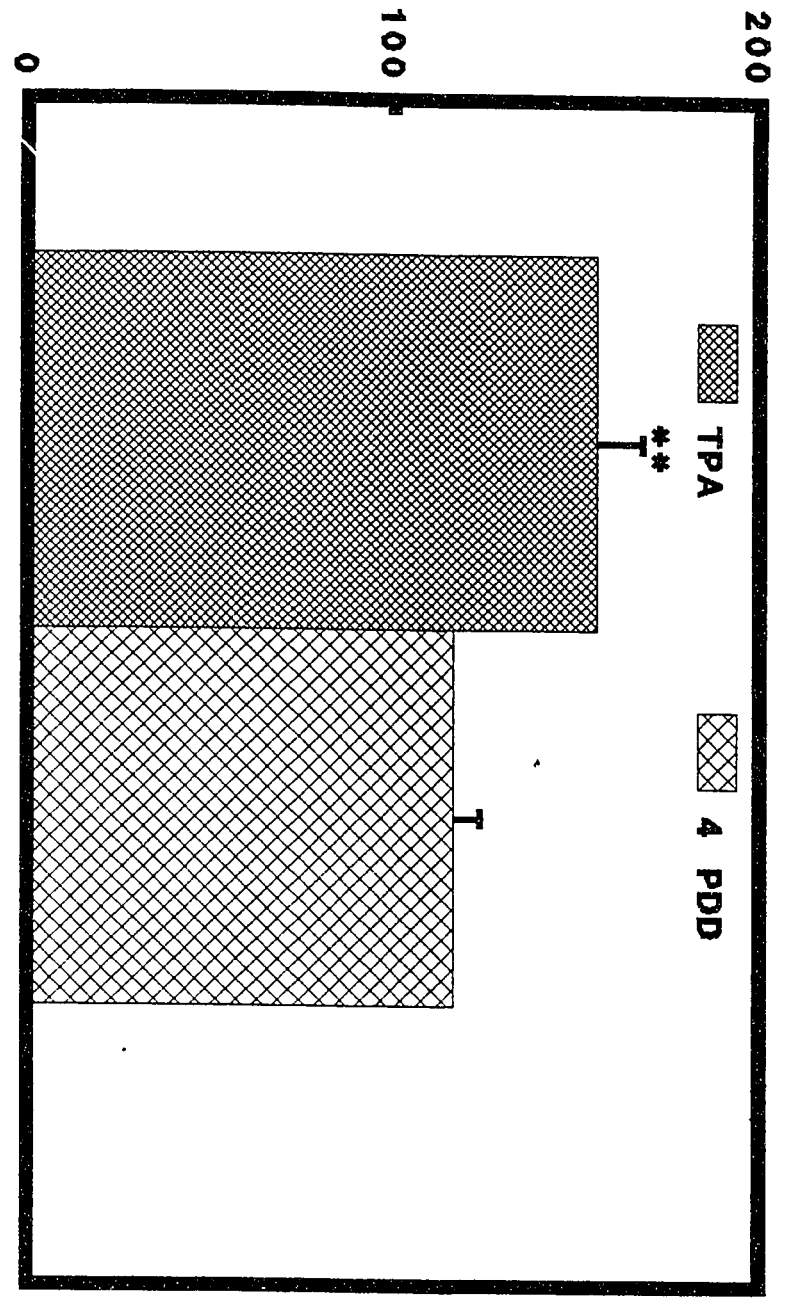

NET SYNTHESIS 24,25(OH)2D (% BASAL)
0
100
200
TPA
4 PDD
**

FIGURE 4.5.1.b.1.4 Effect of PKC down regulation on TPA mediated inhibition of net $1,25(OH)_2D_3$ synthesis.
Data are mean ± SEM, n = 2-3 separate fresh tubule isolations with at least triplicate incubations for each isolation. Tubules were incubated for 18 hours with 160 nM TPA (down regulated) or vehicle (Basal) after which 5 µM $25(OH)D_3$ and 160 nM TPA or 160 nM 4αPDD were added and the tubules were further incubated for 1 hour. Reactions were terminated by addition of equivolume acetonitrile and $1,25(OH)_2D_3$ was isolated and quantitated as described under "Materials and Methods". Data are expressed as % 4αPDD treated tubule net $1,25(OH)_2D_3$ synthesis. No significant difference in net $1,25(OH)_2D_3$ synthesis was observed between tubules pre-incubated with vehicle or 160 nM TPA for 18 hours and subsequently treated with 4αPDD for 1 hour (data not shown). 160 nM TPA significantly (*) ($p<0.05$) decreased net synthesis of $1,25(OH)_2D_3$ in tubules pre-incubated for 18 hours with vehicle (Basal) whereas no significant decrease in net $1,25(OH)_2D_3$ synthesis was observed in tubules pre-incubated for 18 hours with 160 nM TPA (down regulation).

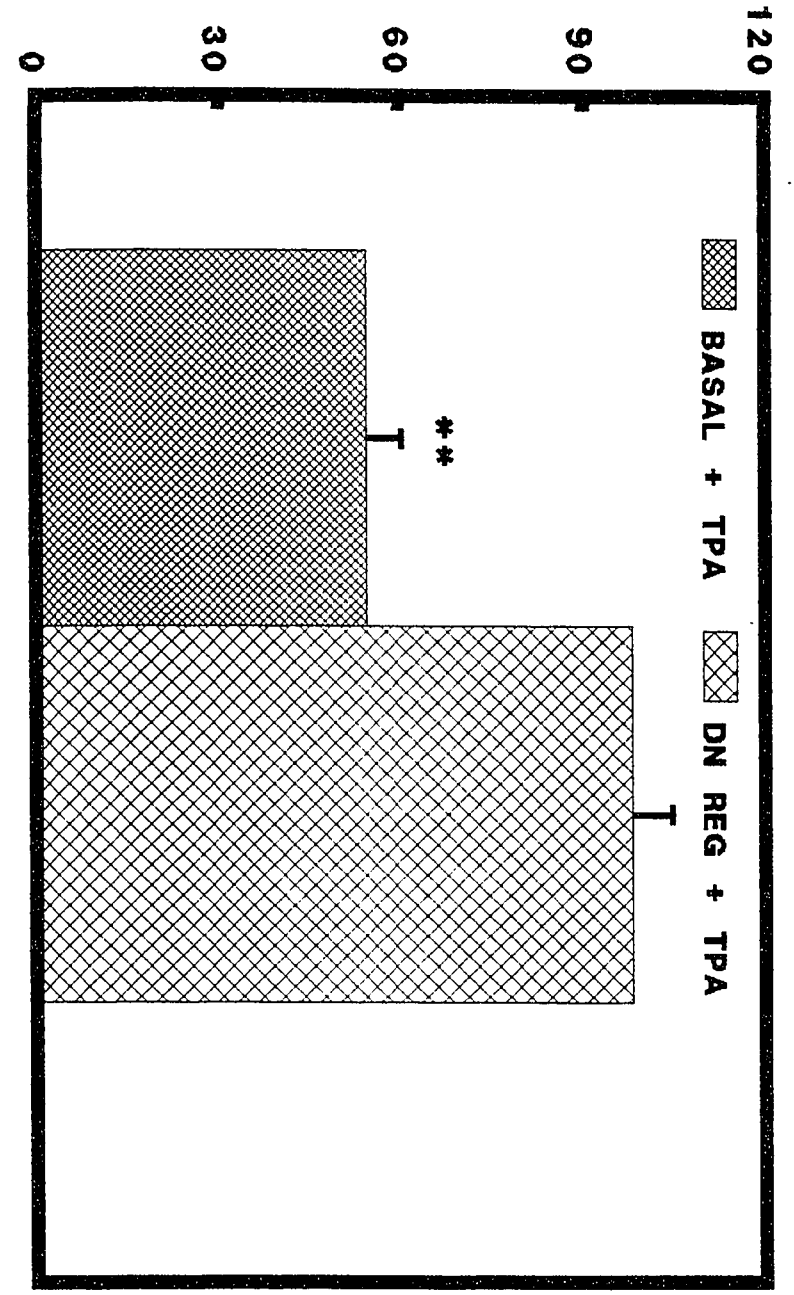

NET SYNTHESIS 1,25(OH)2D (% VEHICLE)
0
30
60
90
120
BASAL + TPA
DN REG + TPA
**

FIGURE 4.5.1.b.1.5 Effect of staurosporine pre-incubation on TPA mediated inhibition of net $1,25(OH)_2D_3$ synthesis.
Data are mean ± SEM, n = duplicate or triplicate incubations of at least 2 separate tubule isolations. Tubules were pre-incubated for 18 hours with the indicated concentration of staurosporine, followed by incubation for 1 hour with 160 nM TPA or vehicle and 5 μM $25(OH)D_3$. Reactions were terminated by addition of equivolume acetonitrile and $1,25(OH)_2D_3$ was isolated and quantitated as described under "Materials and Methods". Data are expressed as % of basal $1,25(OH)_2D_3$ produced in tubules pre-incubated with the indicated concentration of staurosporine and exposed to vehicle rather than TPA during the enzyme assay. * Significantly ($p<0.05$) difference for tubules pre-incubated with same concentration of staurosporine for 18 hours and exposed to vehicle during enzyme assay.

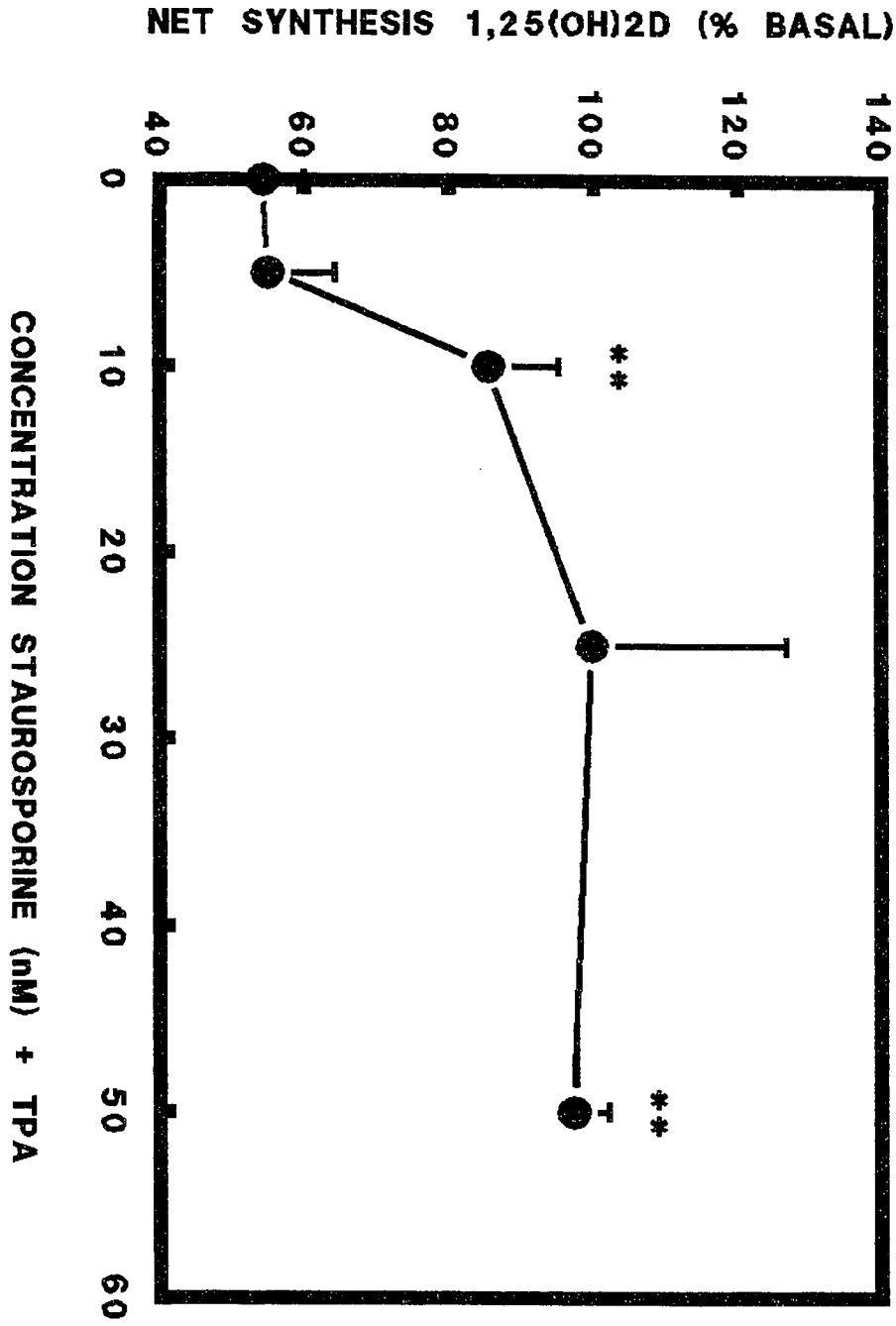

NET SYNTHESIS 1,25(OH)2D (% BASAL)
40
60
80
100
120
140
0
10
20
30
40
50
60
**
**
CONCENTRATION STAUROSPORINE (nM) + TPA

FIGURE 4.5.1.b.1.6 Effect of TPA on renal tubular subcellular PKC activity.

Data are mean ± SEM, n = triplicate incubations from 3 separate tubule isolations for membrane and 5 separate tubule isolations for cytosol. Renal tubules were incubated with 160 nM TPA for the indicated times, after which PKC activity was measured in membrane and cytosolic fractions as phosphorylation of a PKC specific peptide as described in "Materials and Methods". Incubation time did not affect PKC activity in cytosol or membrane fractions from vehicle treated controls. Data are therefore expressed relative to basal activity measured in vehicle treated controls, which were averaged and taken as 100%. ** Significantly ($p<0.05$) different from basal values.

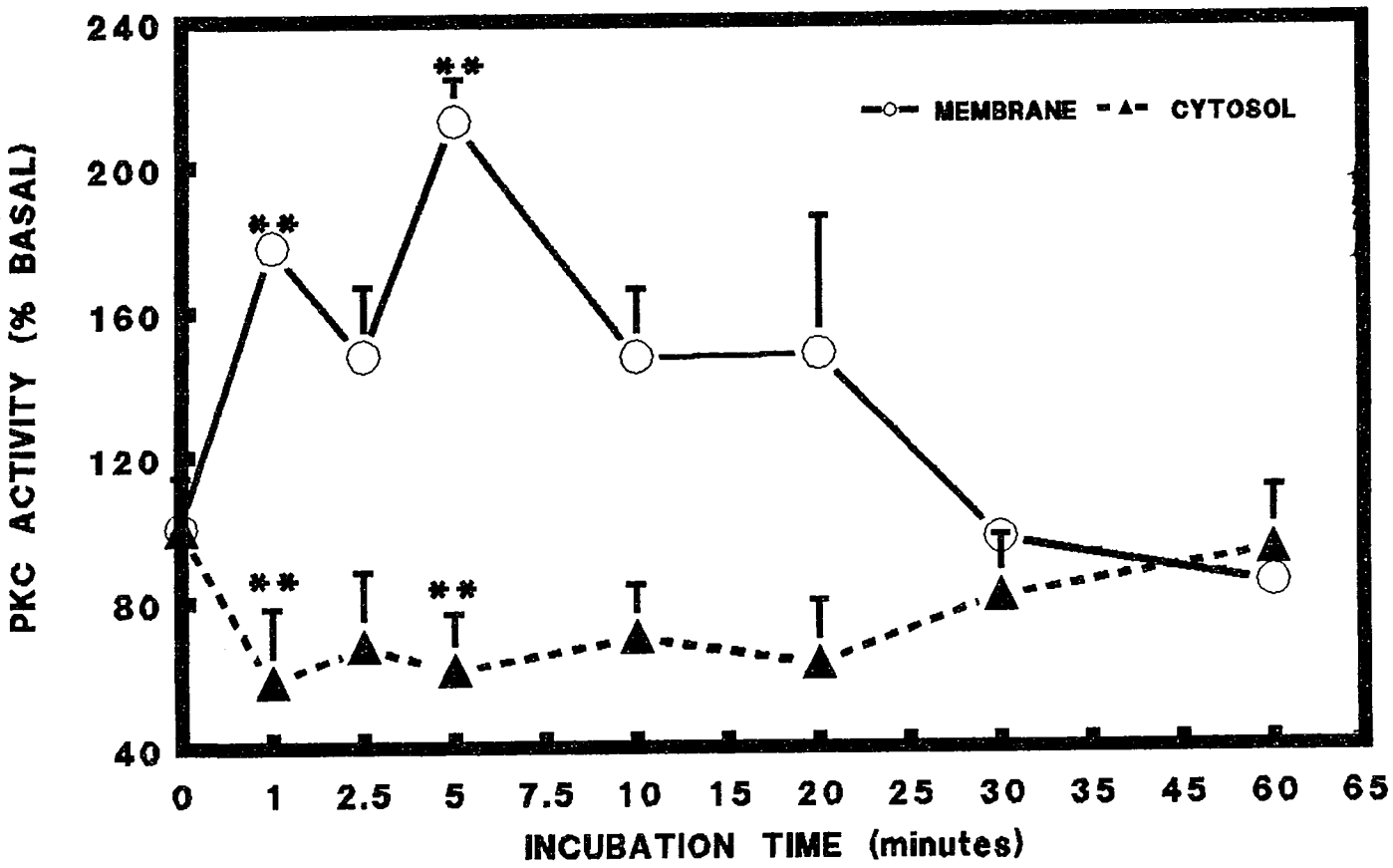

240
200
160
120
80
40
PKC ACTIVITY (% BASAL)
MEMBRANE
CYTOSOL
**
**
**
**
0
1
2.5
5
7.5
10
15
20
25
30
35
45
60
65
INCUBATION TIME (minutes)

TABLE 4.5.1.b.1.2

Effect of PKC down regulation on basal and TPA stimulated PKC activity in cytosol and membrane of renal tubules

	PKC ACTIVITY[1]	
	MEMBRANE	CYTOSOL
4αPDD[2]	19.3 ± 2.9	29.7 ± 8.3
DOWN REGULATION[3]	8.9 ± 1.4*	16.6 ± 1.9*
DOWN REGULATION + TPA[4]	10.1 ± 1.7*	20.7 ± 3.1

Proximal tubules were pre-incubated for 18 hours with TPA, 4αPDD or vehicle. After pre incubation, basal PKC activity was measured in membrane and cytosol fractions derived from tubule homogenates as described in "Materials and Methods".

[1]Expressed as pmoles peptide phosphorylated per min per mg protein. Data shown are mean ± sem, n = 3 separate tubule isolations assayed in duplicate.
[2]Pre-incubated with 160nM 4αPDD for 18 hours prior to measurement of PKC activity.
[3]Pre-incubated with 160nM TPA for 18 hours prior to measurement of PKC activity.
[4]Pre-incubated with 160nM TPA for 18 hours, followed by 5 minutes re-exposure to 160nM TPA prior to measurement of PKC activity.
*Significantly different from 4αPDD treated.

4.5.2.b.2. Protein Kinase C activity and Insulin Dependent Diabetes

Renal tubule PKC activity

Renal tubule membrane PKC activity was higher in diabetic rats seven days after diabetes induction compared to vehicle injected controls (Table 4.5.1.b.2.1). No statistical analyses of this data were made due to the limited number of experimental values (STZ diabetic n=3, Vehicle control n=2). No differences were observed in cytosolic PKC activity between the two groups (STZ diabetic n=4, Vehicle control n=4) (Table 4.5.1.b.2.1).

TABLE 4.5.1.b.2.1

Subcellular PKC activity in renal tubules isolated from STZ diabetic and control rats

	PKC ACTIVITY[1]	
	MEMBRANE	CYTOSOL
STZ DIABETIC[3]	2.77 ± 0.51	11.57 ± 0.45
CONTROL	2.10 ± 0.30	11.88 ± 1.01

[1]Expressed as pmoles peptide phosphorylated per min per mg protein.
[2]Data are mean ± SEM, n = 2-3 for membranes and n = 4 for cytosols.
[3]Streptozotocin injected diabetic rats.

4.5.3. DISCUSSION

4.5.3.a. ADENYLATE CYCLASE SIGNAL TRANSDUCTION AND INSULIN DEPENDENT DIABETES

These studies revealed no apparent defects in the efficacy of in vitro PTH to increase basal cAMP synthesis and 1αhydroxylase activity in tubules from diabetic rats. Likewise, these studies demonstrated that diabetes did not compromise stimulation of 1αhydroxylase activity in response to low dietary Ca.

The efficacy of PTH to increase intracellular cAMP in STZ diabetic tubules was comparable to that achieved in control tubules, consistent with previous reports (Wongsurawat et al., 1985, Rao and Kung, 1988). However, basal and PTH stimulated cAMP synthesis were higher in diabetic than in control tubules although forskolin enhanced cAMP synthesis was comparable. Jointly these results suggest an alteration in one or more of the adenylate cyclase components during insulin deficiency. Increased cAMP synthesis may be due to either elevated basal adenylate cyclase activity or decreased phosphodiesterase activity. Elevated adenylate cyclase activity may result from alterations induced in synthesis rates or number of catalytic units, decreased inhibition (induced by G_i) or increased stimulation (induced by G_s) (Houslay, 1991). Alterations in G_s seem unlikely since diabetes did not affect PTH induced cAMP stimulation, which functions through the G_s pathway (Slatolpolsky et al., 1990). However, a more thorough examination of this possibility would require PTH dose response curves in diabetic and control renal tubules. An increase in the number of adenylate cyclase catalytic units also appears unlikely since "maximal" cAMP synthesis in STZ diabetic and control rat tubules (in response to 10μM forskolin) were comparable. However, it is possible that 10μM forskolin did not maximally stimulate adenylate cyclase activity, therefore dose response curves with forskolin should be conducted to verify this conclusion. Wongsurawat, Armbrecht and Seigel (1991) reported comparable basal and PTH stimulated protein kinase A activity in

diabetic and control renal slices, suggesting decreased phosphodiesterase activity is not present in the diabetic kidney. An alteration in G_i in STZ diabetes could increase basal adenylate cyclase activity without altering the PTH receptor G_s pathway and could explain the elevated basal adenylate cyclase activity observed in these experiments. A relationship between PKC activity and the activity of G_i has been demonstrated (Katada et al., 1985) and the data presented in this thesis and reported by others suggest that diabetes is associated with increased renal membrane PKC activity (Hise and Mehta, 1988, figure 4.6.2.11). Further work to compare the effect of pertussis toxin, which inactivates G_i, in control and diabetic tubules is warranted.

While both exogenous and endogenous elevations of PTH increased 1αhydroxylase activity in STZ diabetic proximal tubules, the relative differences between diabetic and control enzyme rates remained unchanged. Thus while a low Ca diet and in vitro PTH increased both control and diabetic 1αhydroxylase activity to 200-300% basal and 130% basal activity respectively, stimulated diabetic 1αhydroxylase activity remained approximately 50% of stimulated control activity in both instances. Therefore stimulation of 1αhydroxylase activity, through the adenylate cyclase signal transduction pathway, fails to override defective $1,25(OH)_2D_3$ synthesis in diabetes. These results are in agreement with observations described by Wilson, Horst and Schedl (1982) and Ikeda et al. (1987). The failure to override the altered diabetic $1,25(OH)_2D_3$ synthesis may be due to decreased steady state levels of one or more 1αhydroxylase components, the absence of an auxiliary stimulatory or modulating factor (putative steroid transcription factor or putative phosphatase or kinase) which may affect 1αhydroxylase enzyme levels or activity, or enhanced inhibition of $1,25(OH)_2D_3$ synthesis. However, the in vitro PTH studies described in this thesis examined only one dosage of PTH and were conducted in the presence of insulin. These conditions may have masked any differences existing between PTH stimulation of 1αhydroxylase activity in diabetic and control rats in vivo. Although similar results were

obtained when diabetic animals were subjected to low dietary Ca where stimulation of 1αhydroxylase activity is mediated by PTH. Both the in vitro and in vivo studies examined chronic PTH mediated 1αhydroxylase stimulation, while both acute and chronic PTH mediated enhancement have been documented (Kremer and Goltzman, 1982, Siegal, Wongsurawat and Armbrecht, 1986, Henry, 1981, Korkor et al., 1987). Alterations in acute and/or chronic PTH induced 1αhydroxylase activity may exist in diabetes. In support of a potential defect in acute PTH stimulated $1,25(OH)_2D_3$ synthesis, Wongsurawat et al. (1991) observed failure of in vitro PTH to dephosphorylate renoredoxin, a component of the 1αhydroxylase system. Acute stimulation of 1αhydroxylase by PTH is associated with dephosphorylation of renoredoxin which occurs within five minutes (Siegel, Wongsurawat and Armbrecht, 1986). In contrast to the studies described in this thesis, Wongsurawat et al. (1991) failed to observe in vitro PTH or forskolin mediated 1αhydroxylase stimulation in diabetic renal slices, in which renoredoxin was not dephosphorylated by PTH. The discrepancy between their results and the results described in this thesis may be explained by differences in experimental protocols: $1x10^{-7}$ M PTH, phosphodiesterase inhibitor, 5 ug/ml insulin control diet, isolated purified proximal tubules (these studies) versus 5 U/ml PTH, thyroparathyroidectized rats, renal slices (no indication of phosphodiesterase inhibitor or insulin addition for in vitro 1αhydroxylase assay) (Wongsurawat et al., 1991 studies).

Phosphate restriction elevates $1,25(OH_2D_3$ synthesis and circulating $1,25(OH)_2D_3$ independently of PTH (Deluca, 1984). While phosphate restriction elevated circulating $1,25(OH)_2D_3$ in control rats, circulating $1,25(OH)_2D_3$ was not elevated in STZ diabetic rats fed the low P diet. These results are in agreement with those reported by Matsomato et al. (1986). If the defect in diabetes was solely at the level of the PTH adenylate cyclase pathway, phosphate restriction should have increased circulating $1,25(OH)_2D_3$. Future studies should further examine the effects of phosphate restriction on 1αhydroxylase activity in STZ diabetes. Taken in unison, these studies indicate

that the PTH - adenylate cyclase pathway can function to stimulate 1αhydroxylase activity in STZ diabetes.

Although earlier studies have indicated that enhanced metabolic clearance was not evident in diabetic rats fed control diets (Spencer et al., 1980), studies conducted by Wongsurawat et al. (1983) demonstrated increased 24 hydroxylase activity in renal slices from STZ diabetic rats fed low dietary Ca. The results from this thesis demonstrate that while STZ diabetic rats responded to low dietary Ca with increased 1αhydroxylase activity no elevation in circulating $1,25(OH)_2D_3$ was observed. Thus, synthesis of $1,25(OH)_2D_3$ in tubules from STZ diabetic rats fed low Ca did not correlate with circulating $1,25(OH)_2D_3$. This is in contrast to the significant correlation observed between $1,25(OH)_2D_3$ synthesis and circulating levels in STZ diabetic rats fed control diets or in control rats fed either control or low Ca diets. Enhanced metabolic clearance of $1,25(OH)_2D_3$ could explain the lack of correlation between synthesis and circulating levels in the diabetic rats fed low Ca. Enhanced metabolic clearance of $1,25(OH)_2D_3$ in low Ca fed diabetic rats suggests increased 24 hydroxylase activity (Tomon, Tenenhouse and Jones, 1990). However, such a response to low Ca challenge would be abnormal since Ca stress is usually associated with negligible 24 hydroxylase activity (Turner, 1984). These results are consistent, however, with the increased renal 24 hydroxylase activity in low Ca fed diabetic rats observed by Wongsurawat et al. (1983). One question which requires clarification is why control fed diabetics have low 24 hydroxylase activity (Table 4.4.2.5) while low Ca fed diabetics have high 24 hydroxylase activity (Wongsurawat et al., 1983). While control fed diabetics have decreased or normal $1,25(OH)_2D_3$ induced responses, such as D9 and D28 calbindins and 24 hydroxylase activity (Stone et al., 1991a&b) it is possible that low Ca diet modulates vitamin D induced proteins and target tissue responses in diabetes. Future experiments should examine 24 hydroxylase activity, calbindin and circulating $24,25(OH)_2D_3$ in both control and low Ca fed diabetic rats to clarify the situation.

4.5.3.b. PROTEIN KINASE C ACTIVITY AND INSULIN DEPENDENT DIABETES

These studies demonstrate that TPA inhibited $1,25(OH)_2D_3$ production in freshly isolated rat renal tubules within 20 minutes; an effect that was sustained for up to 60 minutes. These results complement those of Henry and Luntao (1989), who reported that four hours incubation with TPA inhibited $1,25(OH_2D_3$ production in vitamin D deficient chick renal tubule cultures. In contrast to studies of Henry and Luntao (1989) the temporal correlation between inhibition of $1,25(OH)_2D_3$ production and PKC activity was assessed. The data indicate that the dose of TPA (160 nM) which rapidly inhibited $1,25(OH)_2D_3$ production induced an increase in membrane PKC activity within five minutes; ie., prior to it's effect on $25(OH)D_3$ hydroxylation. The increase in membrane PKC appeared to be the result of translocation, since a parallel decrease in cytosolic PKC activity was observed. Recently, Mandla, Boneh and Tenenhouse (1990) reported that ten minute exposure of mouse renal tubules to 1.6 μM TPA resulted in translocation of PKC activity to mitochondria. This is of special interest since the vitamin D hydroxylases have been localized to mitochondria. Both membrane and cytosolic PKC activity returned to baseline levels within 60 minutes (Figure 4.5.1.b.1.7). The transient nature of TPA's effect on PKC activity, combined with the apparent translocation from cytosol to membrane, offers an explanation for the reported lack of effect of TPA on total cellular phorbol ester binding sites in chick renal tubules, measured after four hours of incubation with TPA (Henry and Luntao, 1989).

Since TPA-induced translocation of PKC activity preceded it's effects on $1,25(OH)_2D_3$ synthesis, agents and manipulations known to modulate PKC activity were tested in the model system, to determine if they also affected $1,25(OH)_2D_3$ production. Down regulation of PKC activity by prolonged incubation with phorbol esters is a useful tool for probing the role of PKC in cellular events. The results from the studies presented in this thesis indicate that pre-incubation of proximal tubules with 160 nM TPA for 18 hours reduced

both membrane and cytosolic PKC activity and, more importantly, rendered proximal tubules insensitive to acute stimulation by TPA (Table 4.5.1.b.1.2). The same conditions which resulted in down regulation of PKC activity and loss of sensitivity to TPA abolished the effect of TPA on $1,25(OH)_2D_3$ production (Figure 4.5.1.b.1.4). These data support the hypothesis that TPA mediated inhibition of $1,25(OH)_2D_3$ production is related to it's ability to modulate PKC activity. This hypothesis is further supported by the experiments with staurosporine, a PKC inhibitor. In the model system, pre-incubation of tubules with 10 nM staurosporine abolished the inhibitory effect of TPA on $1,25(OH)_2D_3$ production (Figure 4.5.1.b.1.5). Although staurosporine is not a specific PKC inhibitor, the observation that staurosporine negated the effects of TPA is consistent with a role for PKC in mediating TPA's inhibition of $1,25(OH)_2D_3$ production.

Translocation of PKC and inhibition of $1,25(OH)_2D_3$ production occurred within 20 minutes of TPA incubation - a time course consistent with a role for PKC in acute regulation of $25(OH)D_3$ hydroxylation by phosphorylation:dephosphorylation. PKC phosphorylates its substrates on serine and threonine residues (Parker et al., 1989) and serine/threonine phosphorylation of the renoredoxin component of the 1αhydroxylase has been shown to inhibit $1,25(OH)_2D_3$ production in a reconstituted system (Nemani et al., 1989). Boneh and Tenenhouse (1988) have demonstrated enhanced phosphorylation of several substrates in mitochondria derived from mouse renal tubules exposed to TPA. Taken in unison, the available data is consistent with direct inhibition of 1αhydroxylation via PKC dependent phosphorylation, possibly of renoredoxin, however, confirmation will require positive identification of PKC substrates in proximal tubules. Further, the physiological relevance of PKC activation remains dependent on the demonstration that endogenous regulators of 1αhydroxylation exert their effects via modulation of PKC.

Consistent with the known reciprocity between 1α and 24 hydroxylases, 160 nM TPA rapidly stimulated $24,25(OH)_2D_3$ production in rat renal tubules (Figure 4.5.1.b.1.3). Similar effects have been reported in fresh mouse renal tubules (Mandla, Boneh and Tenenhouse, 1990) and cultured chick renal tubules (Henry and Luntao, 1989) although different doses and incubation times were employed in those studies. Since the 1α and the 24 hydroxylases act on the same substrate, and only net production of the dihydroxylated metabolites has been measured in these and previous studies, it was not possible to determine whether TPA affects one or both enzymes. However, the time course data for TPA obtained from the studies conducted for this thesis indicated that inhibition of net $1,25(OH)_2D_3$ production occurred prior to stimulation of $24,25(OH)_2D_3$ production. This suggests that the inhibitory effect of TPA on 1αhydroxylation is not secondary to TPA mediated enhancement of 24 hydroxylation. The data described in this thesis does not exclude the possibility that enhanced 24 hydroxylase activity contributes to inhibition of net $1,25(OH)_2D_3$ production by TPA, however, since 24 hydroxylation of $1,25(OH)_2D_3$ to $1,24,25(OH)_3D_3$ can occur (DeLuca, 1984) and the latter metabolite was not measured in these studies.

The time course data is also compatible with independent effects of TPA on each hydroxylase. This hypothesis was originally suggested by Henry and Luntao (1989) who observed differential effects of PKC activators on renal $25(OH)D_3$ hydroxylations. The observations presented in this thesis that higher doses of TPA were required to modulate $24,25(OH)_2D_3$ production compared to $1,25(OH)_2D_3$ production also support this theory. However, the mechanism whereby TPA might exert an effect on 24 hydroxylase activity which is independent of an effect on 1αhydroxylase activity remains to be elucidated.

The potential relevance of PKC mediated 1αhydroxylase inhibition and defective $1,25(OH)_2D_3$ synthesis during diabetes was assessed next. While statistical inferences could not be made concerning membrane PKC activity in diabetic versus control renal tubules, the data suggested that diabetic

membrane PKC activity was elevated relative to control membrane activity. These observations are consistent with an earlier report of elevated PKC activity in renal basolateral membranes from 3-4 week STZ diabetic rats (Hise and Mehta, 1988). Diabetes is associated with renal hypertrophy (SeyerHansen, 1976, refer to section 4.4.2 table 4.4.2.9) and increased PKC activity is correlated with renal hypertrophy following unilateral nephrectomy (Hise and Mehta, 1989 and Carmelo et al., 1988). Diabetic renal hypertrophy and elevated basolateral membrane PKC activity are both reversible by insulin therapy (Stone et al., 1991a, Hise and Mehta, 1988). Thus elevated PKC activity may be associated with renal hypertrophy. The relevance of renal hypertrophy to the aberrant vitamin D metabolism in insulin dependent diabetes is unknown. However, Almond et al. (1989) reported that 1αhydroxylase activity was inversely correlated with the mass of the remnant kidney after uninephrectomy. Therefore it is possible that renal hypertrophy and 1αhydroxylation may be coordinately regulated. The positive correlation between renal hypertrophy and PKC activity are intriguing when considered in conjunction with the observations presented in this thesis that PKC activation is associated with inhibition of $1,25(OH)_2D_3$ production. It is possible that increased PKC activity associated with renal hypertrophy (in insulin dependent diabetes or following unilateral nephrectomy) comprises a negative feedback loop to repress $1,25(OH)_2D_3$ synthesis thereby preventing overproduction of $1,25(OH)_2D_3$ and associated hypercalcaemia. In diabetes inappropriate operation of this feedback loop may provide an explanation for the observations of reduced $1,25(OH)_2D_3$ synthesis, decreased circulating $1,25(OH)_2D_3$ and aberrant Ca homeostasis.

The signals involved in the induction of the renal hypertrophy and/or PKC activation may provide a key to understanding the aetiology of the aberrant vitamin D metabolism in insulin dependent diabetes.

4.5.4. CONCLUSIONS

4.5.4.a. ADENYLATE CYCLASE SIGNAL TRANSDUCTION AND INSULIN DEPENDENT DIABETES

To summarize, PTH stimulation of adenylate cyclase activity and 1αhydroxylase activity were comparable in STZ diabetic and control rats under the conditions tested. Despite comparable sensitivity of adenylate cyclase to PTH, however, the absolute rate of $1,25(OH)_2D_3$ synthesis and circulating $1,25(OH)_2D_3$ remained significantly lower in diabetic rats in comparison to control rats. STZ diabetic rats fed low P also exhibited significantly lower circulating $1,25(OH)_2D_3$, indicating the defect cannot be attributed solely to a defect in PTH signalling. Finally the lack of correlation between circulating $1,25(OH)_2D_3$ and in vitro $1,25(OH)_2D_3$ in STZ diabetic rats exposed to low dietary Ca suggests that the effect of diabetes on vitamin D metabolism is not limited to $1,25(OH)_2D_3$ synthesis.

4.5.4.b. PROTEIN KINASE C ACTIVITY AND INSULIN DEPENDENT DIABETES

The in vitro studies demonstrated that TPA activation of PKC inhibited $1,25(OH)_2D_3$ production and stimulated $24,25(OH)_2D_3$ production in rat renal tubules. Down regulation of PKC activity or pre-incubation of tubules with the PKC inhibitor staurosporine prevented the TPA mediated inhibition of $1,25(OH)_2D_3$ production. These results suggest a relationship between active PKC and inhibition of $1,25(OH)_2D_3$ production. Impaired $1,25(OH)_2D_3$ production in STZ diabetic rats was associated with an increase in renal tubule membrane PKC activity. In the next series of studies the temporal relationship between PKC activity, renal hypertrophy and altered vitamin D metabolism was investigated.

4.6 TEMPORAL CHANGES IN VITAMIN D METABOLISM ASSOCIATED WITH INSULIN DEFICIENCY

4.6.1. EXPERIMENTAL PROTOCOL

Male weanling Sprague Dawley rats were fed and housed as described

under "Materials and Methods". They received AIN semi-purified normal rat diet and tap water ad libitum. Diabetes induction and insulin treatment were conducted as described previously. Diabetes induction studies consisted of monitoring the changes in vitamin D metabolism, renal physiology and Ca homeostasis subsequent to STZ injection and subsequent to diabetes onset. Diabetes withdrawal studies consisted of monitoring the changes in vitamin D metabolism, renal physiology and Ca homeostasis subsequent to termination of precise insulin therapy in a group of STZ injected diabetic rats.

Diabetes Induction

Two acute induction studies were conducted. The first study examined indices of diabetes and vitamin D metabolism 24, 48, 72, 96 hours and five days post STZ injection with three to five STZ diabetic and two to four control animals sacrificed per time point. The second study examined indices of diabetes and vitamin D metabolism at basal, 12, 48, 72, 96 hours and eight days post STZ injection with four to eight STZ diabetic and four to six control animals sacrificed per time point. Food consumption and body weights were measured. At sacrifice serum was frozen for determination of glucose, insulin and $1,25(OH)_2D_3$. Kidneys were removed, weighed, renal tubules isolated and PKC activity assayed in PNF membrane and cytosolic fractions.

Insulin Withdrawal

One insulin withdrawal study examined changes in vitamin D metabolism and Ca homeostasis after insulin was withdrawn from STZ diabetic rats which had been insulin treated, with two to four STZ diabetic and three to four controls animals per time point. Diabetic insulin treated animals were used for this study only if they exhibited trace glucosuria and weight gain for at least three consecutive days. Body weights and urinary glucose were assayed on each animal daily. Animals were fasted for 12 hours prior to sacrifice when serum was frozen for determination of glucose, insulin, $1,25(OH)_2D_3$, $24,25(OH)_2D_3$ and PTH. Kidneys were removed, weighed, a portion removed for protein and DNA determination, and renal proximal

tubules isolated and assayed for net $1,25(OH)_2D_3$ production.

4.6.2. RESULTS

Diabetes Induction and Insulin Withdrawal

Indices of Diabetes

Within 12 hours of STZ injection, animals exhibited significantly lower circulating insulin compared to vehicle injected controls. Serum insulin decreased further and levelled off at 30-35% of control values after 48 hours. Insulin therapy significantly elevated circulating insulin in STZ diabetics, with values exceeding endogenous insulin levels in control rats. 24 hours after insulin withdrawal, circulating insulin was significantly lower in diabetic than in control rats and remained at 30-35% of control values for the duration of the study (Figure 4.6.2.1). Consistent with the changes in circulating insulin, a significant elevation in circulating glucose was apparent within 12 hours of STZ injection. Hyperglycaemia persisted until insulin treatment was initiated; thereafter glucose was normalized unless insulin was withdrawn (Figure 4.6.2.2). Following STZ treatment, rats reduced their food intake temporarily, lost weight and were therefore significantly smaller than their control counterparts (who continued to gain weight) within 48 hours. Food intake became normalized after 48 hours and hyperphagia became apparent within five days post injection (data not shown). Diabetic rats grew at a much slower rate than did controls and therefore maintained significantly lower body weights throughout the study unless insulin therapy was initiated. Insulin treated diabetic rats had body weights comparable to their control counterparts. Insulin withdrawal did not result in acute weight loss or reduced food intake, however diabetic rats again grew at a much slower rate than controls and therefore had significantly lower body weights within 48 hours of insulin withdrawal (Figure 4.6.2.3).

Indices of Vitamin D Metabolism

Circulating $1,25(OH)_2D_3$ was transiently elevated (280% of control values) 12 hours after STZ injection; thereafter levels of $1,25(OH)_2D_3$ were

comparable to control until 72 hours when levels decreased significantly (75% of control). A further decrease was observed at 96 hours to 45-50% of control levels; thereafter circulating $1,25(OH)_2D_3$ remained unchanged unless insulin therapy was initiated. Insulin treated STZ diabetic rats had circulating $1,25(OH)_2D_3$ and in vitro 1αhydroxylase activity comparable to controls. Insulin withdrawal resulted in a significant decrease in circulating $1,25(OH)_2D_3$ (50-55% of control) after 72 hours (Figure 4.6.2.5). Following insulin withdrawal, net synthesis of $1,25(OH)_2D_3$ in vitro was comparable to control rates until 72 hours, when $1,25(OH)_2D_3$ synthesis declined (45% of control). Net synthesis of $1,25(OH)_2D_3$ declined further to (30-35% of control) by seven days after insulin withdrawal (Figure 4.6.2.6). Changes in $1,25(OH)_2D_3$ synthesis paralleled the changes in circulating $1,25(OH)_2D_3$ (See figure 4.6.2.4; insulin withdrawal section). Circulating $24,25(OH)_2D_3$ was 50% lower in insulin treated diabetic rats compared to their control counterparts and was normalized after insulin withdrawal. However no significant difference between circulating $24,25(OH)_2D_3$ in diabetics and controls was observed at any time point examined (Figure 4.6.2.7). Circulating PTH in insulin treated diabetic rats were comparable to control rats. Following insulin withdrawal, circulating PTH was significantly lower in diabetics than controls at 48 and 72 hours after withdrawal; however, thereafter PTH in diabetic rats was comparable to control values (Figure 4.6.2.8).

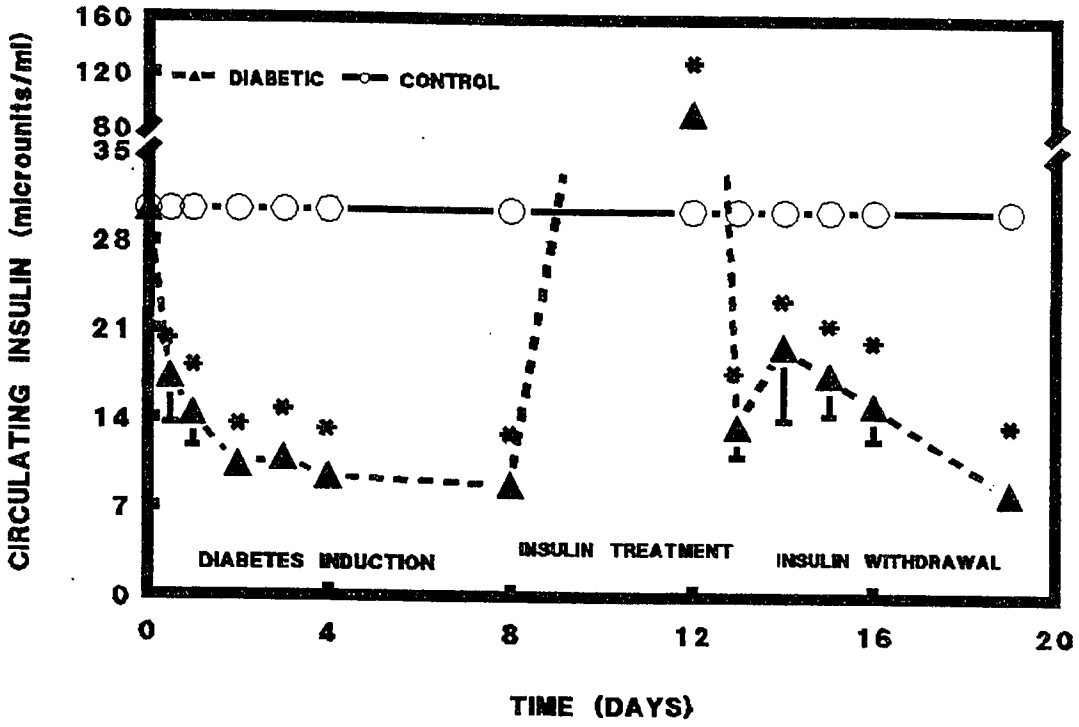
Indices of Renal Physiology

Renal mass increased linearly with diabetes onset or following insulin withdrawal and was significantly elevated relative to control kidneys by 48 hours. Renal somatic index was significantly greater in diabetic rats within 48 hours of STZ injection or insulin withdrawal; insulin therapy normalized renal growth (Figure 4.6.2.4). Total kidney protein increased with time in both control and diabetic rats. The rate of increase in renal protein was greater in diabetic than control rats, such that seven days after insulin withdrawal, untreated diabetics had higher total kidney protein content than their control

counterparts although the difference was not significant (Figure 4.6.2.9). No significant differences were observed in DNA/protein between diabetic and control rat kidneys even after seven days post insulin withdrawal (Figure 4.6.2.10). PKC activity in renal tubule membranes was significantly elevated in diabetic rats 12, 48, 72 hours and eight days after STZ injection. Renal tubule cytosolic PKC activity was significantly elevated 12 hours after STZ injection and significantly lower 72 hours after injection (Figure 4.6.2.11).

FIGURE 4.6.2.1 (top) Temporal changes in circulating insulin following STZ injection, insulin treatment and insulin withdrawal as compared to vehicle injected control rats.
Data are mean ± SEM, n = 3-9 determinations of duplicate circulating insulin measured in serum obtained from STZ injected diabetic rats before, during and after withdrawal of insulin therapy. No significant differences were observed between circulating insulin measured in control rats at any time examined therefore control values were pooled and averaged ($n \geq 20$). A significant (*) ($p<0.05$) decrease in circulating insulin was observed within 12 hours of STZ injection and 24 hours after insulin therapy was discontinued as compared to vehicle injected controls while insulin therapy significantly elevated circulating insulin.

FIGURE 4.6.2.2 (bottom) Temporal changes in circulating glucose following STZ injection, insulin treatment and insulin withdrawal as compared to vehicle injected control rats.
Data are mean ± SEM, n = 3-9 determinations of duplicate circulating glucose measured in serum obtained from STZ injected diabetic rats before, during and after withdrawal of insulin therapy. No significant differences were observed between circulating glucose measured in control rats at any time examined, therefore control values were pooled and averaged ($n \geq 20$). A significant (*) ($p<0.05$) elevation in circulating glucose was observed 12 hours following STZ injection and 24 hours after insulin therapy was discontinued as compared to vehicle injected controls while insulin therapy normalized circulating glucose.


CIRCULATING INSULIN (microunits/ml)
160
120
80
35
28
21
14
7
0
DIABETIC
CONTROL
DIABETES INDUCTION
INSULIN TREATMENT
INSULIN WITHDRAWAL
0
4
8
12
16
20
TIME (DAYS)

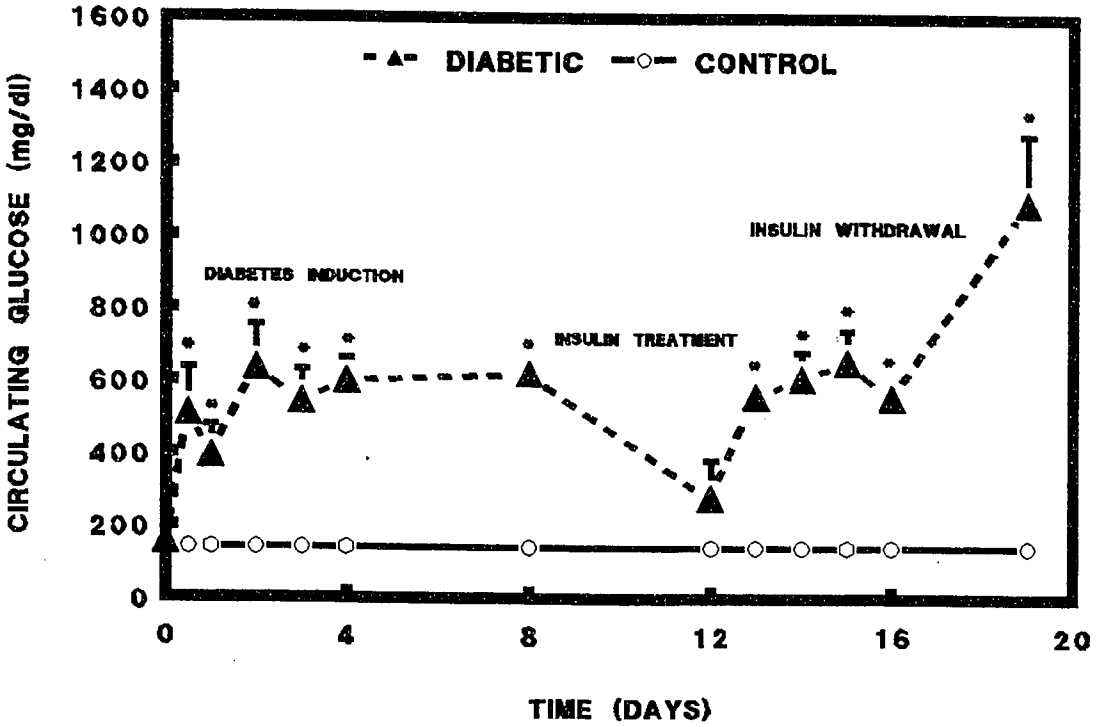
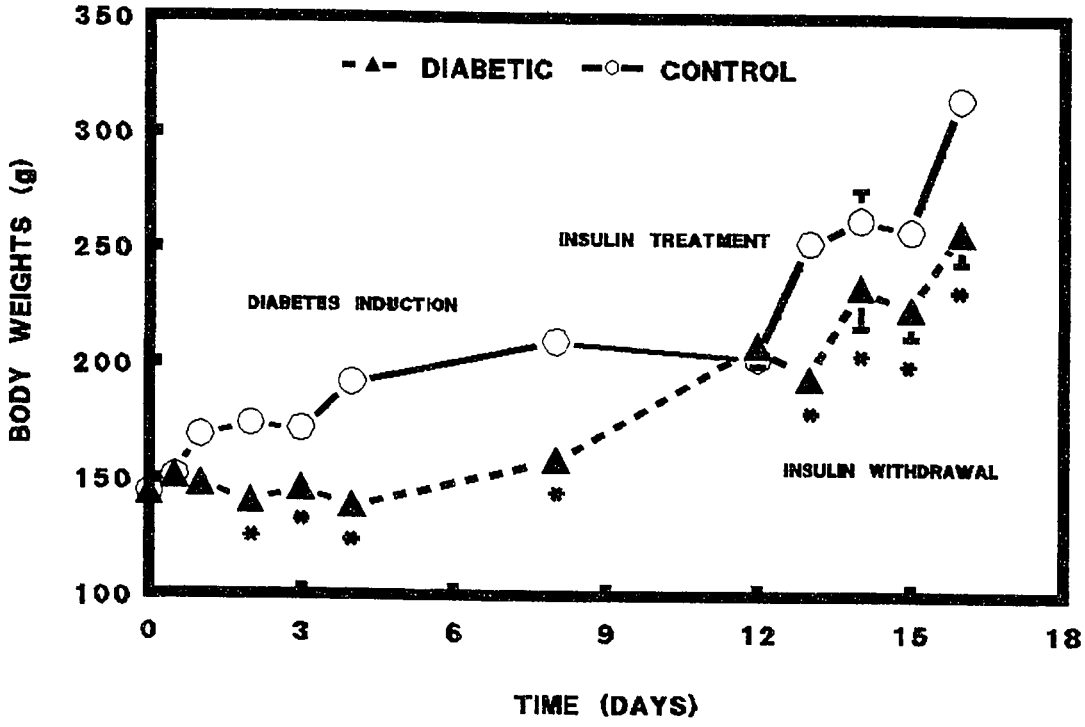

CIRCULATING GLUCOSE (mg/dl)
1600
1400
1200
1000
800
600
400
200
0
DIABETIC
CONTROL
DIABETES INDUCTION
INSULIN TREATMENT
INSULIN WITHDRAWAL
0
4
8
12
16
20
TIME (DAYS)

FIGURE 4.6.2.3 (top) Temporal changes in body weights following STZ injection, insulin treatment and insulin withdrawal as compared to vehicle injected control rats.
Data are mean ± SEM, n = 3-9 determinations of body weights of STZ injected diabetic rats before, during and after withdrawal of insulin therapy as compared to vehicle injected control rats. STZ diabetic rats grew at a slower rate than did vehicle injected control rats. STZ diabetic body weights were significantly (*) ($p<0.05$) lower 48 hours after STZ injection and remained lower unless insulin therapy was initiated (in comparison to age matched vehicle injected controls). Insulin therapy normalized STZ diabetic body weight as compared to age matched vehicle injected controls. Following withdrawal of insulin therapy growth rates declined in STZ diabetic rats and body weights were significantly lower as compared to controls.

FIGURE 4.6.2.4 (bottom) Temporal changes in renal somatic index following STZ injection, insulin therapy and insulin withdrawal as compared to vehicle injected control rats.
Data are mean ± SEM, n = 3-9 determinations of body weight and renal mass of STZ diabetic rats before, during and following insulin treatment in comparison to values obtained in vehicle injected control rats. A significant (*) ($p<0.05$) increase in renal somatic index was observed in STZ diabetic rats 48 hours following STZ injection or discontinuation of insulin treatment, while insulin therapy normalized diabetic renal somatic index as compared to values obtained in age matched vehicle injected controls.


DIABETIC
CONTROL
BODY WEIGHTS (g)
350
300
250
200
150
100
INSULIN TREATMENT
DIABETES INDUCTION
INSULIN WITHDRAWAL
0
3
6
9
12
15
18
TIME (DAYS)

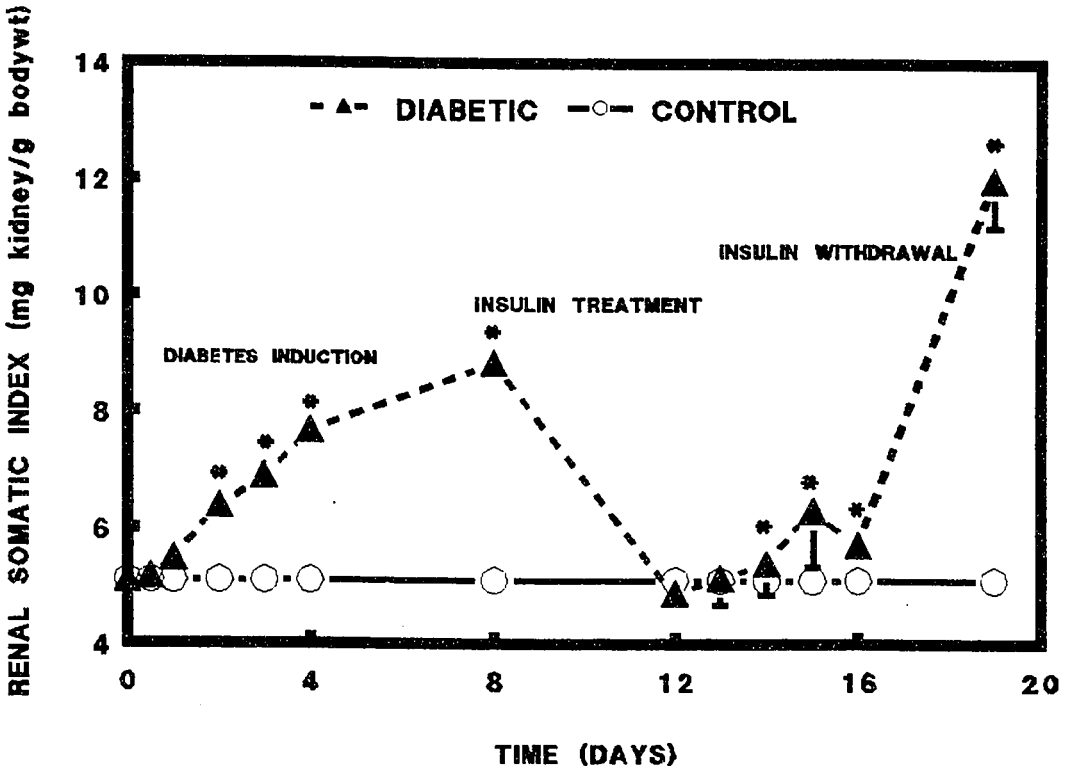
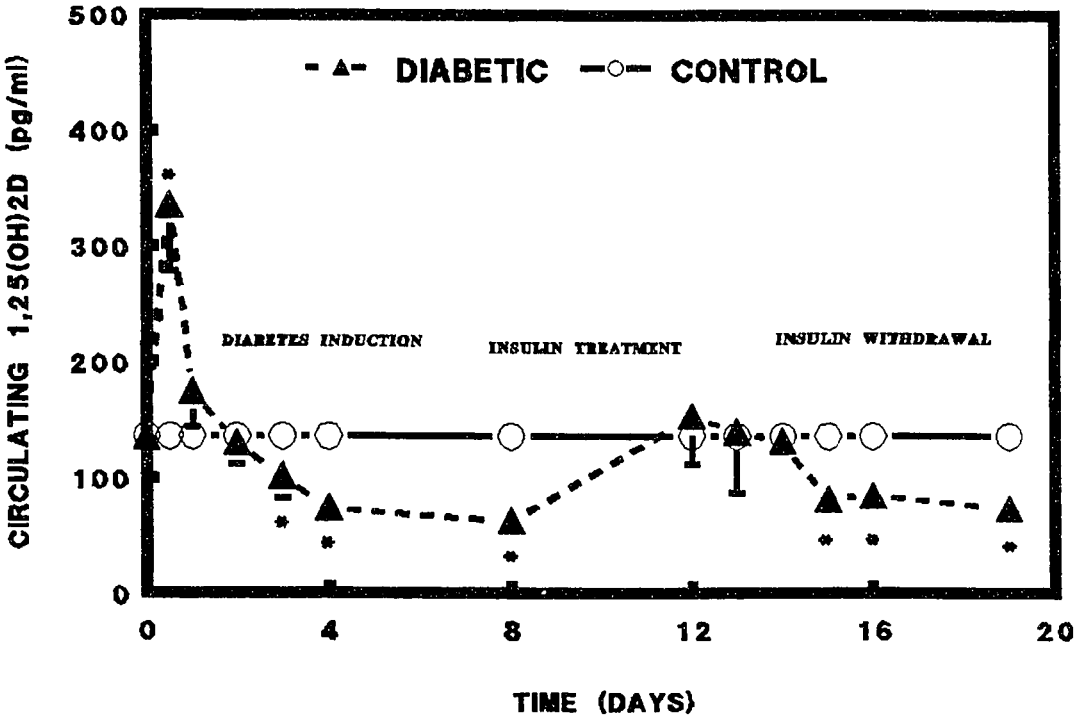

DIABETIC
CONTROL
RENAL SOMATIC INDEX (mg kidney/g bodywt)
14
12
10
8
6
4
INSULIN WITHDRAWAL
INSULIN TREATMENT
DIABETES INDUCTION
0
4
8
12
16
20
TIME (DAYS)

FIGURE 4.6.2.5 (top) Temporal changes in circulating $1,25(OH)_2D_3$ following STZ injection, insulin treatment and insulin withdrawal as compared to vehicle injected control rats.
Data are mean ± SEM, n = 3-9 determinations of duplicate circulating $1,25(OH)_2D_3$ measured in serum from STZ diabetic rats before, during and following insulin therapy. No significant differences in circulating $1,25(OH)_2D_3$ were observed at any time examined therefore control values were pooled and averaged ($n \geq 20$). Significantly (*) ($p<0.05$) elevated circulating $1,25(OH)_2D_3$ was observed in STZ diabetic rats 12 hours following STZ injection. Circulating $1,25(OH)_2D_3$ was significantly decreased 72 hours following STZ injection or discontinuation of insulin therapy while insulin therapy normalized STZ diabetic circulating $1,25(OH)_2D_3$ as compared to vehicle injected controls.

FIGURE 4.6.2.6 (bottom) Net synthesis of $1,25(OH)_2D_3$ in renal proximal tubules isolated from STZ diabetics following insulin treatment and insulin withdrawal compared to vehicle injected control rats.
Data are mean ± SEM, n = 2-7 duplicate determinations of net $1,25(OH)_2D_3$ synthesis measured in renal proximal tubules isolated from STZ diabetic rats treated with insulin for 3-5 days and following insulin withdrawal. No significant differences in net $1,25(OH)_2D_3$ were observed between tubules isolated from vehicle injected controls at any time examined therefore control values were pooled and averaged ($n \geq 12$). In vivo insulin therapy normalized net $1,25(OH)_2D_3$ synthesis in tubules isolated from STZ diabetic rats and synthesis rates declined 72 hours following insulin withdrawal and were significantly (*) ($p<0.05$) decreased seven days after insulin treatment was discontinued as compared to rates measured in tubules isolated from vehicle injected controls.


DIABETIC
CONTROL
CIRCULATING 1,25(OH)2D (pg/ml)
500
400
300
200
100
0
DIABETES INDUCTION
INSULIN TREATMENT
INSULIN WITHDRAWAL
0
4
8
12
16
20
TIME (DAYS)

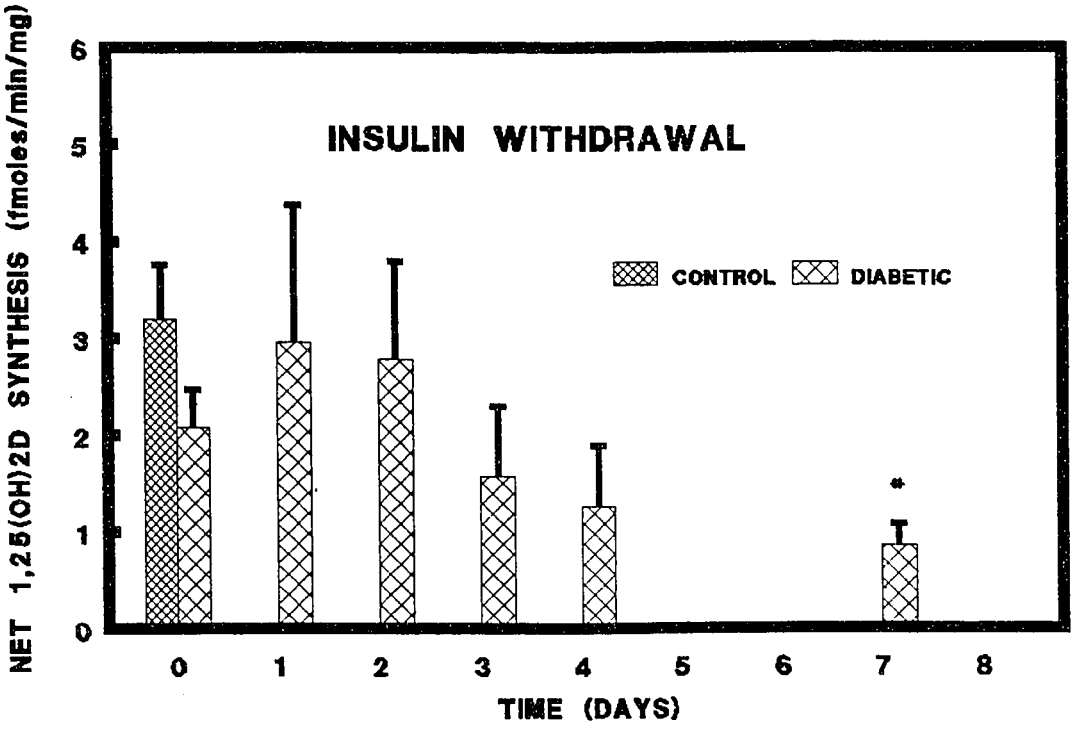
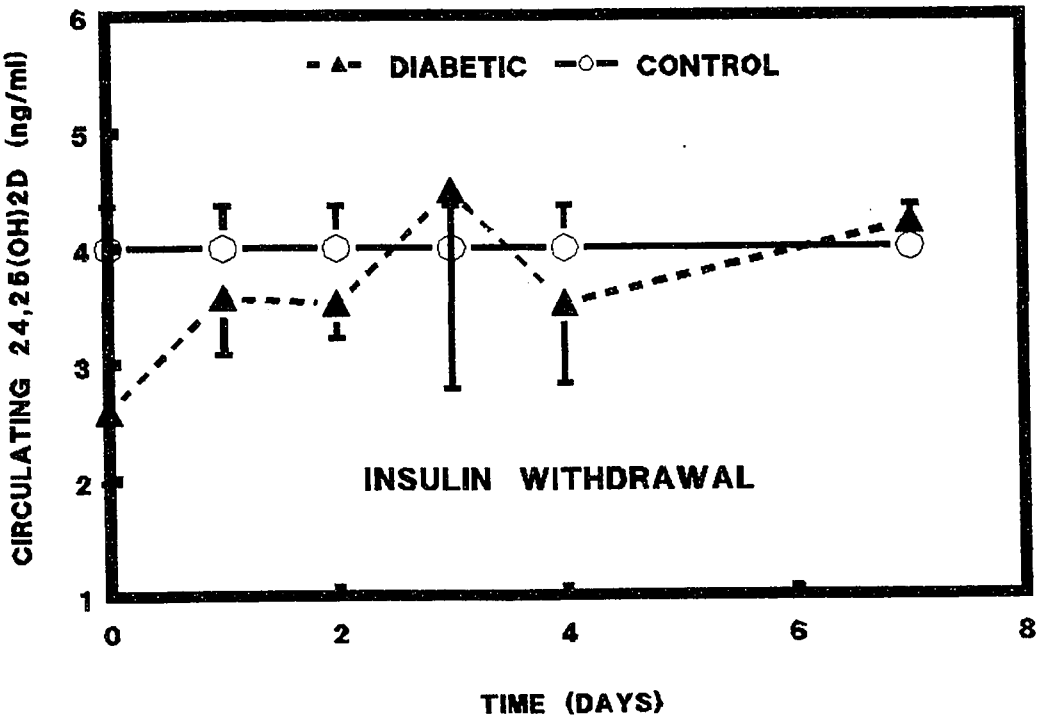

INSULIN WITHDRAWAL
CONTROL
DIABETIC
NET 1,25(OH)2D SYNTHESIS (fmoles/min/mg)
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
8
TIME (DAYS)

FIGURE 4.6.2.7 (top) Circulating $24,25(OH)_2D_3$ in STZ diabetic rats following insulin treatment and insulin withdrawal compared to vehicle injected control rats.
Data are mean ± SEM, n = 2-7 duplicate determinations of circulating $24,25(OH)_2D_3$ measured in serum obtained from STZ diabetic rats treated with insulin for 3-5 days and following insulin withdrawal. No significant differences in circulating $24,25(OH)_2D_3$ measured in vehicle injected controls were observed at any time examined therefore control values were pooled and averaged ($n \geq 12$). No significant differences between circulating $24,25(OH)_2D_3$ in STZ diabetics and vehicle injected controls were observed at any time.

FIGURE 4.6.2.8 (bottom) Circulating PTH in STZ diabetic rats following insulin treatment and insulin withdrawal compared to vehicle injected control rats.
Data are mean ± SEM, n = 2-7 determinations of circulating PTH measured in serum obtained from STZ diabetic rats treated with insulin for 3-5 days and following insulin withdrawal. No significant differences in circulating PTH measured in vehicle injected controls were observed at any time examined therefore control values were pooled and averaged ($n \geq 12$). Circulating PTH was significantly (*) ($p<0.05$) decreased 48 and 72 hours following discontinuation of insulin therapy as compared to vehicle injected controls, thereafter circulating PTH returned to control levels and was not significantly different.


DIABETIC
CONTROL
CIRCULATING 24,25(OH)2D (ng/ml)
INSULIN WITHDRAWAL
TIME (DAYS)

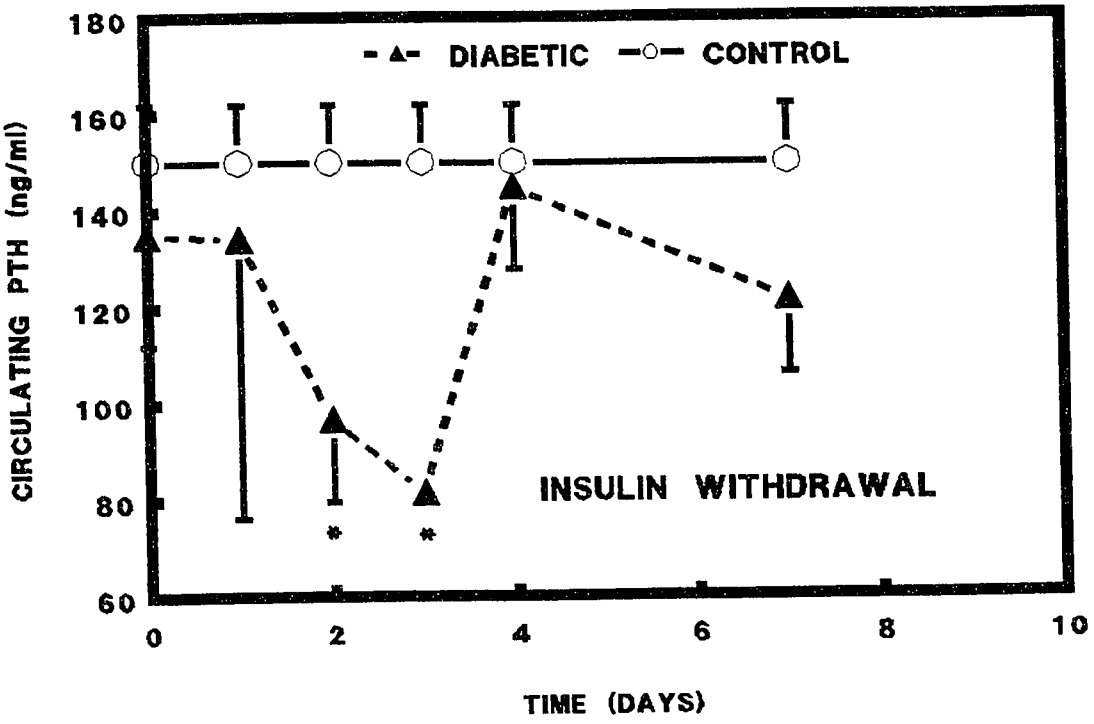
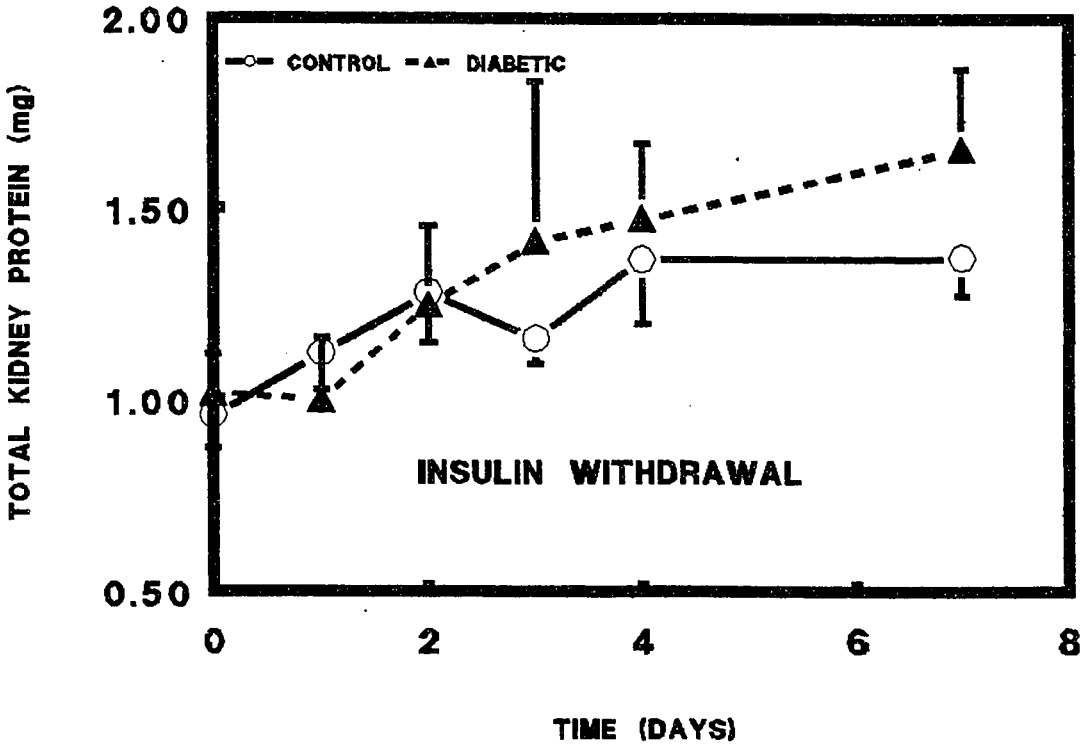

DIABETIC
CONTROL
CIRCULATING PTH (ng/ml)
INSULIN WITHDRAWAL
TIME (DAYS)

FIGURE 4.6.2.9 (top) Renal protein in STZ diabetic rats following insulin treatment and insulin withdrawal compared to vehicle injected control rats.
Data are mean ± SEM, n = 2-7 determinations of duplicates of protein measured in kidneys from STZ diabetic rats following insulin treatment and insulin withdrawal compared to controls. No significant differences were observed between STZ diabetic and controls at any time examined.

FIGURE 4.6.2.10 (bottom) Renal DNA/protein ratio in STZ diabetic rats following insulin treatment and insulin withdrawal compared to vehicle injected control rats.
Data are mean ± SEM, n = 2-7 determinations of duplicates of DNA and protein measured in kidneys from STZ diabetic rats follow insulin treatment and insulin withdrawal compared to controls. Data is expressed as μgDNA/mg kidney protein. No significant differences were observed between STZ diabetic and controls at any time examined.


2.00
1.50
1.00
0.50
TOTAL KIDNEY PROTEIN (mg)
CONTROL
DIABETIC
INSULIN WITHDRAWAL
0
2
4
6
8
TIME (DAYS)

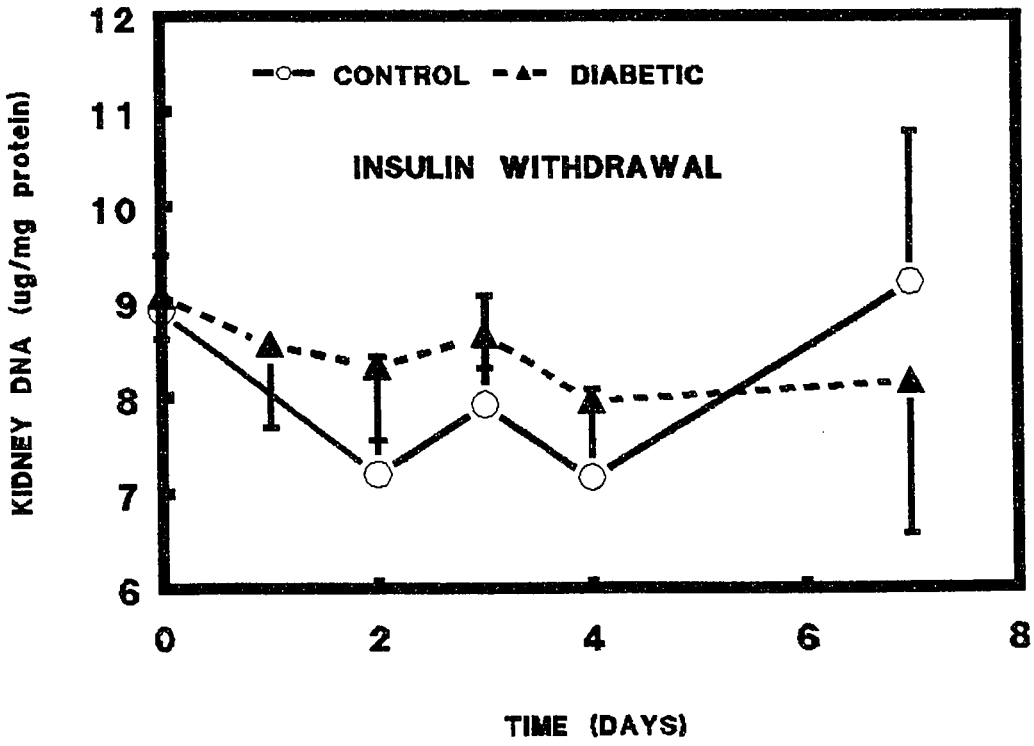

12
11
10
9
8
7
6
KIDNEY DNA (ug/mg protein)
CONTROL
DIABETIC
INSULIN WITHDRAWAL
0
2
4
6
8
TIME (DAYS)

FIGURE 4.6.2.11 Temporal changes in subcellular PKC activity in post nuclear fractions of renal tubules isolated from STZ diabetic and control rats.

Data are mean ± SEM, n = 3-4 with duplicate or triplicate determinations of PKC activity measured in 10 ug membrane protein and 25 ug cytosol protein prepared from post nuclear fractions of renal tubules isolated from STZ diabetic or vehicle injected control rats. Data is expressed as % control PKC activity. A significant (*) ($p<0.05$) elevation in STZ diabetic membrane PKC activity was observed 12, 48, 72 hours and seven days following STZ injection. A significant elevation in STZ diabetic cytosolic PKC activity was observed 12 hours following STZ injection and a significant decrease in STZ diabetic cytosolic PKC activity was observed 72 hours following STZ injection.

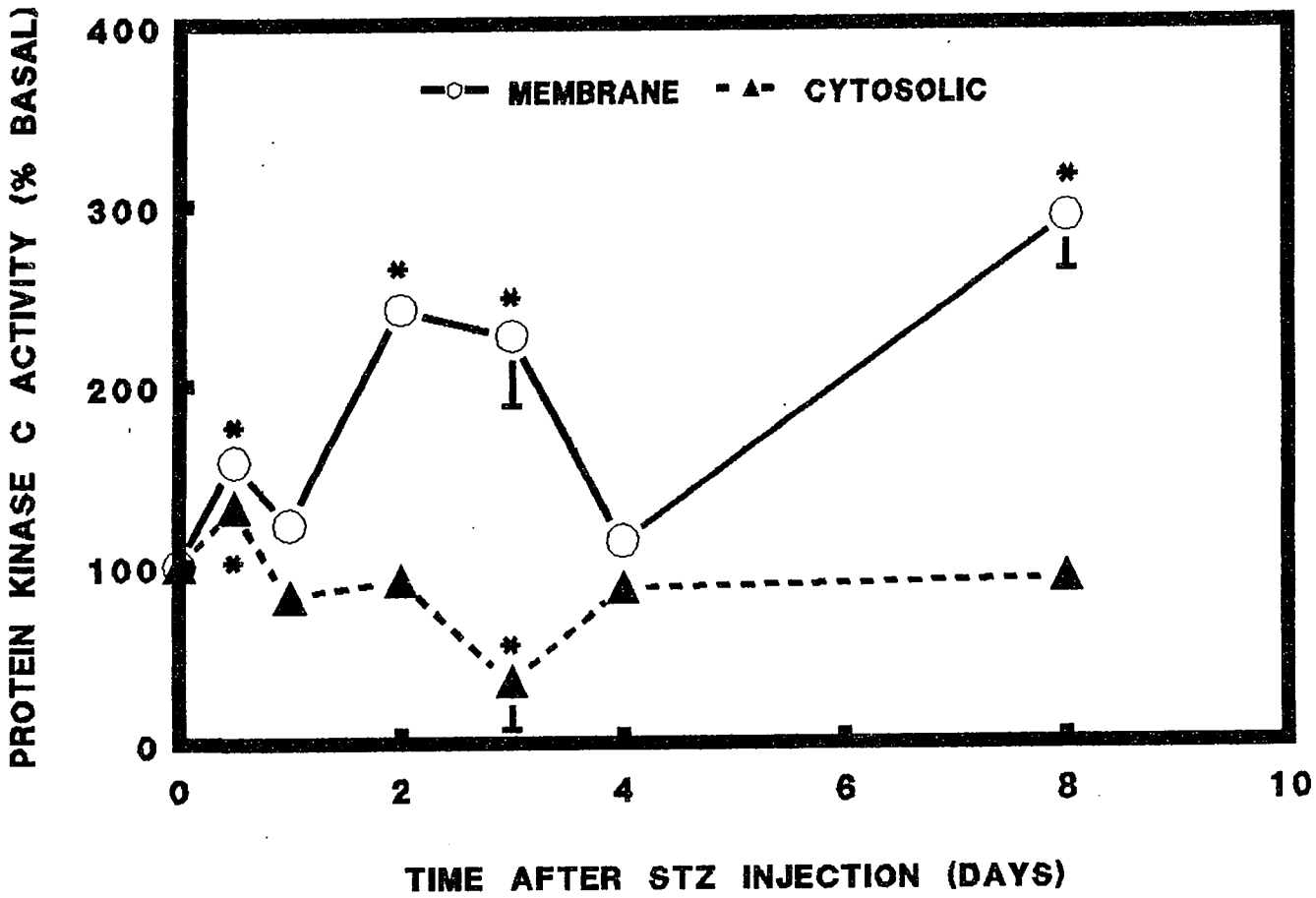
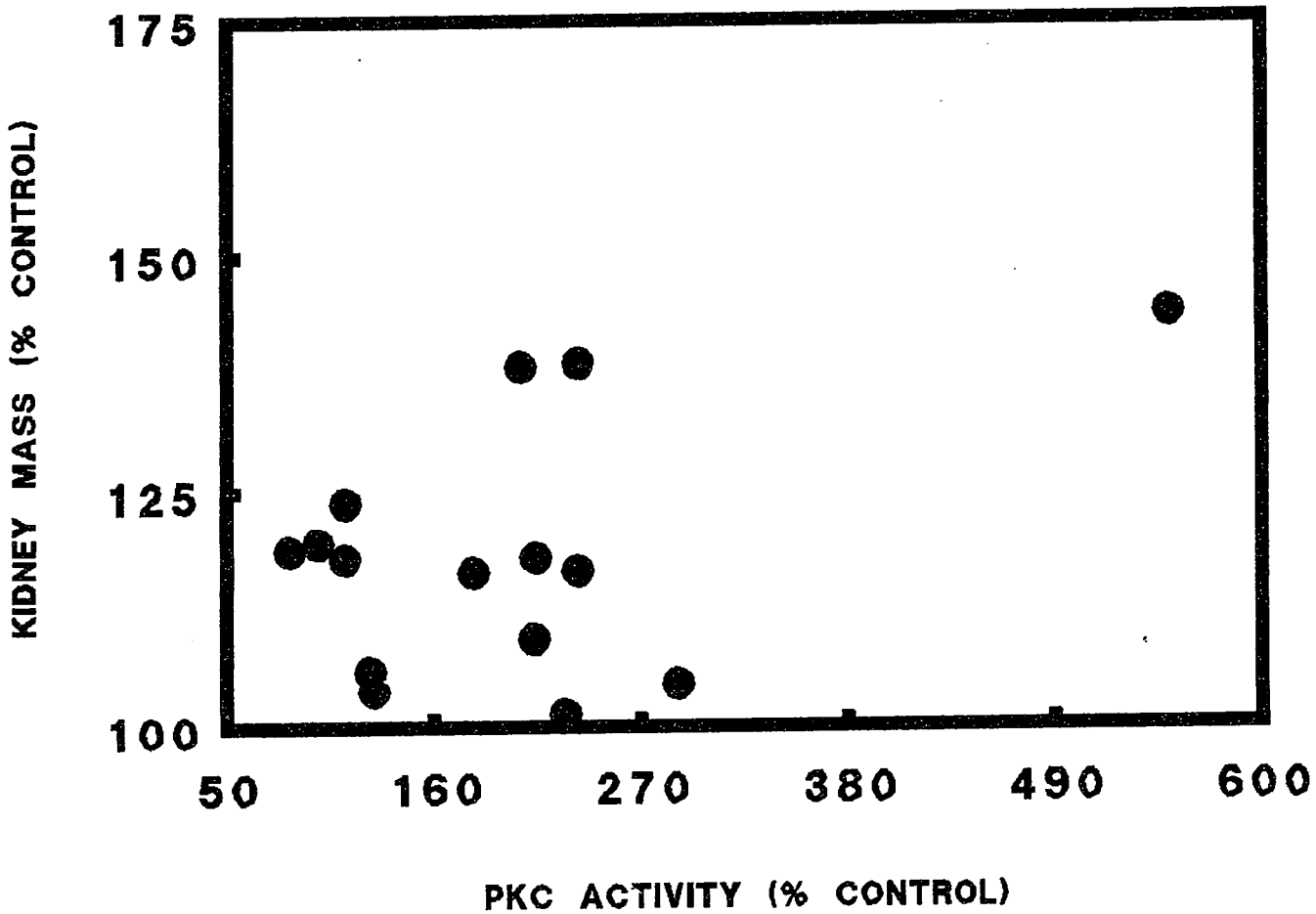

MEMBRANE
CYTOSOLIC
PROTEIN KINASE C ACTIVITY (% BASAL)
400
300
200
100
0
0
2
4
6
8
10
TIME AFTER STZ INJECTION (DAYS)

FIGURE 4.6.2.12 Correlation of renal membrane PKC activity and renal mass in STZ diabetic rats.
Data are duplicate or triplicate determinations of PKC activity measured in separate preparations of renal tubule membrane vs renal mass determined from matched STZ diabetic animals. The data on membrane PKC activity is obtained from figure 4.6.2.11 and the data on kidney mass was obtained from data used to calculate renal somatic index for figure 4.6.2.4. A positive correlation was observed between PKC activity and renal mass.


175
150
125
100
KIDNEY MASS (% CONTROL)
50
160
270
380
490
600
PKC ACTIVITY (% CONTROL)

4.6.3. DISCUSSION

These studies demonstrate that circulating $1,25(OH)_2D_3$ was significantly lower 72 hours after STZ injection or following insulin withdrawal. The reduced $1,25(OH)_2D_3$ was paralleled by reductions in renal proximal tubule 1αhydroxylase activity; these changes occurred at least 48-60 hours following onset of hypoinsulinemia and hyperglycaemia. Thus the alterations in vitamin D metabolism associated with diabetes do not appear to be the consequence of acute insulin absence. Although insulin deficiency could rapidly induce metabolic changes, such as availability of the 1αhydroxylase cofactor NADPH, which modulate $1,25(OH)_2D_3$ synthesis, results described in this thesis and reported by others (Section 4.5.1a&b, Ikeda et al., 1987, and Wongsurawat and Armbrecht, 1985) demonstrate that $1,25(OH)_2D_3$ synthesis in STZ diabetics can increase in response to low dietary Ca or PTH infusion. Since low dietary Ca does not alter serum insulin or glucose in diabetic rats, 1αhydroxylase induction would not be feasible if renal metabolic integrity were severely compromised by diabetes. Similarly Wongsurawat et al. (1983) demonstrated enhanced 24 hydroxylase activity in renal slices excised from chronically diabetic animals; the 24 hydroxylase enzyme requires similar metabolic conditions as the 1αhydroxylase.

Insulin may be required as a trophic factor for the maintenance of steady state levels of one or more of the 1αhydroxylase components or an auxiliary factor required for 1αhydroxylation or stimulation. The turnover time of adrenal steroid hydroxylase proteins is three to five days (Waterman, Mason and Simpson, 1988); if the vitamin D 1α and 24 hydroxylases follow a similar pattern, this would offer an explanation for the decrease in net synthesis of $1,25(OH)_2D_3$ observed three days after onset of hypoinsulinemia in diabetic rats. Such a role for insulin is consistent with earlier data presented in this thesis on the stimulatibility of $1,25(OH)_2D_3$ synthesis during diabetes (Section 4.5.1a&b). These studies demonstrated that while the % stimulation of basal 1αhydroxylase activity was comparable between STZ diabetic and control

animals, absolute net synthesis of $1,25(OH)_2D_3$ remained proportionately lower in the diabetics than in controls. Further investigations require the use of antibodies and cDNA probes for the renal vitamin D hydroxylases. The recent cloning of the 24 hydroxylase (Ohyama et al., 1991) and the possibility that 1αhydroxylase clones have been identified (Henry et al., 1991, Ghazarian et al., 1991) will be important tools for answering these questions. Although speculative, insulin may modulate the levels of various transcription factors (such as SHIP's) which may be required to elicit expression of chronic 1αhydroxylase stimulation (by [PTH induced] intracellular cAMP elevations) as has been demonstrated for several other steroid hydroxylases (Waterman, Mason and Simpson, 1988). Consistent with this hypothesis insulin was permissive for the PTH mediated increase in net $1,25(OH)_2D_3$ synthesis in avian renal tubule cultures (Henry, 1981). The chronic PTH mediated enhancement of net $1,25(OH)_2D_3$ synthesis in mammalian renal tubule cultures was correlated with total cellular cAMP and required new protein synthesis (Korkor et al., 1987). Experiments conducted during this thesis suggested that insulin was required for PTH stimulation of $1,25(OH)_2D_3$ synthesis in freshly isolated or primary cultures of proximal tubules (data not shown). Indeed Korkor et al. (1987) reported protein synthesis was necessary to demonstrate PTH stimulation of 1αhydroxylase activity. If insulin is required for chronic PTH stimulation of 1αhydroxylase via cAMP increased SHIP protein levels or activity or the induction of another auxiliary factor such as a putative phosphatase or kinase this could explain why decreased 1αhydroxylase activity persisted in low Ca fed diabetic animals compared to nondiabetic animals fed low Ca (Section 4.5.1.a.2).

The observation that 1αhydroxylase activity decreased in parallel with circulating $1,25(OH)_2D_3$ after insulin withdrawal suggests that increased metabolic clearance of $1,25(OH)_2D_3$ was not present in diabetes. Consistent with this suggestion, no significant changes in circulating $24,25(OH)_2D$ were observed between diabetic and control rats at any time following insulin

withdrawal. These observations agree with our data of reduced 24 hydroxylase activity and decreased or normal circulating $24,25(OH)_2D_3$ levels in established acute diabetes (Section 4.4.2). While there was a significant decrease in circulating PTH prior to the decreases in circulating $1,25(OH)_2D_3$ and net synthesis of $1,25(OH)_2D_3$ the decrease in PTH was transient, returning to control levels by 96 hours following insulin withdrawal in contrast to the depressed circulating $1,25(OH)_2D_3$ and 1αhydroxylase activity. Interestingly, reduced circulating PTH was also present in our studies of acutely diabetic BB rats. In that model reduced PTH was postulated to be due to Ca overload secondary to decreased requirements and increased Ca intake due to hyperphagia. While hyperphagia has been documented in insulin dependent diabetes (Nyomba et al., 1989), hyperphagia did not develop in STZ injected animals in these temporal studies until four to five days after diabetes induction and did not precede or correlate with altered vitamin D metabolism.

Renal hypertrophy, quantified as a significant increased renal somatic index and total protein, was observed by 48 hours of diabetes induction or insulin withdrawal. These changes in renal growth occurred 24 hours prior to the reductions in 1αhydroxylase activity and circulating $1,25(OH)_2D_3$. While total renal protein was increased in diabetics compared to controls, no significant decrease in renal protein/DNA ratio was apparent even after seven days of insulin absence. Thus the decrease in 1αhydroxylase activity cannot be attributed to alterations in 1αhydroxylase specific activity.

Renal hypertrophy was associated with a significant increase in renal tubule membrane PKC activity. The changes in kidney mass and PKC activity were temporally associated with decreased synthesis of $1,25(OH)_2D_3$ and reduced circulating $1,25(OH)_2D_3$ in diabetic rats. These observations support the suggestion that increases in renal tubule membrane PKC activity are associated with diabetic hypertrophy and might be related to impaired $1,25(OH)_2D_3$ synthesis. However, while temporal association provides circumstantial evidence for this hypothesis, further studies to confirm a

relationship are required and could include an assessment of insulin effects on PKC activity and $1,25(OH)_2D_3$ synthesis in diabetes by: demonstration of the reversibility of increased renal membrane PKC activity by insulin in vivo/in vitro and demonstration of correction of the compromised $1,25(OH)_2D_3$ synthesis in diabetes by inhibition of PKC activity in vitro. For example, during preparation of this thesis, it was observed that high doses of staurosporine (>50nM) actually stimulated net synthesis of $1,25(OH)_2D_3$ in isolated renal proximal tubules from low Ca fed rats however, further studies were not conducted to investigate the phenomena (data not shown). It would be interesting to determine if incubation of renal proximal tubules from diabetic animals, overnight with 100nM stauroporine, would correct the compromised 1αhydroxylase activity observed in these animals. In addition, the role of renal hypertrophy in conjunction with elevated PKC activity could be examined by the use of growth factor analogues (sandostatin) which prevent renal hypertrophy secondary to diabetes (Flyvbjerg et al., 1989).

The increased membrane PKC activity in association with decreased cytosolic PKC activity measured in renal tubules from STZ diabetic rats implies that translocation of PKC from cytosol to membrane occurred (Jaken, 1989). However, while decreased cytosolic activity was apparent at 24 and 48 hours and was significantly reduced 72 hours after STZ injection thereafter cytosolic PKC was not significantly different from controls. In addition, 12 hours following STZ injection, cytosolic PKC activity was significantly elevated relative to that measured in renal tubule cytosolic fractions isolated from vehicle injected control rats. Also, with one exception (96 hrs) PKC activity was elevated throughout the study, indicating that down regulation of PKC in response to chronic stimulation was not apparent. This implies that diabetes is associated with: continual translocation of cytosolic PKC to membrane, decreased degradation of membrane PKC, or increased amount of membrane PKC (possibly secondary to increased synthesis). Since increased membrane PKC activity was not always associated with decreased cytosolic PKC activity

it is probable that increased total cellular steady state levels of PKC are present in hypertrophied kidneys of STZ diabetic animals. In support for this hypothesis, Hise and Mehta (1989) demonstrated that total soluble, cytosolic and proximal tubule brush border membrane PKC activity was significantly elevated 4, 24 and 48 hours following unilateral nephrectomy in the hypertrophied remnant kidney. Measurement of total cellular PKC activity, assay of immunoreactive PKC and quantification of steady state mRNA levels in diabetic kidneys would be required to properly address these questions.

The PKC assay employed in these studies quantifies total activatible PKC activity since TPA, Ca and DAG were all present in the incubation. Thus, although the results indicate that diabetes is associated with increased PKC activity under maximally stimulated conditions, these results may not reflect PKC activity in situ which would depend on the prevailing concentration of endogenous PKC activators and inhibitors. Further studies examining non stimulated native membrane PKC activity in diabetic renal tubules, using a method such as that described by Chakravarthy et al. (1989), should be considered. In addition the concept that various isozymes of PKC (Parker et al., 1989) may be differentially affected by diabetes requires clarification.

While enhanced renal PKC activity in diabetes provides an explanation for the reduced synthesis of $1,25(OH)_2D_3$ and decreased circulating $1,25(OH)_2D_3$, the issue of 24 hydroxylase activity requires clarification. The in vitro studies described in this thesis (Section 4.5.1.b.2) demonstrated that decreased 1αhydroxylase activity and increased 24 hydroxylase activity were associated with PKC activation. Thus increased PKC activity should be associated with decreased 1αhydroxylase and increased 24 hydroxylase activity in diabetes and increased metabolic clearance of $1,25(OH)_2D_3$ would also be expected (Tomon, Tenenhouse and Jones, 1990). While reductions in both 1αhydroxylase activity and circulating $1,25(OH)_2D_3$ are consistently observed during diabetes (Hough et al., 1983, Schneider et al., 1977), 24 hydroxylase activity has been reported as increased (Wongsurawat et al., 1983) or reduced

(Section 4.4.2) and circulating 24,25$(OH)_2D_3$ normal (Rodland et al., 1985) or reduced (Imura, Seino and Ishida, 1985, Section 4.4.2). In addition, increased metabolic clearance of 1,25$(OH)_2D_3$ has not been observed in diabetic animals fed normal diets (Spencer et al., 1980). One explanation is that the 24 hydroxylase and 1αhydroxylases are differentially regulated by PKC, as suggested and supported by Henry and Luntao (1989) and by the in vitro PKC data presented previously in this thesis (Section 4.5.1.b.2) which may be facilitated by different PKC isozymes. Alternately, as previously discussed (4.4.2 and 4.5.1), 24 hydroxylase activity in diabetes may reflect target tissue effects of circulating 1,25$(OH)_2D_3$, which remains low in STZ diabetic animals on normal diets. Finally it is possible that more than one 24 hydroxylase protein exists, exemplified by the recent cloning of unique 24 hydroxylase cDNA's (Ghazarian et al., 1991, Ohyama et al., 1991). The existence of 24 hydroxylase isozymes could explain variable PKC modulation.

A transient, but significant, rise in circulating 1,25$(OH)_2D_3$ was observed 12 hours after STZ injection. At this time, circulating insulin was significantly reduced and significant hyperglycaemia was evident. Discussion of the aetiology of this surge in 1,25$(OH)_2D_3$ is purely speculative, however it is of interest that 1,25$(OH)_2D_3$ synthesis also rises transiently in the remnant kidney following unilateral nephrectomy (Almond et al., 1989). Alternately, changes in renal sensitivity to insulin may occur in the initial stages of diabetes onset facilitating stimulation of 1,25$(OH)_2D_3$ production. Future studies will be required to ascertain if the increase in circulating 1,25$(OH)_2D_3$ is paralleled by an increase in 1,25$(OH)_2D_3$ synthesis, if it is also observed at 12 hours following insulin withdrawal and if the surge is associated with renal hypertrophy.

4.7. OVERALL SUMMARY AND CONCLUSIONS

4.7.1. MG DEFICIENCY AND VITAMIN D METABOLISM

Mg deficient chicks fed adequate Ca exhibited normal circulating 1,25$(OH)_2D_3$ and 1αhydroxylase activity with elevated bone Ca despite

significant hypocalcaemia. This is an inappropriate response as compared to control chicks fed Ca deficient diets who exhibited elevated circulating $1,25(OH)_2D_3$ and increased 1αhydroxylase activity, with decreased bone Ca and maintenance of extracellular Ca. Chicks consuming diets low in Ca concomitant with or subsequent to Mg depletion had elevated circulating $1,25(OH)_2D_3$ and decreased bone Ca and maintained normocalcaemia. Since Mg deficient chicks supplemented with $1,25(OH)_2D_3$ also had elevated circulating $1,25(OH)_2D_3$ and normal extracellular Ca these results suggest that alterations in vitamin D metabolism in Mg deficiency are pivotal to the genesis of hypomagnesaemic hypocalcaemia.

The inappropriate responsiveness of the 1αhydroxylase to the hypocalcaemia of Mg deficiency was not associated with decreased circulating $25(OH)D_3$, increased circulating $24,25(OH)_2D_3$, increased metabolic clearance of $1,25(OH)_2D_3$ or decreased renal viability. Nor was renal PTH resistance indicated as the cause of the refractoriness of 1αhydroxylase to the hypocalcaemia, evidenced by intact in vitro PTH adenylate cyclase and 1αhydroxylase responsiveness and an in vivo elevation in circulating $1,25(OH)_2D_3$ during low dietary Ca. Further evidence for intact target tissue responsiveness to PTH, despite Mg deficiency, was demonstrated by decreased bone mass and Ca content in low Ca fed Mg deficient chicks. Finally vitamin D target tissue resistance during Mg deficiency was not indicated since no changes in plasma and bone alkaline phosphatase activity or intestinal calbindin D28k were observed during Mg deficiency, and bone Ca content and intestinal calbindin D28k increased comparably following $1,25(OH)_2D_3$ supplementation in control and Mg deficient chicks. Finally, the data demonstrates that $1,25(OH)_2D_3$ is not a major regulator of Mg homeostasis.

4.7.2. INSULIN DEPENDENT DIABETES AND VITAMIN D METABOLISM

These studies demonstrated reduced net synthesis of $1,25(OH)_2D_3$ and decreased circulating $1,25(OH)_2D_3$ in both chemically induced and spontaneous

diabetes. These changes were apparent in the STZ diabetic model 48-60 hours following onset of hypoinsulinemia and hyperglycaemia. The impaired $1,25(OH)_2D_3$ synthesis and reduced circulating $1,25(OH)_2D_3$ was corrected in both models by in vivo insulin therapy. The defect in $1,25(OH)_2D_3$ synthesis was not corrected in chemically induced diabetes by up to 18 hours exposure to insulin, in vitro. The low circulating $1,25(OH)_2D_3$ appears to be due primarily to reduced net $1,25(OH)_2D_3$ synthesis. However, increased metabolic clearance of $1,25(OH)_2D_3$ may also be involved as was indicated when diabetic rats were subjected to diets low in either Ca or P. While hypoparathyroidism was observed in established BB diabetes and transiently after insulin withdrawal from STZ diabetic rats, there was no correlation between serum PTH levels and circulating $1,25(OH)_2D_3$ or 1αhydroxylase activity. Thus while PTH insufficiency may contribute to decreased 1αhydroxylase activity and lower circulating $1,25(OH)_2D_3$ in diabetes, other factors must also participate since 1αhydroxylase and circulating $1,25(OH)_2D_3$ are consistently low in insulin dependent diabetes. These studies indicate that target tissue resistance to PTH does not explain the decreased $1,25(OH)_2D_3$ synthesis observed in diabetes since PTH stimulation of cAMP synthesis and 1αhydroxylase activity was comparable in renal tubules isolated from STZ diabetic and control rats. In addition % 1αhydroxylase stimulation in response to low dietary Ca was comparable in vivo between STZ diabetic and controls rats. Instead these results suggest that stimulation of 1αhydroxylase activity fails to overcome the compromised net synthesis of $1,25(OH)_2D_3$ in diabetes. Thus decreased net synthesis of $1,25(OH)_2D_3$ during diabetes appears to be due to either decreased steady state levels of one or more components of the 1αhydroxylase system or increased inhibition of 1αhydroxylation. A consistent observation in both models of diabetes, corrected by insulin therapy, was renal hypertrophy, which was apparent 48 hours following diabetes induction or insulin withdrawal in STZ diabetes. Diabetic renal hypertrophy was evident 24 hours prior to low circulating $1,25(OH)_2D_3$ and was associated with increased renal membrane

PKC activity. Since these studies also demonstrated that PKC activation decreased net synthesis of $1,25(OH)_2D_3$, collectively these observations form the basis of the hypothesis that elevated PKC activity in insulin dependent diabetes, is related to decreased 1αhydroxylase activity. Future studies will be required to test this hypothesis and to ascertain the precise role of insulin on vitamin D metabolism. The quantitation of hydroxylase mRNA and protein levels using cDNA probes and antibodies should elucidate whether insulin exerts trophic actions on the hydroxylase enzyme system. Finally, further examination of the role of PTH signal transduction in the regulation of renal vitamin D metabolism, will be necessary for a comprehensive assessment of the aetiology of the aberrant vitamin D metabolism associated with insulin dependent diabetes.

4.7.3. ASSAY OF NET $1,25(OH)_2D_3$ SYNTHESIS

The effects of alterations in extracellular Mg and insulin as well as in vitro insulin, PTH and phorbol esters on net $1,25(OH)_2D_3$ synthesis in avian (chick) and mammalian (rat) isolated renal tubule suspensions were assessed in these studies. In both species, quantitation of $1,25(OH)_2D_3$ produced, by renal tubules, was achieved by radioreceptor assay following isolation of $1,25(OH)_2D_3$ on C18 and Silica Sep Paks and HPLC. Chick studies used fresh mixed renal tubule preparations while rat studies used fresh purified proximal preparations. Neither system relied on dietary or surgical manipulations to obtain measurable enzyme activity. Unique from reports by other labs, these studies were conducted in preparations from individual animals. A significant correlation between circulating $1,25(OH)_2D_3$ and 1αhydroxylase activity was obtained for both species when preparations were obtained from individual animals. Tubules isolated from low Ca (rats, chicks) and vitamin D deficient animals (rats) had high 1αhydoxylase activity and tubules isolated from diabetic animals (STZ, BB rats) had low 1αhydroxylase activity relative to controls. Freshly isolated rat tubule preparations maintained viability, demonstrated intact PTH and vasopressin receptors, PKC and 1αhydroxylase

activity and adenylate cyclase stimulatibility for up to 18-20 hours and could be successfully cultured. Therefore these results demonstrate the validity of the isolated tubule preparations from both chicks and rats as in vitro models for studying vitamin D metabolic regulation.

Collectively these studies demonstrated that extracellular Mg is not a major regulator of $1,25(OH)_2D_3$ net synthesis and insulin does not regulate 1αhydroxylase activity directly nor does insulin acutely regulate 1αhydroxylase activity. Activation of PKC was associated with acute decreases in 1αhydroxylase activity and increases in 24 hydroxylase activity while in vitro PTH acutely enhanced in vitro 1αhydroxylase activity in chick tubules and chronically enhanced in vitro 1αhydroxylase activity, in the presence of insulin, in rat tubules.

The failure to observe acute PTH stimulation of 1αhydroxylase activity in rat tubules described in this thesis may reflect species differences. Since various kinases and phosphatases have been implicated in acute PTH mediated stimulation of 1αhydroxylase activity it is possible that failure to observe acute PTH stimulation in rats was due to differential expression of these proteins in rats vs chicks.

$1,25(OH)_2D_3$ synthesis rates measured in mixed renal tubules isolated from low Ca fed chicks were 17x (1111 fmoles/min/mg tubule protein vs 67 fmoles/min/mg tubule protein) higher at a substrate concentration 50x (100nM vs 5,000nM $25(OH)D_3$) lower than that measured in proximal renal tubules isolated from low Ca fed rats. These results are consistent with previous reports of high avian 1αhydroxylase activity at low substrate concentrations and low rat 1αhydroxylase activity at high substrate concentrations (Booth, Tsai and Morris, 1985, Turner et al., 1982). Since both systems employed isolated renal tubules, utilized comparable tubule protein levels and exhibited similar product recovery rates these differences cannot be attributed to characteristics inherent in the assay system.

It has been suggested that the presence of an inhibitory protein (such

as transcalciferin) is responsible for the lower 1αhydroxylase activity and higher substrate requirement demonstrated by rats (Botham et al., 1976). Indeed rat serum possesses high vitamin D binding capacity and transcalciferin levels (Shephard and DeLuca, 1980). Addition of vitamin D binding protein to guinea pig mitochondrial preparations decreased 1αhydroxylase activity (Simboli, 1988). The capacity of rat serum to bind vitamin D metabolites is increased by vitamin D deficiency (Shephard and DeLuca, 1980), thus if the low in vitro activity and high substrate concentration requirements observed in rat tubules are due solely to the presence of transcalciferin, it would be anticipated that assay of 1αhydroxylase activity in tubules from vitamin D deficient rats would require higher substrate concentrations to overcome this effect. However the experiments described in this thesis and in the literature demonstrate comparable 1αhydroxylase disassociation constants (Km 1μM) values for tubules excised from low Ca fed and vitamin D deficient rats which were exposed to increasing substrate concentration.

Alternately differences in 1αhydroxylase activity and substrate requirements may reflect higher vitamin D catabolic rates in renal tubule preparations from rats (Turner, 1984). Vitamin D deficiency is associated with low 24 hydroxylase activity, which has been implicated in vitamin D catabolism. However tubules isolated from vitamin D deficient rats had 1αhydroxylase activity 52x lower than chicks fed low Ca diets; 3.2x lower than tubules isolated from low Ca fed rats. Thus the differences between 1αhydroxylase activity in chicks and rats must reflect the presence of other, as yet unidentified, inhibitors, the absence of an auxiliary factor necessary for maintenance of elevated 1αhydroxylase activity or inherent genetic species differences. Protein and nucleic acid sequence analysis of avian and rat 1αhydroxylase P450s will be required to clarify this issue.

The 1αhydroxylase systems, described in this thesis are consistent with existing reports. Thus concerning avian 1αhydroxylase characteristics:

Trechsel, Bonjour and Fleisch (1979) reported a Km of 125nM for chick primary cultures and Delvin and Dussault (1985) reported a Km of 605nM and Vmax of 40 pmole/30min/mg mitochondrial proteins from guinea pigs while Engstrom et al. (1984) reported a Km of 445nM and Vmax of 286 pmoles/min/g guinea pig kidney protein which is consistent with the Km of 100nM and Vmax of 1.111 pmoles/min/mg renal tubule protein from low Ca fed chicks reported in this thesis. Concerning mammalian (rodent, rat) 1αhydroxylase characteristics: Korkor et al. (1987) reported a Km of 1.4μM and a Vmax of 2.6 pmoles/hour/mg in mouse primary cultures (D deficient) similar to Turner et al. (1982) who reported a Km of 0.8μM for 1αhydroxylase activity and 2μM for 24 hydroxylase activity in renal cell suspensions from control rats and Lobaugh and Drezner (1986) who reported a Vmax of 0.5 fmoles/min/mg suspended renal cells from control rats, which are all in agreement with the data presented in these experiments of a Vmax of 21 fmoles/min/mg tubule protein for 1αhydroxylase activity from D deficient rats, 67 fmoles/min/mg tubule protein for 1αhydroxylase activity from low Ca fed rats and a Km of 1.0μM. The data concerning mammalian 24 hydroxylase activity is also consistent with the data from this thesis: Ishida et al. (1987) reported a Km of 1.1-1.2μM for 24 hydroxylase activity and a Vmax of 8.2 fmoles/10min/mg kidney protein from control rats which agrees with the Km estimated by these studies of 1.25μM and a Vmax of 500 fmoles/min/mg tubule protein for 24 hydroxylase activity from control rats. In addition, Lobough and Drezner (1986) reported an average product recovery rate of 58 ± 4% for $1,25(OH)_2D_3$ synthesis measured in rat tubule suspensions employing a radioreceptor detection method which is in agreement with an average product recovery of 45-65% for avian 1αhydroxylase, rat 1αhydroxylase and 24 hydroxylase assays reported in this thesis.

Finally, these results demonstrate clear differences between avian and mammalian 1αhydoxylases and underscore the need for caution when extrapolating results from one animal species to another.

OVERALL CONCLUSIONS

The overall conclusions of these studies were as follows:

1. Vitamin D metabolism is altered in primary Mg deficiency. These alterations are critical to the development and/or progression of hypomagnesaemic hypocalcaemia.
2. Decreased circulating $1,25(OH)_2D_3$ in diabetes is due to a defect in $1,25(OH)_2D_3$ synthesis; although increased metabolic clearance may be present during Ca or P deficiency. Decreased $1,25(OH)_2D_3$ synthesis during diabetes appears to be due to decreased steady state levels of 1αhydroxylase components or increased inhibition of $1,25(OH)_2D_3$ synthesis. Alterations in Mg homeostasis exist in diabetes, however Mg deficiency is not the primary cause of altered vitamin D metabolism in diabetes. Although hypomagnesaemia may prove to be a complicating factor, evidence suggests that other factors, such as renal hypertrophy and elevated PKC activity are more relevant to the 1αhydroxylase disturbances.
3. Avian 1αhydroxylase activity is refractory to large fluctuations in extracellular Mg. It is probable that secondary changes, induced by Mg deficiency, are responsible for the refractoriness of the 1αhydroxylase to the hypocalcaemia of Mg deficiency in chicks. Insulin does not directly stimulate mammalian basal $1,25(OH)_2D_3$ production and its absence does not acutely alter $1,25(OH)_2D_3$ synthesis in rats. Collectively these studies suggest that Mg per se is not an important regulator of $1,25(OH)_2D_3$ synthesis whereas insulin is required for the maintenance of in vivo vitamin D metabolism. Finally these studies imply dual regulation of $1,25(OH)_2D_3$ by a stimulatory pathway (adenylate cyclase) (rats and chicks) and an inhibitory pathway (PKC) (rats).

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APPENDIX 1

Materials and Methods - Tables and Figures

TABLE 2.1 Basal chick diet composition

COMPONENT	% OF DIET
Isolated soybean protein	25.00
DL Methionine, 98%	0.60
Glycine	0.40
Refined corn oil	4.00
Glucose monohydrate	60.17
Cellulose (Solka Flok)	3.00
Vitamin premix[a]	1.20
Mg-free mineral premix[b]	5.63

[a]Composition (in amount/100 g diet):
Thiamine HCl, 1.5 mg; Riboflavin, 1.5 mg; Nicotinic acid, 5.0 mg; Folic acid, 0.6 mg; Pyridoxine, 0.6 mg; Biotin, 0.06 mg; Vitamin B_{12}, 2.0 g; Choline chloride, 200.0 mg; d-Calcium pantothenate, 2.0 mg; menadione sodium bisulfate, 0.15 mg; Vitamin E, 5IU; Vitamin D_3, 450 IU; Vitamin A, 450 IU; butylated hydroxytoluene, 10.0 mg; Glucose monohydrate added to make 1.20 g.
[b]See Table 2.2

TABLE 2.2 Composition of Mg free low Ca mineral mix used for basal chick diet

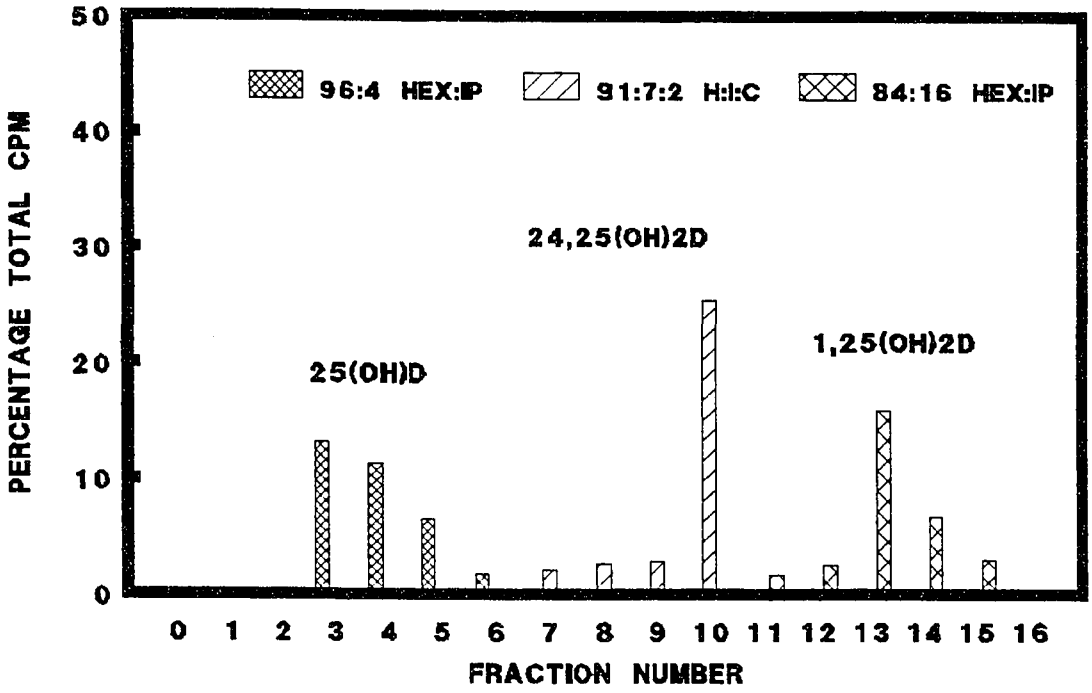
COMPONENT	mg/100g DIET
KH_2PO_4	1000
KCl	100
NaCl	600
$FeSO_4.7H_2O$	50
$MnSO_4.H_2O$	35
$ZnSO_4$	34.3
$CuSO_4.5H_2O$	3
KIO_3	1
$CoCl_2$	0.17
$NaMoO_4.2H_2O$	0.83
Na_2SeO_4	0.02
$CaHPO_4.2H_2O$	
(1.5%) Control Ca	3913
(0.2%) Low Ca	508.7
$CaCO_3$	
(1.5%) Control Ca	1480
or	
(0.2%) Low Ca	192.4
$MgSO_4.7H_2O$	
(0.1%) Control	1014
or	
(0.01%) Mg Deficient	OMIT

FIGURE 2.1 (top) Adsorption silica SEP PAK separation of vitamin D metabolites

Separation of vitamin D metabolites by silica Sep Pak adsorption chromatography. Serum was spiked with equal amounts of tritiated vitamin D metabolites, extracted with acetonitile and polar lipids were purified by C18 affinity chromatography. Purified extract was resuspended in Hexane:Isopropanol (96:4) and applied to pre-washed silica Sep Pak columns and vitamin D metabolites were eluted with 1.5 ml aliquots of solvent mixtures as indicated. Eluent were dried by evaporation in a fumehood and counted for radioactivity by scintillation detection. Data is expressed as % total counts.

FIGURE 2.2 (bottom) Silica HPLC isolation of $24,25(OH)_2D_3$.

HPLC isolation of $24,25(OH)_2D_3$ from serum spiked with tritiated $24,25(OH)_2D_3$, acetonitrile extracted and C18 affinity chromatography purified. Extract was resuspended in 100ul HPLC solvent and separated on a silica (5um) HPLC column using Hexane:isopropanol:methanol (91:7:2) flow rate 2 mls/min. Sample was collected every 0.5 minutes for 12 minutes, dried overnight in a fumehood and counted for radioactivity by scintillation detection. Data is expressed as % total counts.


96:4 HEX:IP
91:7:2 H:I:C
84:16 HEX:IP
24,25(OH)2D
25(OH)D
1,25(OH)2D
PERCENTAGE TOTAL CPM
FRACTION NUMBER

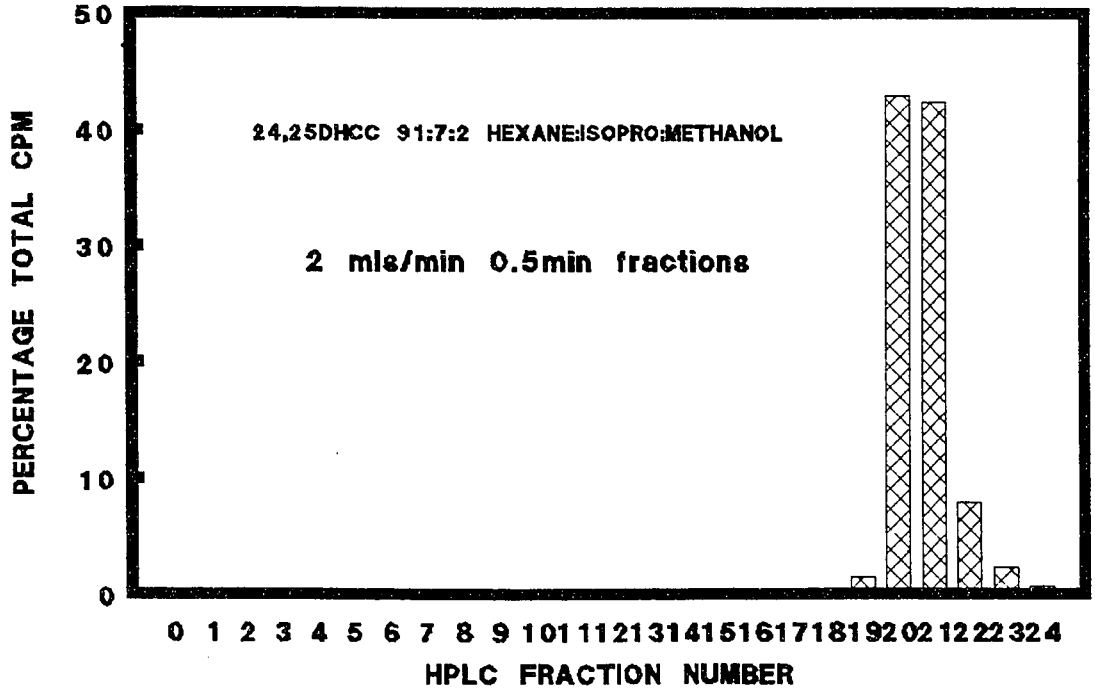
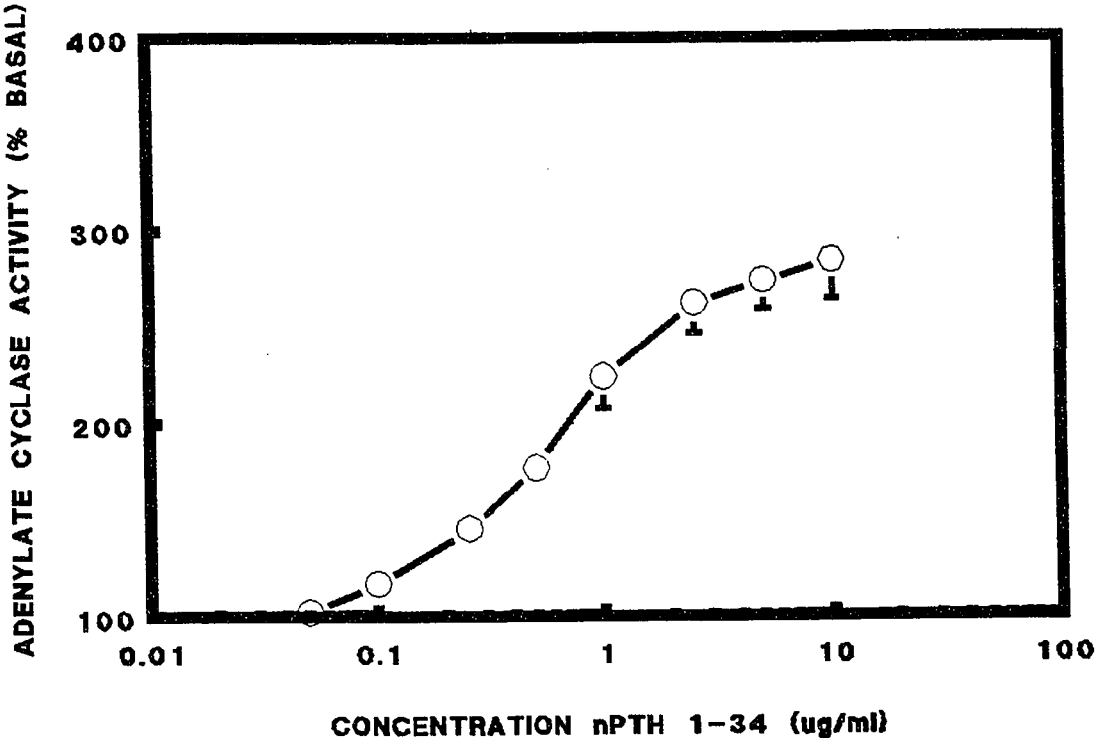

24,25DHCC 91:7:2 HEXANE:ISOPRO:METHANOL
2 mls/min 0.5min fractions
PERCENTAGE TOTAL CPM
HPLC FRACTION NUMBER

FIGURE 2.3 (top) Dose response of PTH on cAMP in isolated chick renal membranes.
Effect of increasing bovine n1-34 PTH on adenylate cyclase activity in the presence of $1x10^{-6}$ GTP and 1.5 mM Mg, measured in renal membranes isolated from chicks fed adequate dietary Ca and Mg. Data is mean ± SEM of duplicates from three separate experiments.

FIGURE 2.4. (bottom) Dose response of GTP on cAMP in isolated chick renal membranes.
Effect of increasing GTP concentration with or without 5 μM bovine n1-34 PTH on adenylate cyclase activity in renal membranes isolated from chicks fed adequate dietary Ca and Mg. Data is mean of duplicates of two separate experiments.


ADENYLATE CYCLASE ACTIVITY (% BASAL)
400
300
200
100
0.01
0.1
1
10
100
CONCENTRATION nPTH 1-34 (ug/ml)

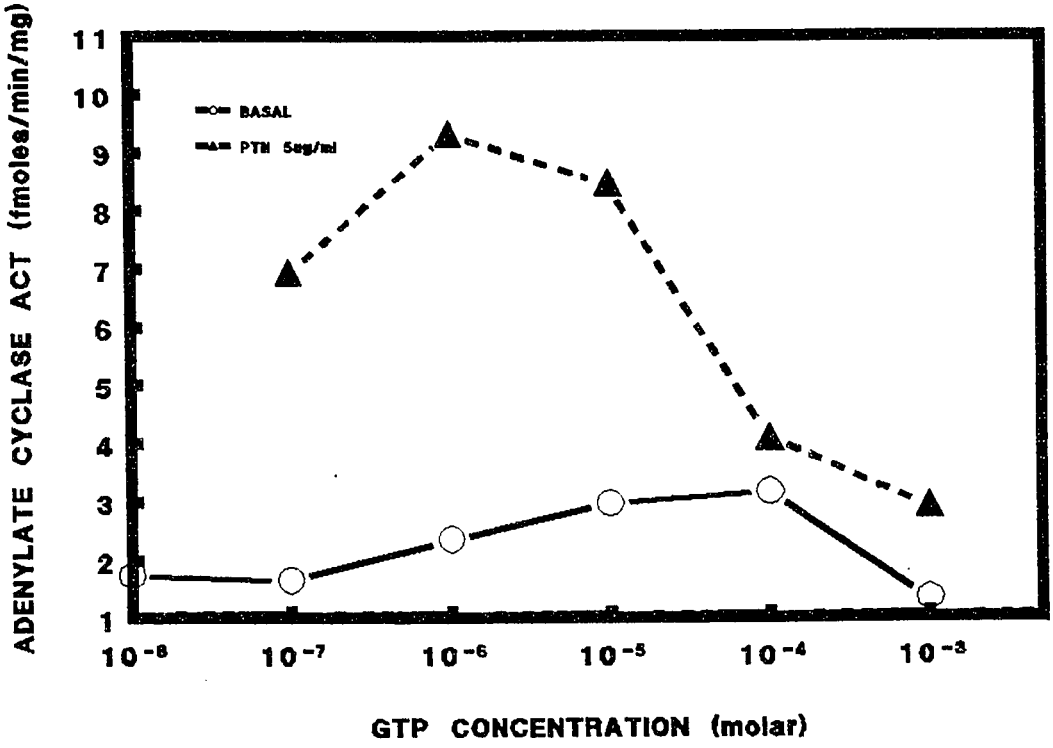
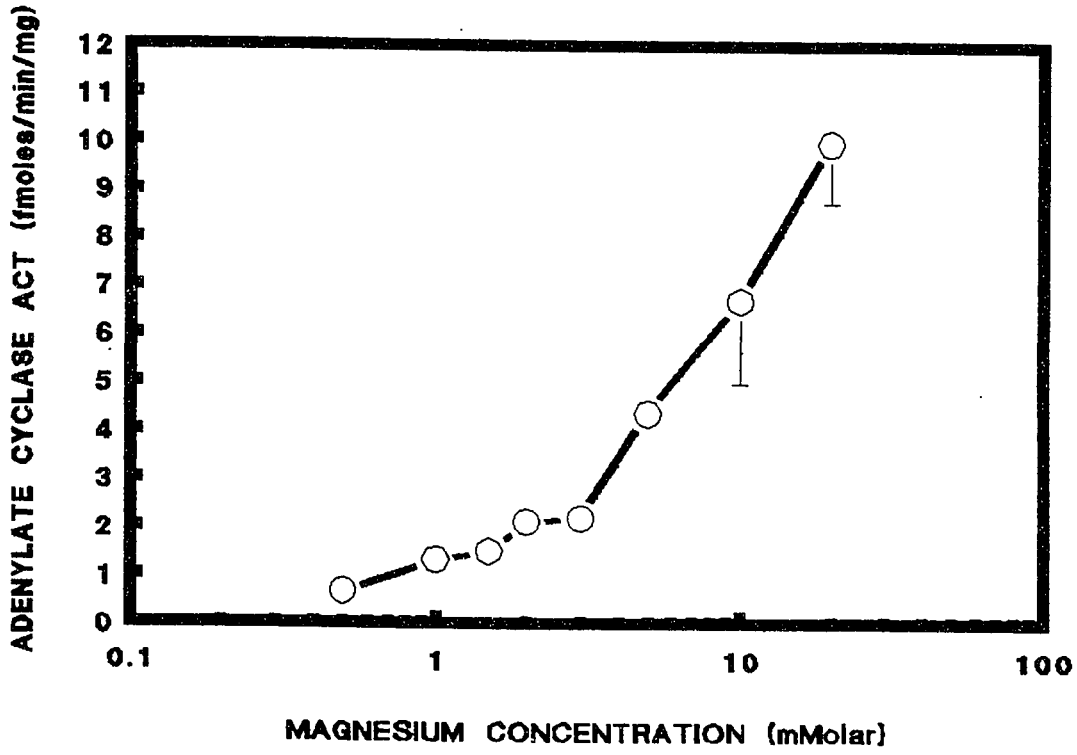

ADENYLATE CYCLASE ACT (fmoles/min/mg)
11
10
9
8
7
6
5
4
3
2
1
BASAL
PTH 5ug/ml
10-8
10-7
10-6
10-5
10-4
10-3
GTP CONCENTRATION (molar)

FIGURE 2.5.a. (top) Effect of Mg concentration on basal cAMP in isolated chick renal membranes.
Effect of increasing Mg concentration on adenylate cyclase activity in renal membranes isolated from chicks fed adequate dietary Ca and Mg. Data is mean ± SEM of duplicates from two separate experiments.

FIGURE 2.5.b. (bottom) Effect of Ca concentration on basal cAMP in isolated chick renal membranes.
Effect of increasing Ca concentration on adenylate cyclase activity in renal membranes isolated from chicks fed adequate dietary Ca and Mg. Data is mean ± SEM of duplicates from two separate experiments.


ADENYLATE CYCLASE ACT (fmoles/min/mg)
12
11
10
9
8
7
6
5
4
3
2
1
0
0.1
1
10
100
MAGNESIUM CONCENTRATION (mMolar)

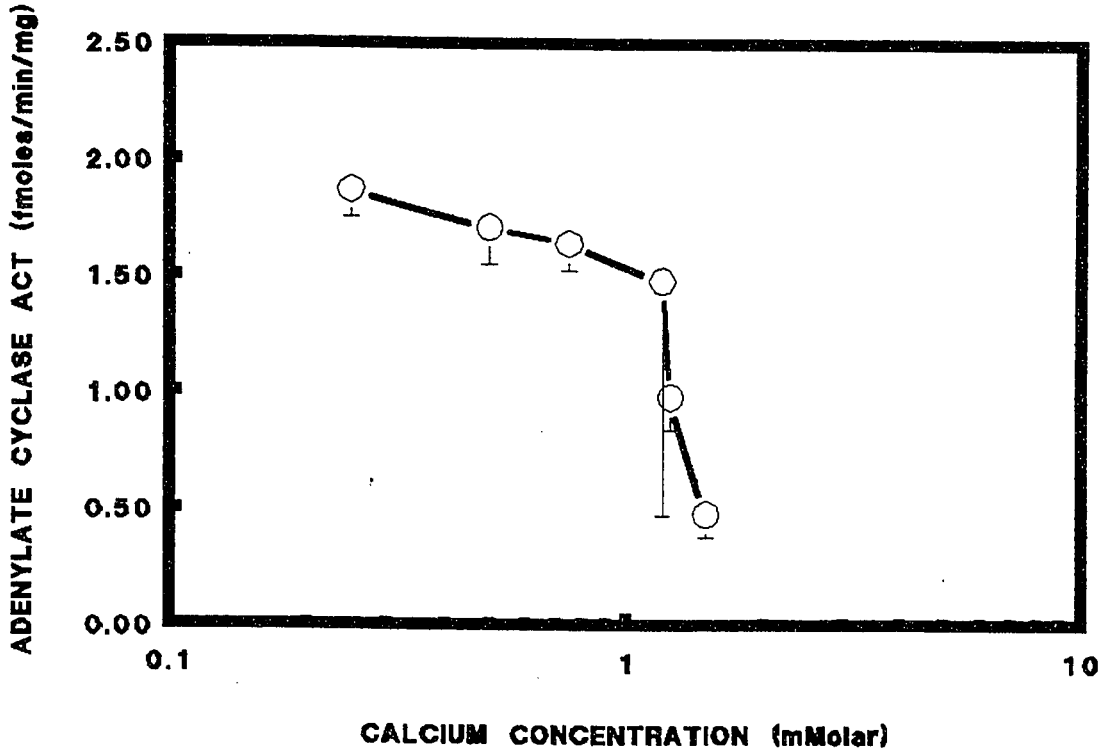
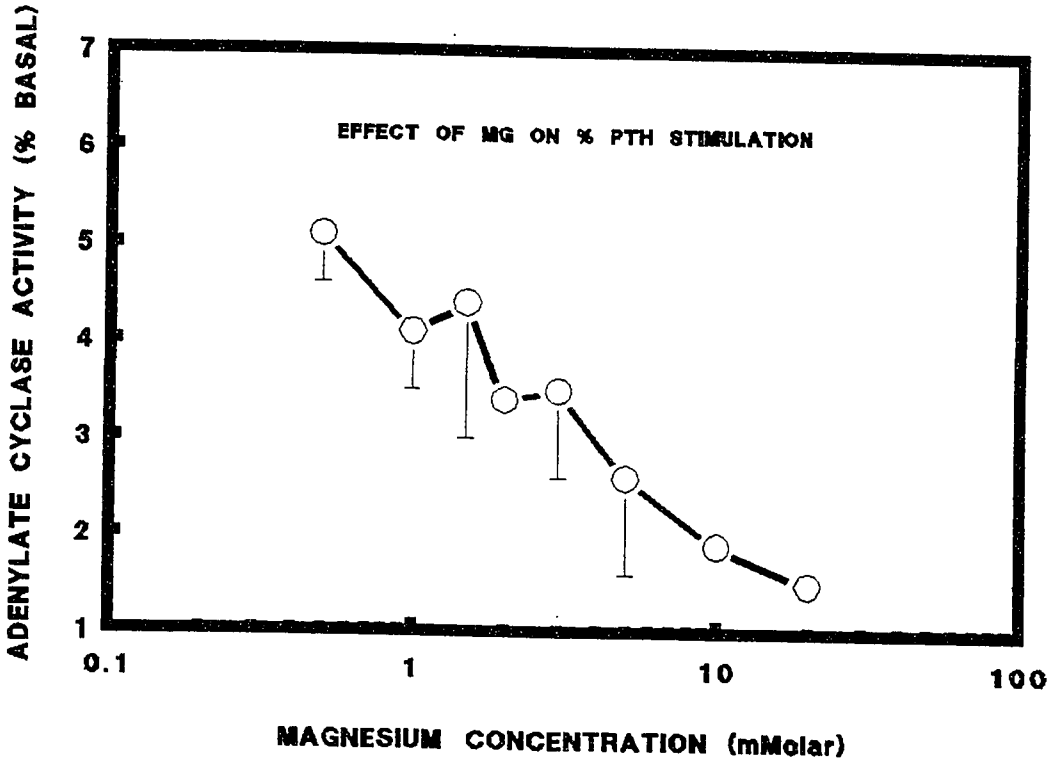

ADENYLATE CYCLASE ACT (fmoles/min/mg)
2.50
2.00
1.50
1.00
0.50
0.00
0.1
1
10
CALCIUM CONCENTRATION (mMolar)

FIGURE 2.6.a. (top) Effect of Mg concentration on PTH stimulated cAMP in isolated chick renal membranes.
Effect of increasing Mg concentration on 5μM bovine n1-34 PTH stimulation of adenylate cyclase activity in renal membranes isolated from chicks fed adequate dietary Ca and Mg. Data are mean ± SEM of duplicates from two separate experiments.

FIGURE 2.6.b. (bottom) Effect of Ca concentration on PTH stimulated cAMP in isolated chick renal membranes.
Effect of increasing Ca concentration on 5μM bovine n1-34 PTH stimulation of adenylate cyclase activity in renal membranes isolated from chicks fed adequate dietary Ca and Mg. Data are mean ± SEM of duplicates from two separate experiments.


EFFECT OF MG ON % PTH STIMULATION
ADENYLATE CYCLASE ACTIVITY (% BASAL)
7
6
5
4
3
2
1
0.1
1
10
100
MAGNESIUM CONCENTRATION (mMolar)

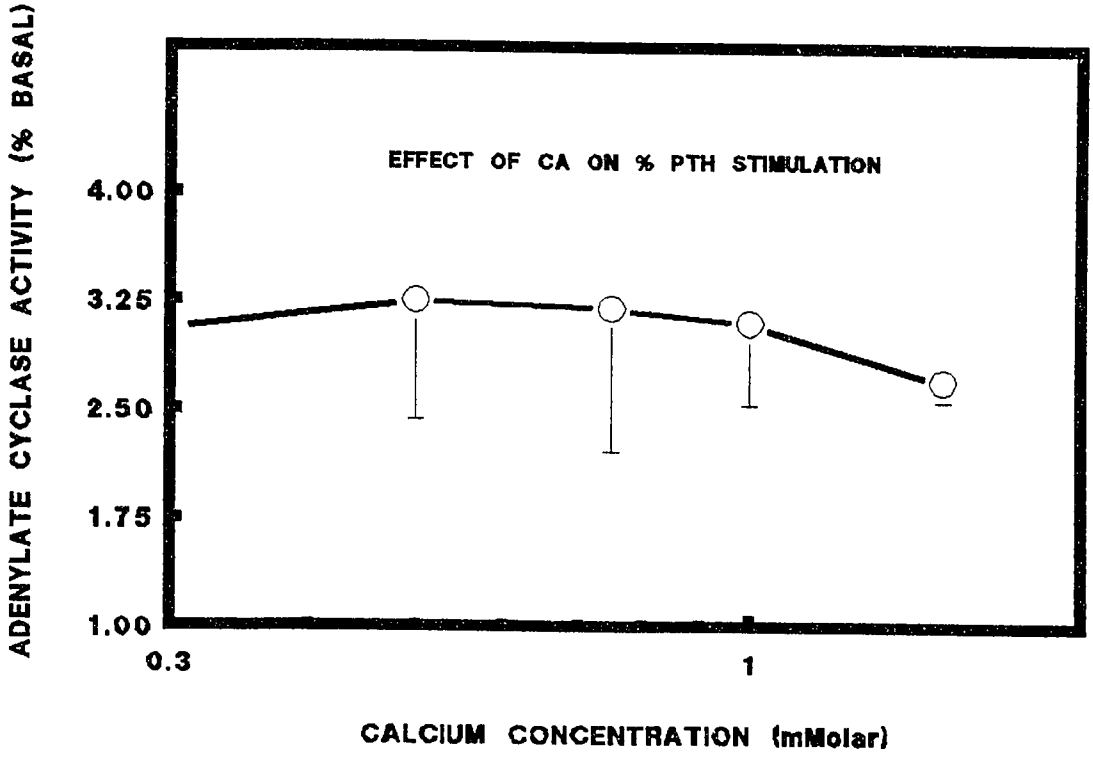
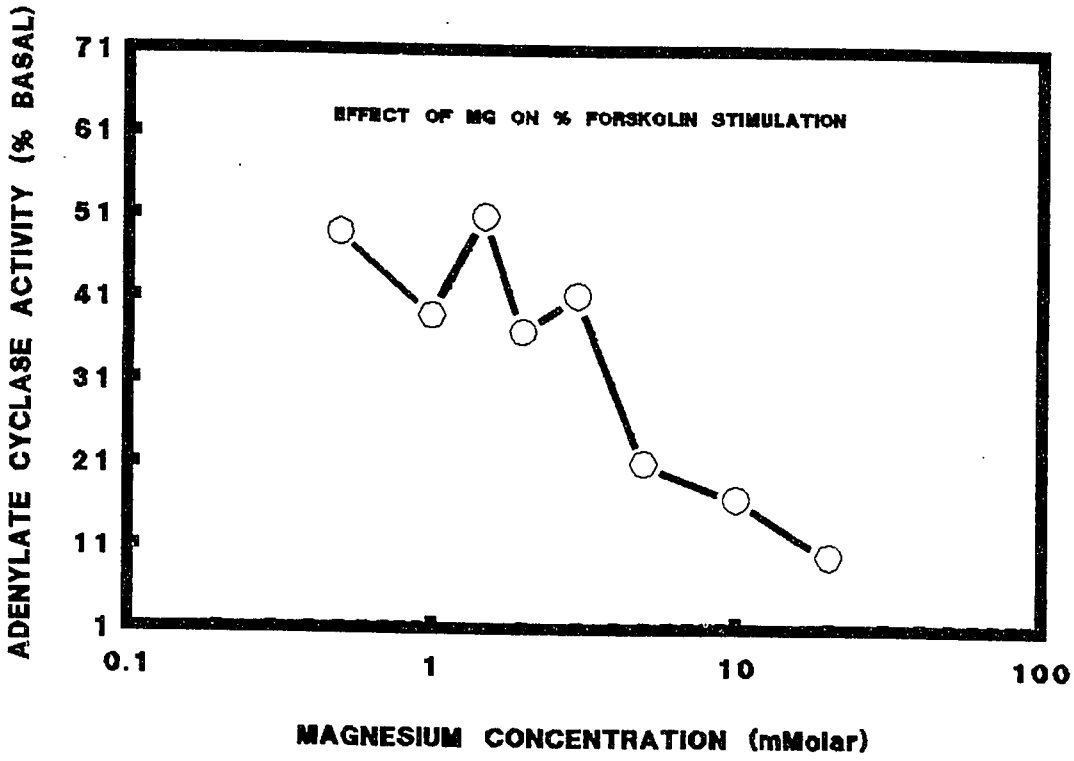

EFFECT OF CA ON % PTH STIMULATION
ADENYLATE CYCLASE ACTIVITY (% BASAL)
4.00
3.25
2.50
1.75
1.00
0.3
1
CALCIUM CONCENTRATION (mMolar)

FIGURE 2.7.a. (top) Effect of Mg concentration on forskolin stimulated cAMP in isolated chick renal membranes.
Effect of increasing Mg concentration on 10^{-4} M forskolin stimulation of adenylate cyclase activity in renal membranes isolated from chicks fed adequate dietary Ca and Mg. Data are mean ± SEM of duplicates from two separate experiments.

FIGURE 2.7.b. (bottom) Effect of Ca concentration on forskolin stimulated cAMP in isolated chick renal membranes.
Effect of increasing Ca concentration on 10^{-4} M forskolin stimulation of adenylate cyclase activity in renal membranes isolated from chicks fed adequate dietary Ca and Mg. Data are mean ± SEM of duplicates from two separate experiments.


EFFECT OF MG ON % FORSKOLIN STIMULATION
ADENYLATE CYCLASE ACTIVITY (% BASAL)
71
61
51
41
31
21
11
1
0.1
1
10
100
MAGNESIUM CONCENTRATION (mMolar)

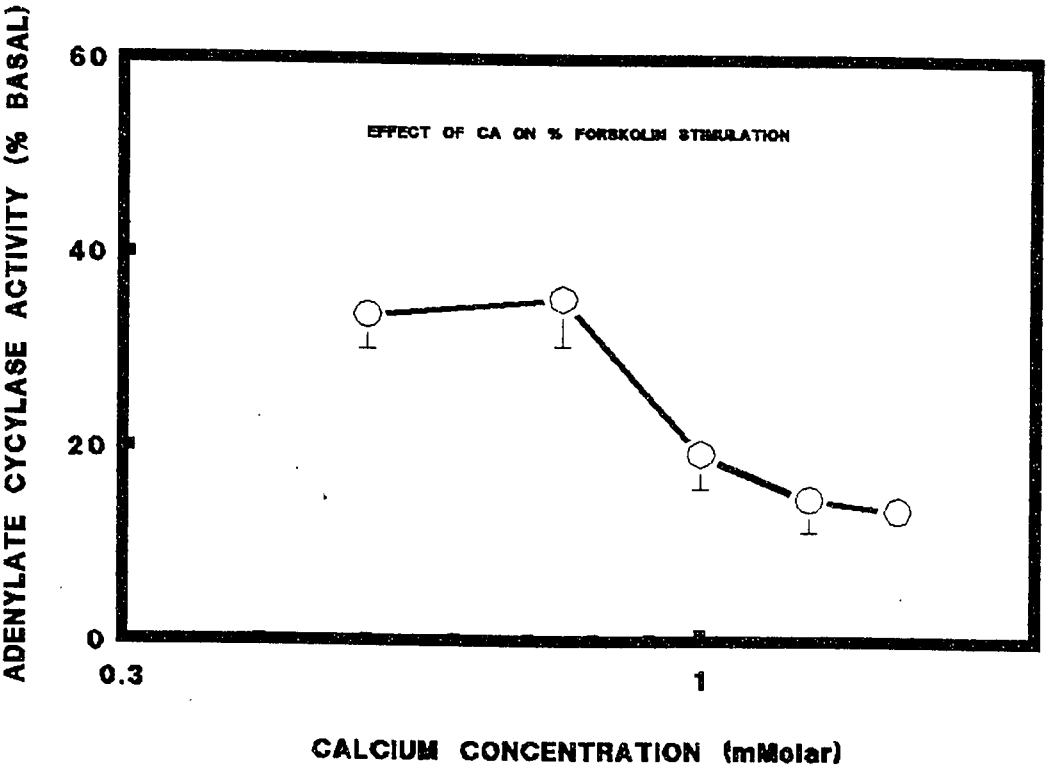
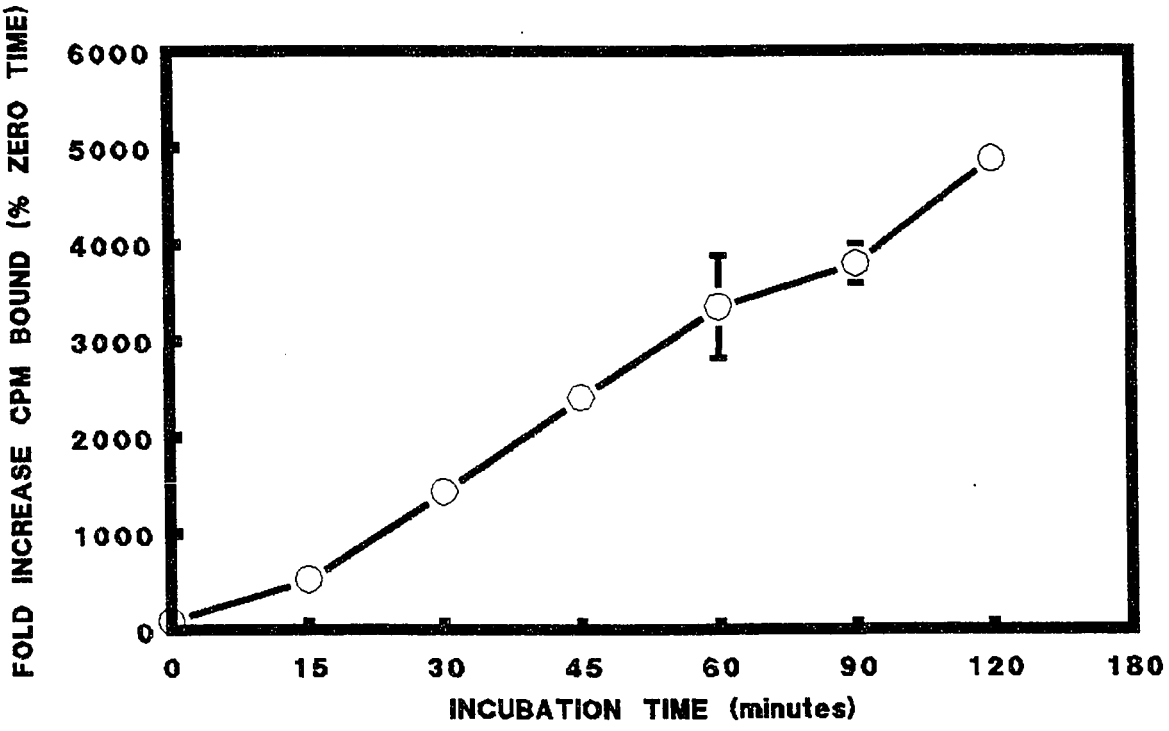

EFFECT OF CA ON % FORSKOLIN STIMULATION
ADENYLATE CYCYLASE ACTIVITY (% BASAL)
60
40
20
0
0.3
1
CALCIUM CONCENTRATION (mMolar)

FIGURE 2.8.a. (top) Viability of avian mixed renal tubules assessed by glucose oxidation.
Metabolism of ^{14}C-Glucose to $^{14}CO_2$ by mixed renal tubules isolated from chicks fed adequate dietary Ca and Mg. Data are mean ± SEM of duplicates or triplicates from three separate tubule isolations. Tubules were viable for at least two hours in a sealed environment.

FIGURE 2.8.b. (bottom) Viability of rat proximal tubules assessed by glucose oxidation.
Metabolism of ^{14}C-Glucose to $^{14}CO_2$ by purified proximal tubules isolated from control rats. Data are mean ± SEM of triplicate determinations from one tubule isolation. Tubules were viable for up to 90 minutes in a sealed environment.


FOLD INCREASE CPM BOUND (% ZERO TIME)
6000
5000
4000
3000
2000
1000
0
0
15
30
45
60
90
120
180
INCUBATION TIME (minutes)

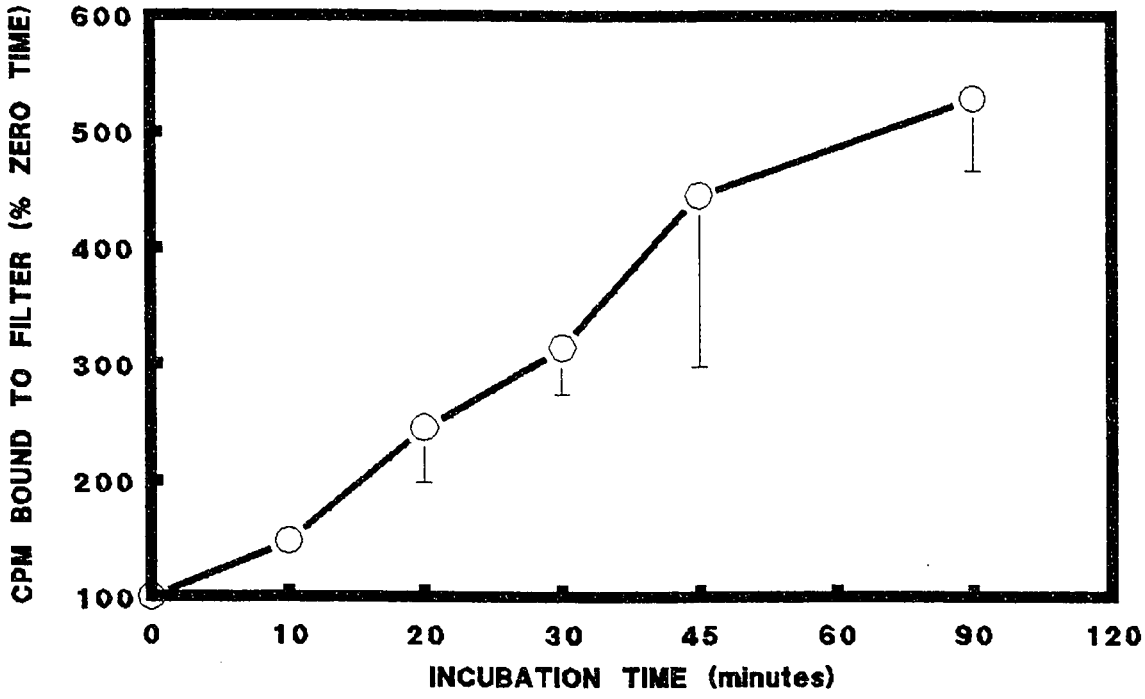

CPM BOUND TO FILTER (% ZERO TIME)
600
500
400
300
200
100
0
10
20
30
45
60
90
120
INCUBATION TIME (minutes)

FIGURE 2.9 Adenylate cyclase response to PTH and vasopressin in mixed mammalian tubules.
Adenylate cyclase activity measured in mixed renal tubules isolated from control rats in response to $1x10^{-7}$ M rat n1-34 PTH, 10 μM forskolin and 10 munits/ml vasopressin. Tubules were prelabelled for four hours with ^{32}P-ATP and stimulated for 2-20 minutes in the presence of phosphodiesterase inhibitor. Newly synthesized cAMP was isolated and quantitated as described in "Materials and Methods". Data are mean ± SEM of triplicates of one representative experiment. The data demonstrate the presence of adenylate cyclase activity and the integrity of PTH and vasopressin receptors in freshly isolated mixed tubule preparations.

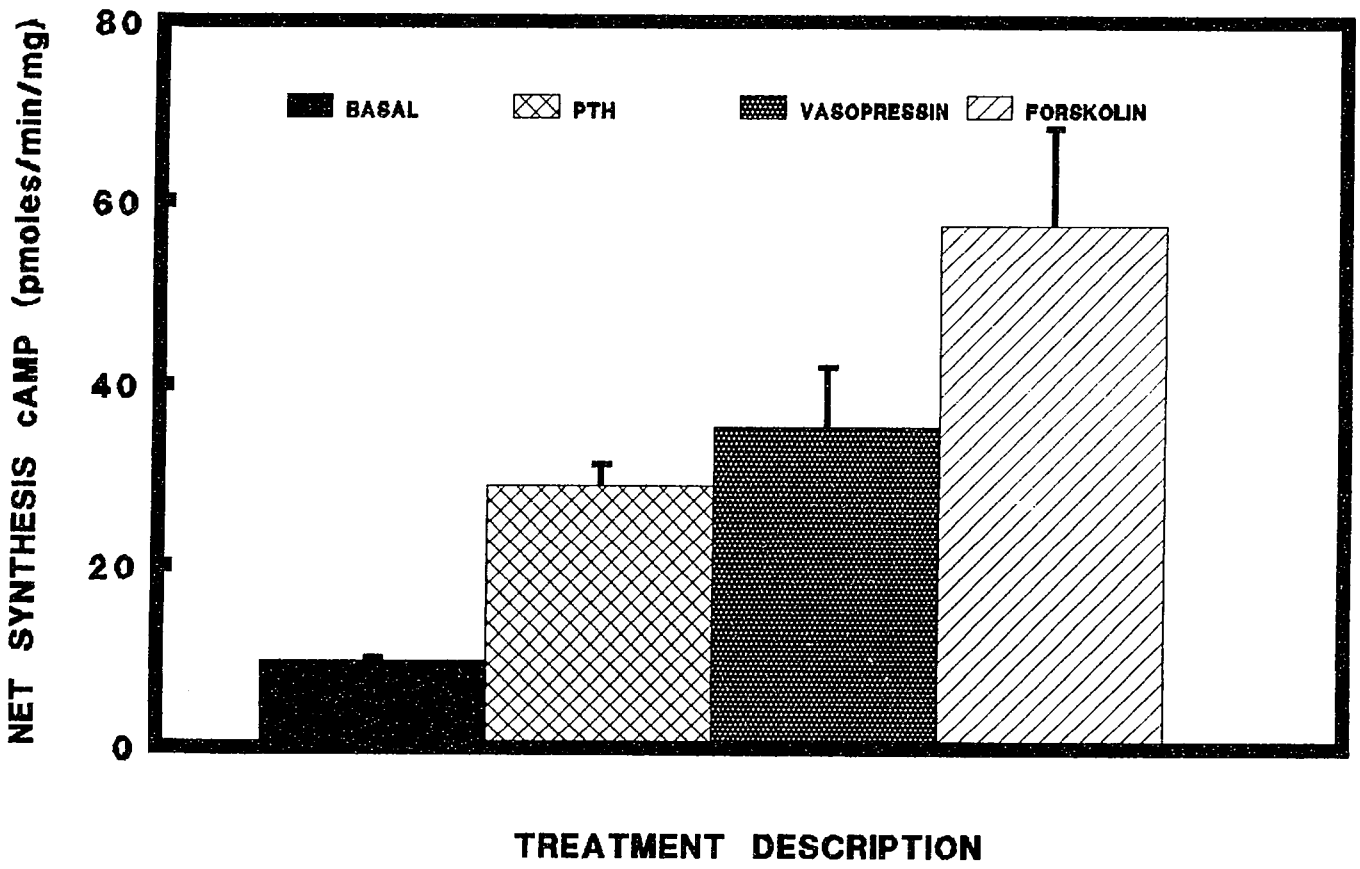

NET SYNTHESIS cAMP (pmoles/min/mg)
80
60
40
20
0
BASAL
PTH
VASOPRESSIN
FORSKOLIN
TREATMENT DESCRIPTION

FIGURE 2.10 Purification of 25(OH)D_3 substrate and UV quantitation. Purification and quantitation of 25(OH)D_3 metabolite to be used as a substrate for vitamin D hydroxylase assays in mixed and proximal tubule suspensions. An aliquot of 25(OH)D_3 in solvent was dried under a stream of nitrogen and the vitamin D metabolite was resuspended in hexane:isopropanol:methanol (91:7:2) and applied in 100 ul aliquots to a straight phase silica bed HPLC column, flow rate 2 mls/min, using the same solvent system. Isolated metabolite was collected as detected by UV absorbance, dried under a stream of nitrogen, resuspended in UV grade diethyl ether and quantitated by UV spectrophotometry, absorbance 265um, extinction coefficient 19,400. Metabolite was subsequently dried down and resuspended in ethanol for use in vitamin D hydroxylase assays. Illustrated is a representative trace of one UV absorbance scan from one aliquot of purifed 25(OH)D_3.

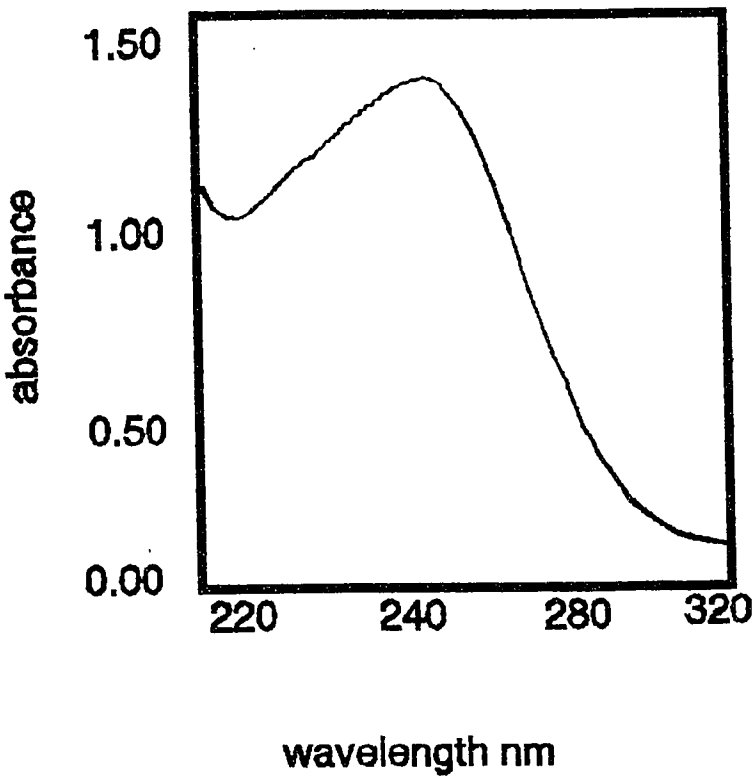
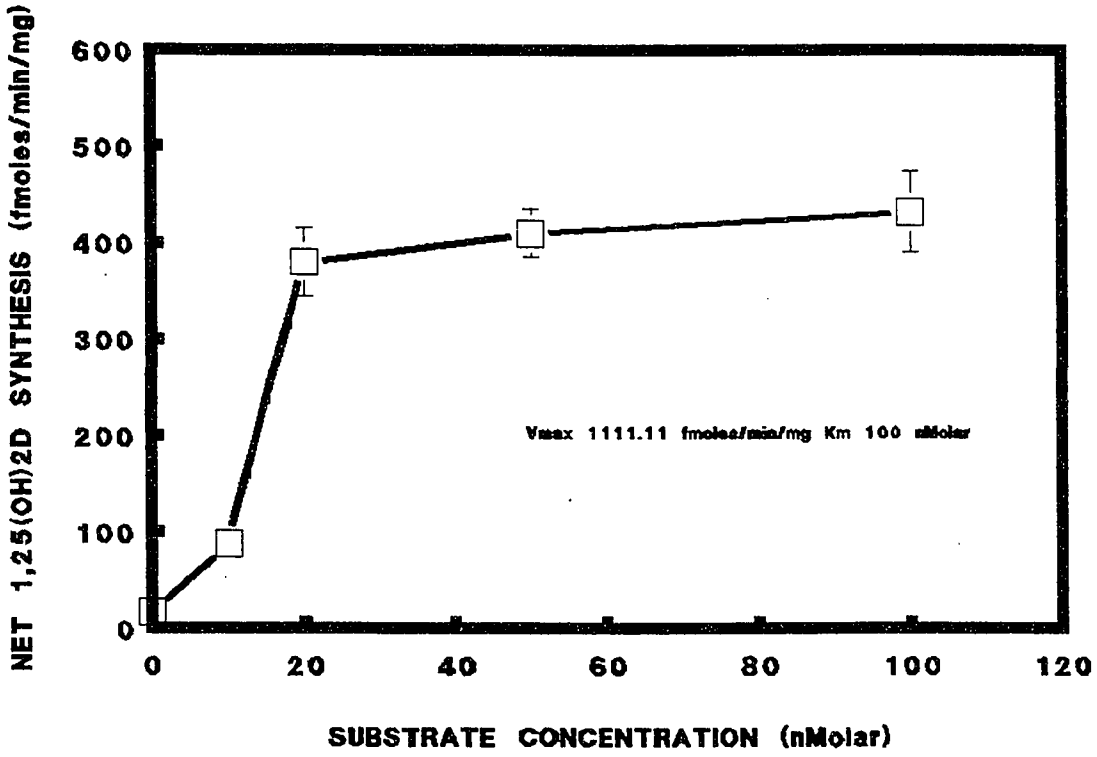

1.50
1.00
0.50
0.00
absorbance
220
240
280
320
wavelength nm

FIGURE 2.11 (top) Substrate concentration curve for 1αhydroxylase in avian tubules from chicks fed low Ca.
Effect of increasing substrate concentration on net synthesis of $1,25(OH)_2D_3$ by renal tubules isolated from chicks fed low dietary Ca. Data are mean ± SEM of duplicate determinations from two tubule preparations. A lineweaver-burke plot revealed a Vmax of 1111 fmoles/min/mg tubule protein and a Km of 100nM.

FIGURE 2.12 (bottom) Effect of avian tubule protein concentration on 1αhydroxylase activity.
Effect of increasing tubule protein on net $1,25(OH)_2D_3$ synthesis by renal tubules isolated from chicks fed adequate dietary Ca and Mg. Data are mean ± SEM of duplicate or triplicate determinations from one experiment. Net $1,25(OH)_2D_3$ synthesis was linear up to and including 4 mg tubule protein.


NET 1,25(OH)2D SYNTHESIS (fmoles/min/mg)
600
500
400
300
200
100
0
0
20
40
60
80
100
120
Vmax 1111.11 fmoles/min/mg Km 100 nMolar
SUBSTRATE CONCENTRATION (nMolar)

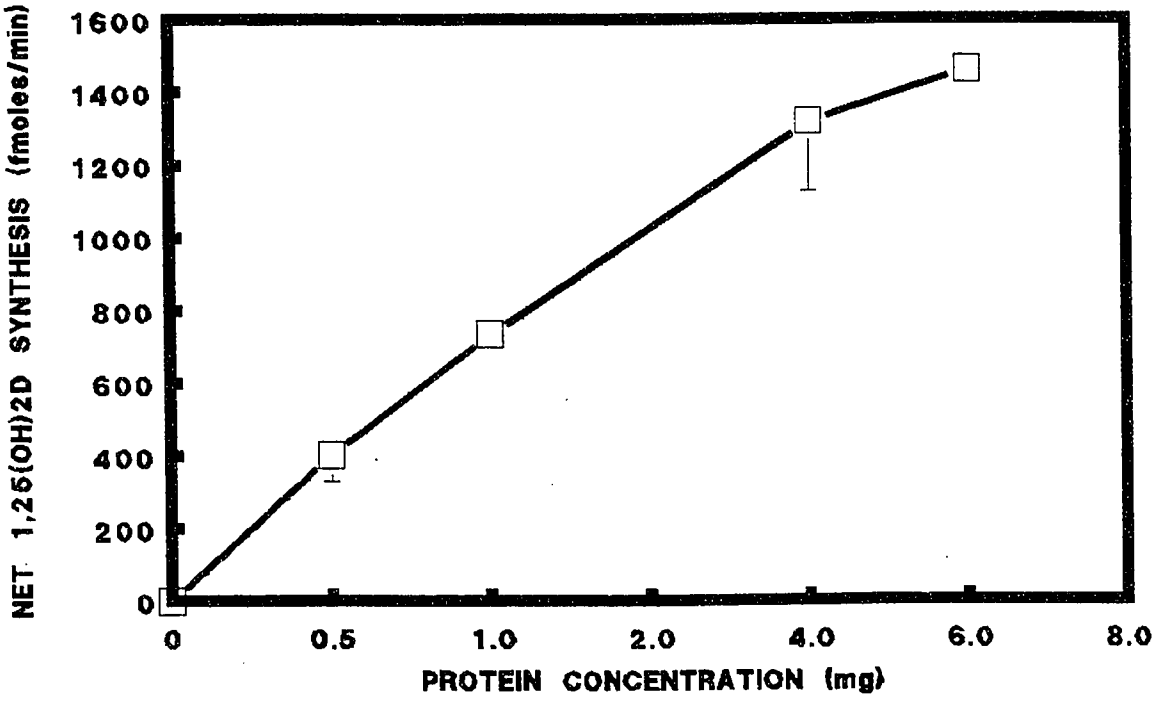
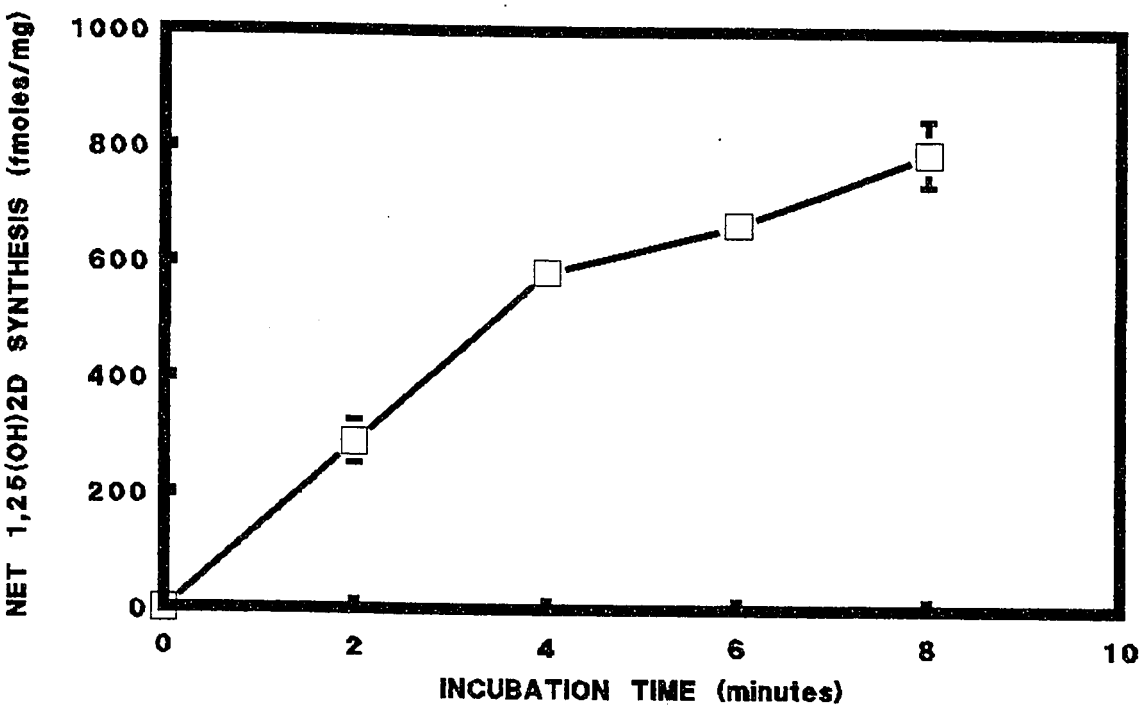

NET 1,25(OH)2D SYNTHESIS (fmoles/min)
1600
1400
1200
1000
800
600
400
200
0
0
0.5
1.0
2.0
4.0
6.0
8.0
PROTEIN CONCENTRATION (mg)

FIGURE 2.13 (top) Time course of 1αhydroxylase activity in avian tubules from chicks fed low Ca.
Effect of incubation time on net $1,25(OH)_2D_3$ synthesis by renal tubules isolated from chicks fed low dietary Ca. Data are mean ± SEM of duplicate determinations from one tubule preparation. Synthesis rates were linear for up to four minutes.

FIGURE 2.14 (bottom) Time course of 1αhydroxylase activity in avian tubules from chicks fed control diets.
Effect of incubation time on net $1,25(OH)_2D_3$ synthesis by renal tubules isolated from chicks fed adequate dietary Ca. Data are mean ± SEM of duplicate determinations from one tubule preparation. Synthesis rates were linear for up to eight minutes.


NET 1,25(OH)2D SYNTHESIS (fmoles/mg)
1000
800
600
400
200
0
0
2
4
6
8
10
INCUBATION TIME (minutes)

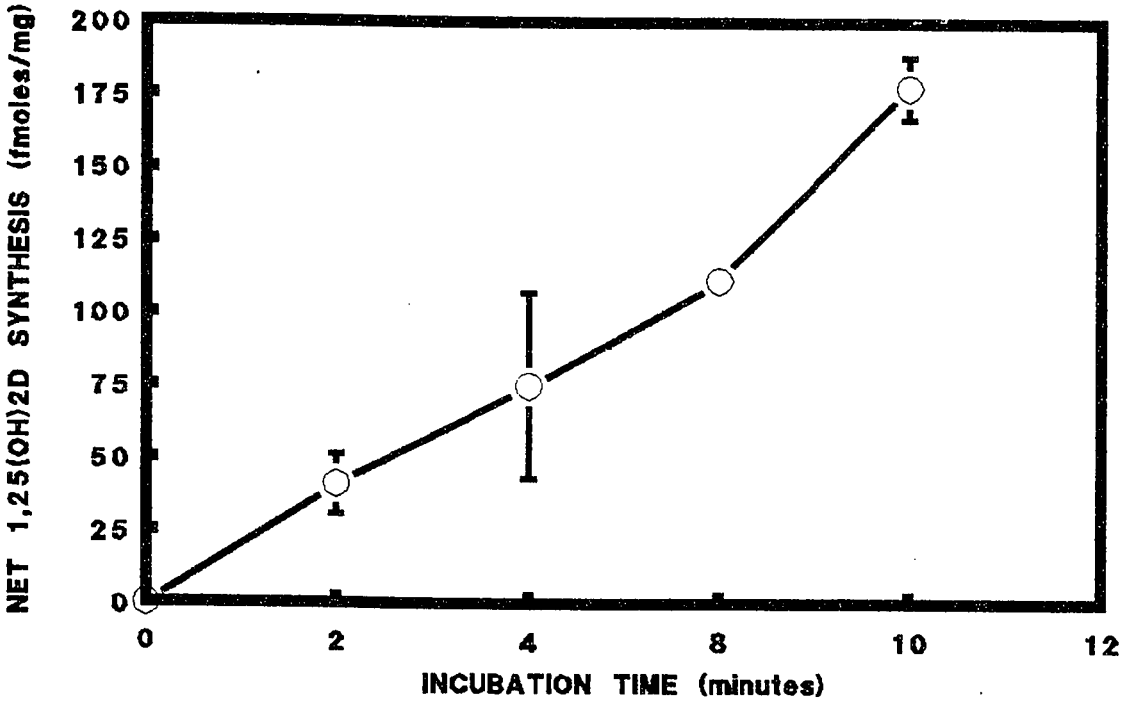

NET 1,25(OH)2D SYNTHESIS (fmoles/mg)
200
175
150
125
100
75
50
25
0
0
2
4
8
10
12
INCUBATION TIME (minutes)

FIGURE 2.15 Effect of substrate concentration on 24 hydroxylase activity in rat renal tubules.
Effect of increasing substrate concentration on net 24,25$(OH)_2D_3$ synthesis by renal tubules isolated from rats fed adequate dietary Ca. Data are mean ± SEM of duplicate determination from one tubule preparation. A lineweaver-burke plot revealed a Vmax of 500 fmoles/min/mg tubule protein and a Km of 1.25μM.

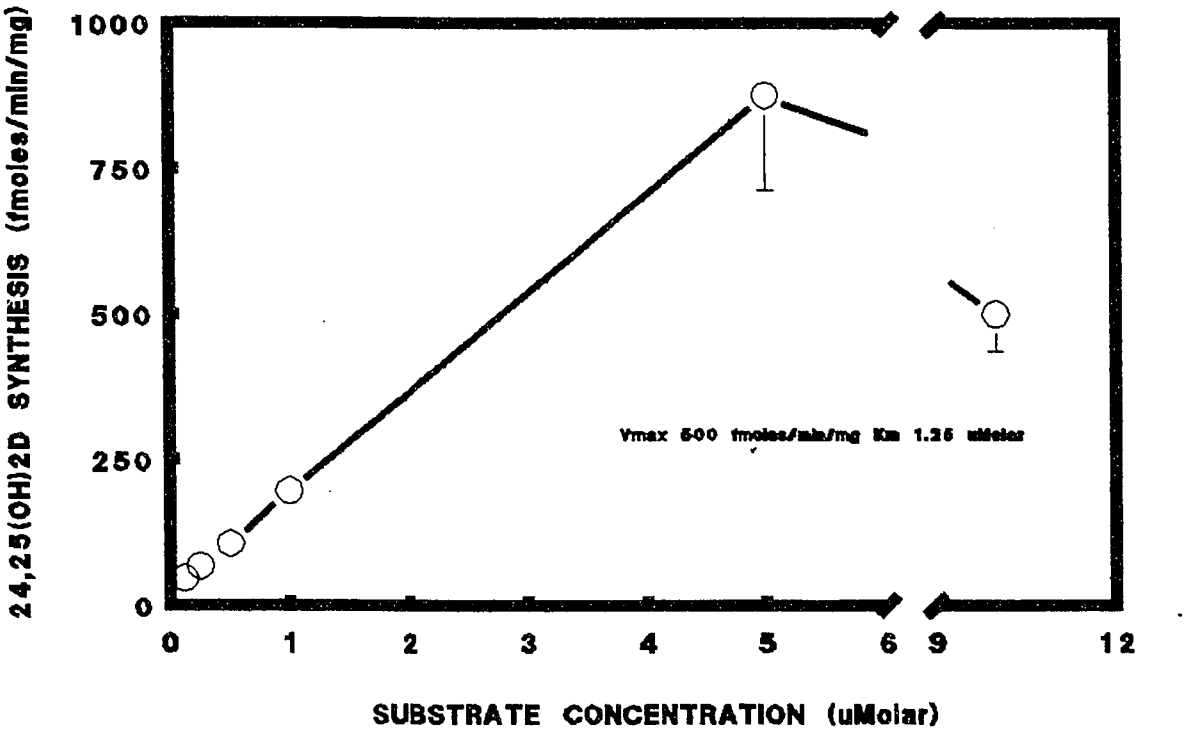
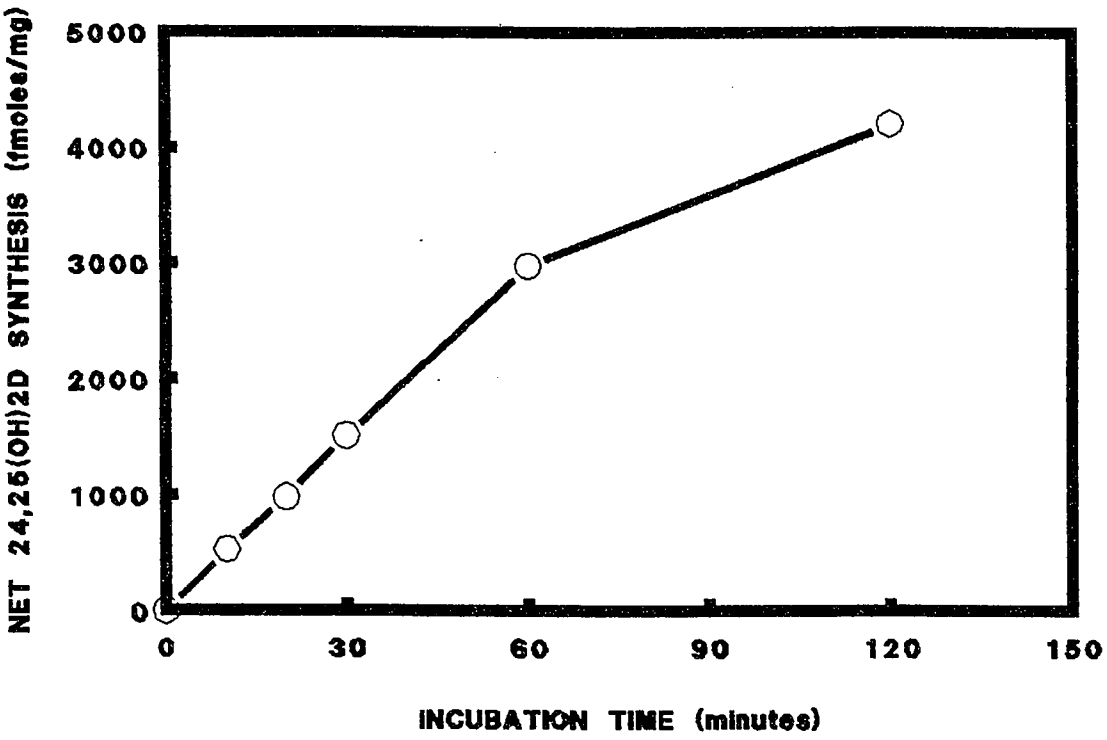

24,25(OH)2D SYNTHESIS (fmoles/min/mg)
1000
750
500
250
0
0
1
2
3
4
5
6
9
12
Vmax 500 fmoles/min/mg Km 1.25 uMolar
SUBSTRATE CONCENTRATION (uMolar)

FIGURE 2.16 (top) Effect of time on 24 hydroxylase activity in rat renal tubules.
Effect of incubation time on net $24,25(OH)_2D_3$ synthesis by renal tubules isolated from rats fed adequate dietary Ca. Data are mean of duplicates from two separate tubule preparations. Synthesis rates were linear for up to 60 minutes.

FIGURE 2.17 (bottom) Effect of tubule protein concentration on 24 hydroxylase activity in rat renal tubules.
Effect of tubule protein on net $24,25(OH)_2D_3$ synthesis by renal tubules isolated from rats fed adequate dietary Ca. Data are mean ± SEM of duplicate or triplicate determinations from one tubule preparation. Synthesis was linear up to and including 6 mg tubule protein.


NET 24,25(OH)2D SYNTHESIS (fmoles/mg)
5000
4000
3000
2000
1000
0
0
30
60
90
120
150
INCUBATION TIME (minutes)

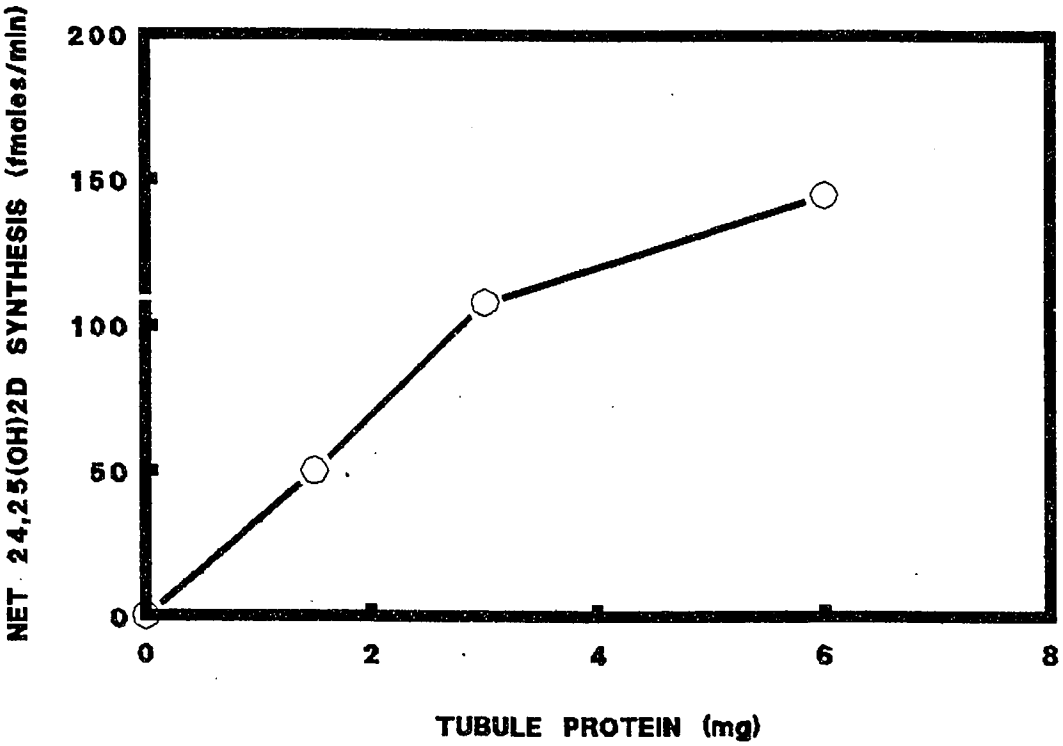

NET 24,25(OH)2D SYNTHESIS (fmoles/min)
200
150
100
50
0
0
2
4
6
8
TUBULE PROTEIN (mg)

FIGURE 2.18 Effect of membrane and cytosolic protein on PKC activity in preparations of rat renal tubules.
Effect of protein on PKC activity assayed in renal tubule membranes and cytosol of PNF isolated from tubules from rats fed adequate dietary Ca. Data are mean ± SEM of duplicate or triplicate determinations from n = 3-4 for membrane protein and n = 5-6 for cytosolic protein. PKC was linear up to and including 25 ug membrane protein and up to and including 50 ug cytosol protein.

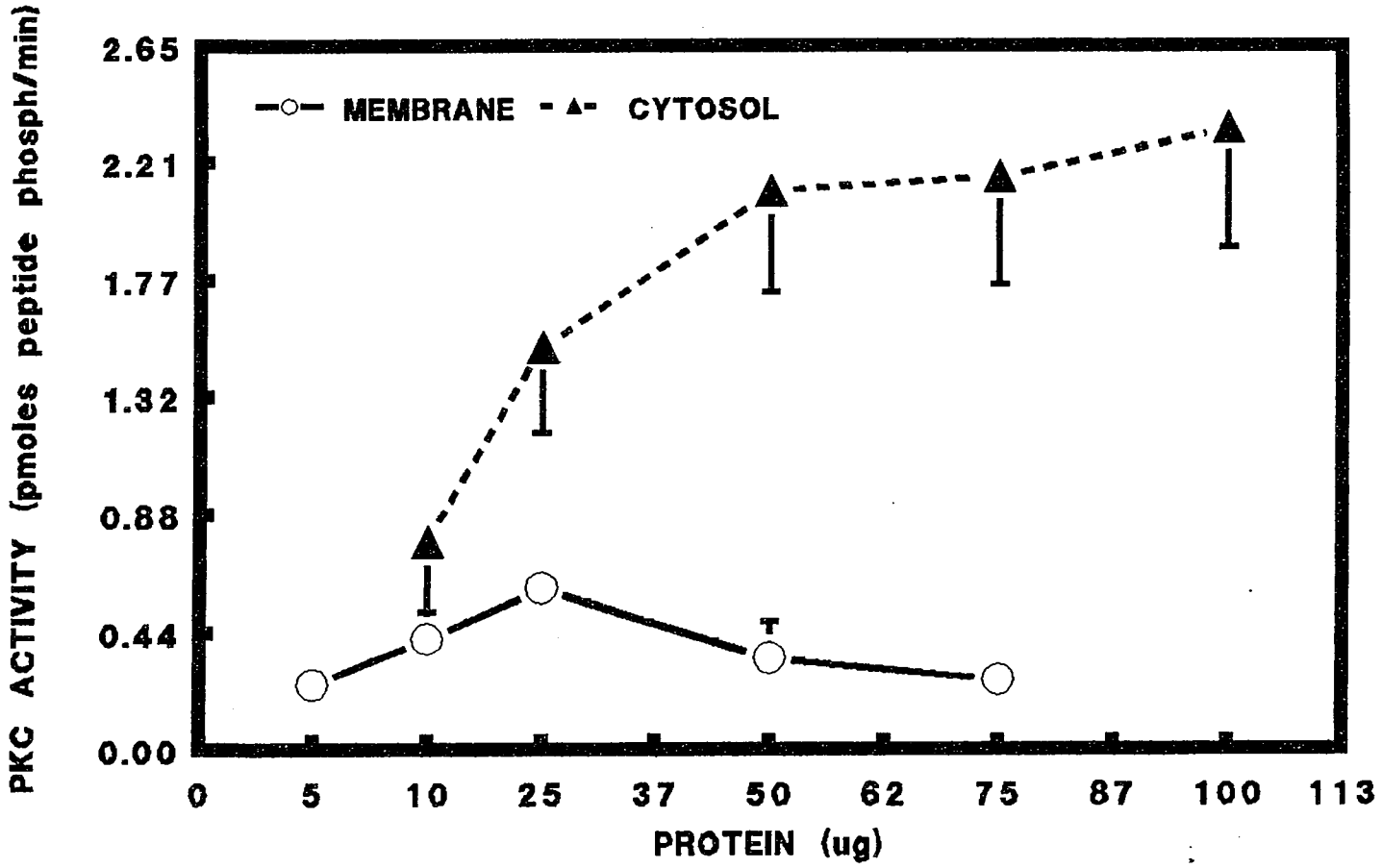

PKC ACTIVITY (pmoles peptide phosph/min)
2.65
2.21
1.77
1.32
0.88
0.44
0.00
MEMBRANE
CYTOSOL
0
5
10
25
37
50
62
75
87
100
113
PROTEIN (ug)

FIGURE 2.19 Effect of renal tubule purification on sensitivity to agonists.
Adenylate cyclase activity measured in mixed, proximal and distal enriched tubule fractions from rats fed adequate dietary Ca in response to $1x10^{-7}$ M rat n1-34 PTH, 10 μM forskolin and 10 munits/ml vasopressin. Data are mean ± SEM of triplicate determinations from one experiment. The data demonstrates that basal and forskolin stimulated adenylate cyclase activity was comparable between all the tubule preparations while proximal enriched tubule fractions exhibited decreased vasopressin and increased PTH stimulated adenylate cyclase activity when compared to mixed or distal enriched tubules.

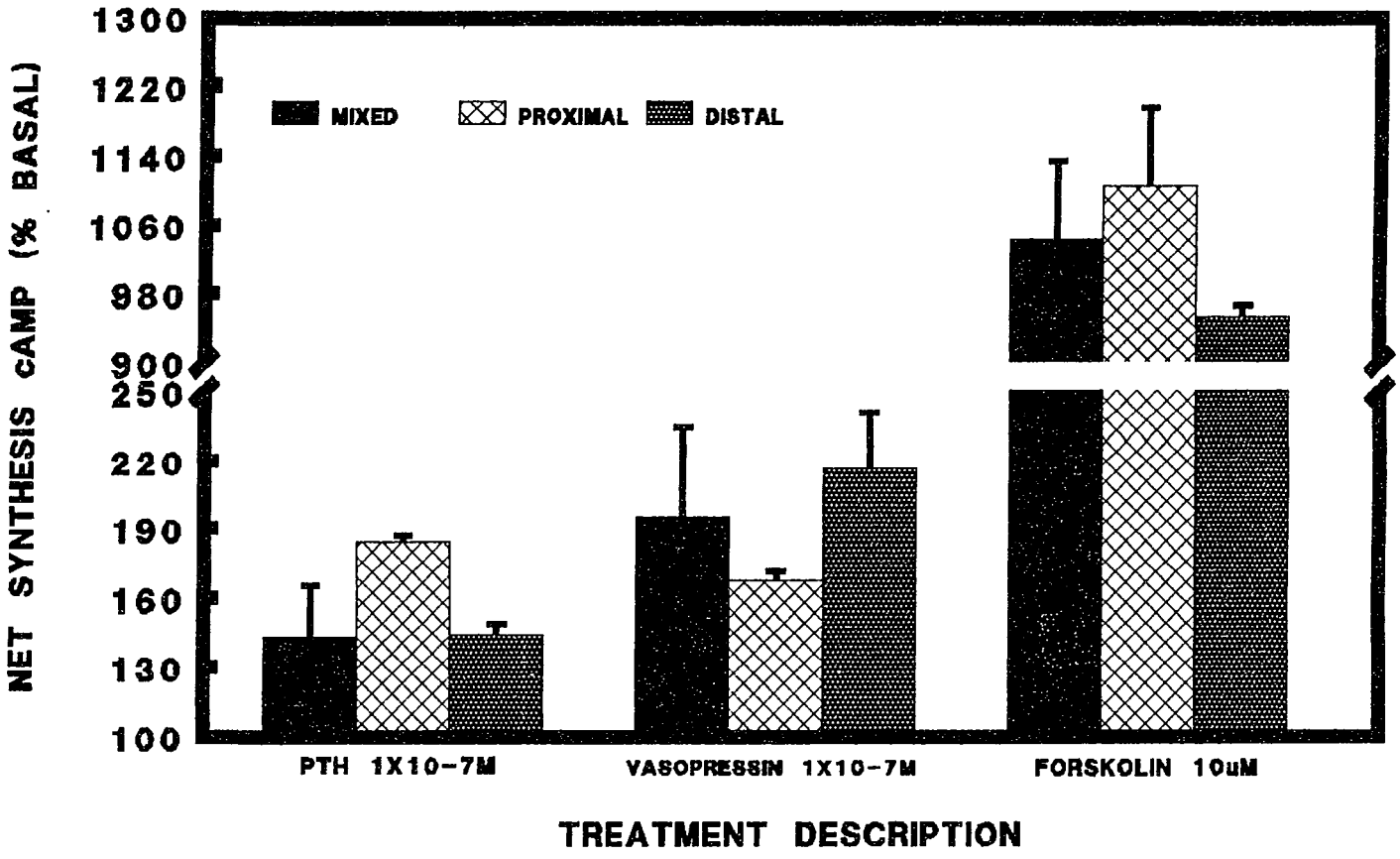
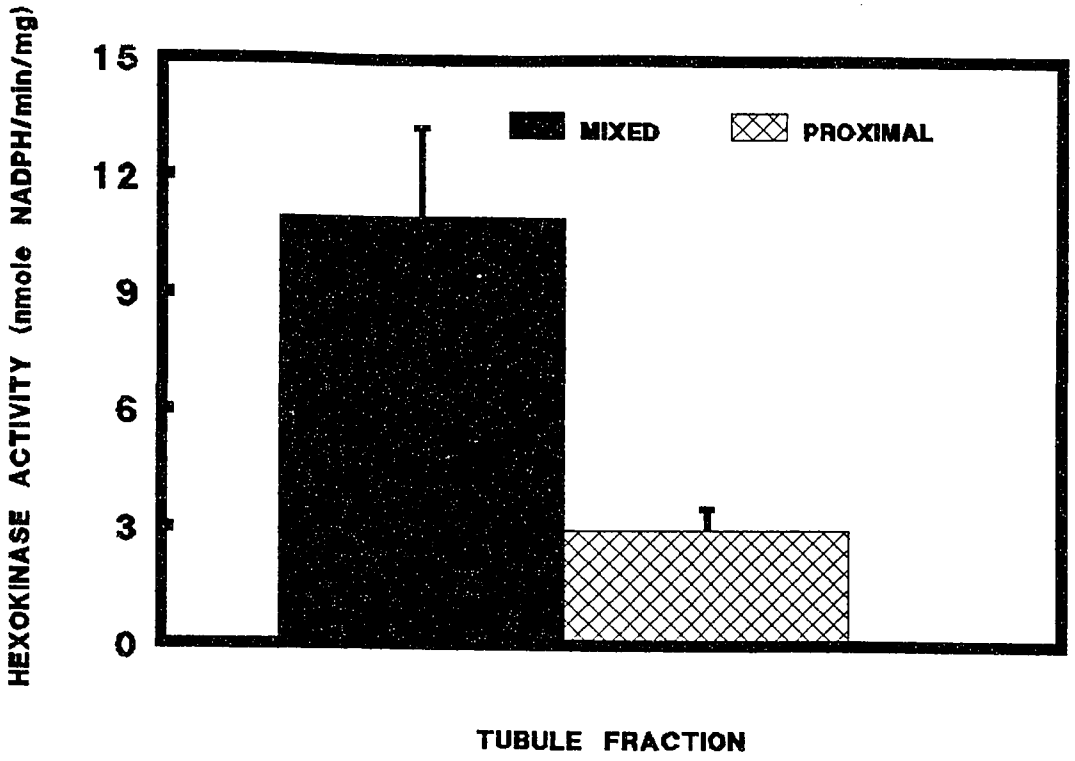

1300
1220
1140
1060
980
900
250
220
190
160
130
100
MIXED
PROXIMAL
DISTAL
NET SYNTHESIS cAMP (% BASAL)
PTH 1X10-7M
VASOPRESSIN 1X10-7M
FORSKOLIN 10uM
TREATMENT DESCRIPTION

FIGURE 2.20 (top) Effect of renal tubule purification on hexokinase activity.
Hexokinase activity was assayed spectrophotometrically as described by Miradj and Jagannathn (1966), by the coupled formation of NADPH, detected at 340nm. Product was quantitated using the extinction coefficient $6.3x10^3$. Data is mean ± SEM of enzyme activity measured in duplicate determinations from three tubule isolations. Hexokinase activity was lower in tubule preparations enriched in proximal segments.

FIGURE 2.21 (bottom) Effect of renal tubule purification on fructose-1,6 bisphosphatase and glucose -6-phosphatase activity.
Fructose 1,6 bisphosphatase was assayed spectrophotometrically as described by Tashima and Miaunumi (1982), by the coupled formation of NADPH, detected at 340nm. Product was quantitated using the extinction coefficient $6.3x10^3$. Data is mean ± SEM of enzyme activity measured in duplicate determinations from three tubule isolations. Fructose 1,6 bisphosphatase activity was higher in tubule preparations enriched in proximal segments when compared to tubule preparations enriched in distal segments.

Glucose 6 phosphatase was assayed spectrophotometrically as described by Alegre et al. (1988) , by the coupled formation of NADH, detected at 340nm. Product was quantitated using the extinction coefficient $6.3x10^3$. Data is mean ± SEM of enzyme activity measured in duplicate determinations from three tubule isolations. Glucose 6 phosphatase activity was higher in tubule preparations enriched in proximal segments when compared to tubule preparations enriched in distal segments.


HEXOKINASE ACTIVITY (nmole NADPH/min/mg)
15
12
9
6
3
0
MIXED
PROXIMAL
TUBULE FRACTION

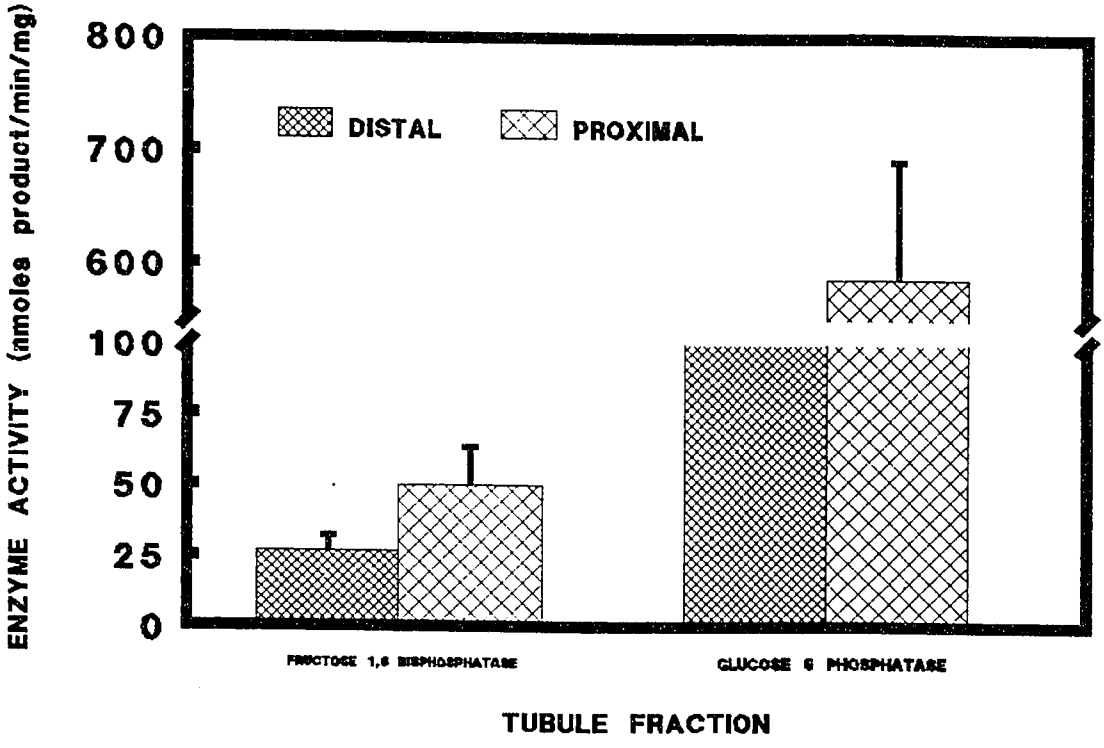
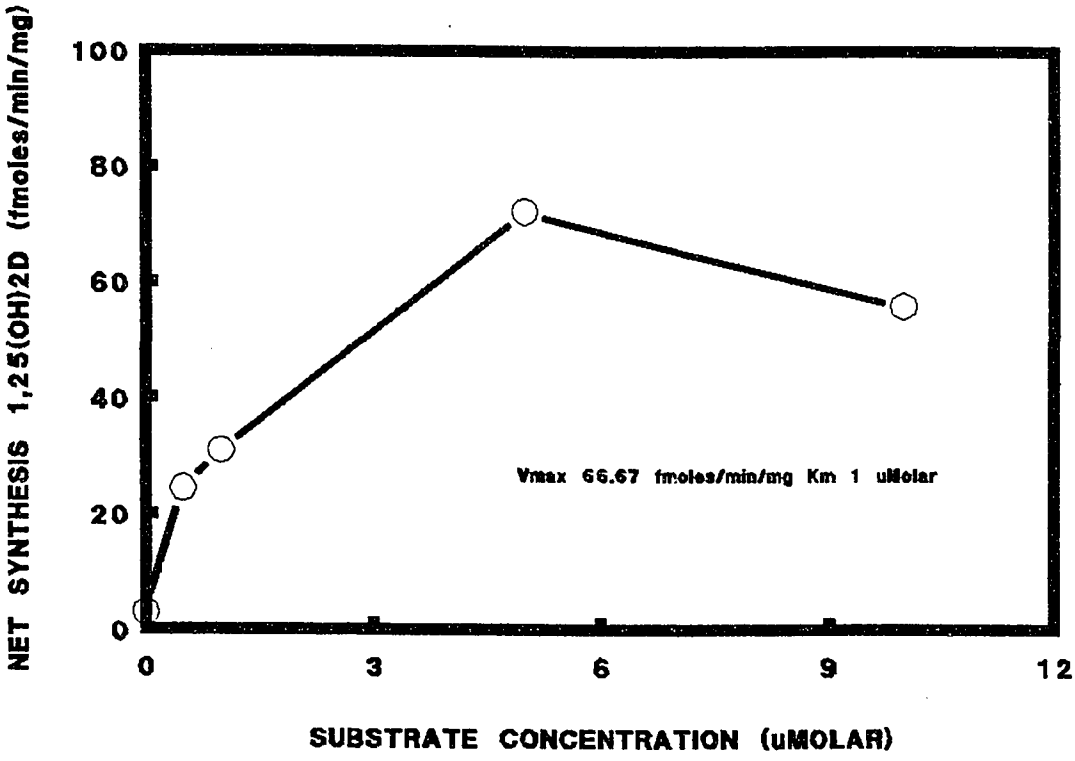

ENZYME ACTIVITY (nmoles product/min/mg)
800
700
600
100
75
50
25
0
DISTAL
PROXIMAL
FRUCTOSE 1,6 BISPHOSPHATASE
GLUCOSE 6 PHOSPHATASE
TUBULE FRACTION

FIGURE 2.22 (top) Effect of substrate on 1αhydroxylase activity in proximal renal tubules from low Ca fed rats.
Effect of increasing substrate concentration on net $1,25(OH)_2D_3$ synthesis by proximal renal tubules isolated from rats fed low dietary Ca. Data are mean ± SEM of duplicate determinations from one experiment. A lineweaver-burke plot revealed a Vmax of 66.67 fmoles/min/mg tubule protein and a Km of 1 μM.

FIGURE 2.23 (bottom) Effect of substrate on 1αhydroxylase activity in proximal renal tubules from vitamin D deficient rats.
Effect of increasing substrate concentration on net $1,25(OH)_2D_3$ synthesis by proximal renal tubules isolated from vitamin D deficient rats. Data are mean ± SEM of duplicate determinations from one experiment. A lineweaver-burke plot revealed a Vmax of 21.98 fmoles/min/mg tubule protein and a Km of 1μM.


NET SYNTHESIS 1,25(OH)2D (fmoles/min/mg)
100
80
60
40
20
0
0
3
6
9
12
Vmax 66.67 fmoles/min/mg Km 1 uMolar
SUBSTRATE CONCENTRATION (uMOLAR)

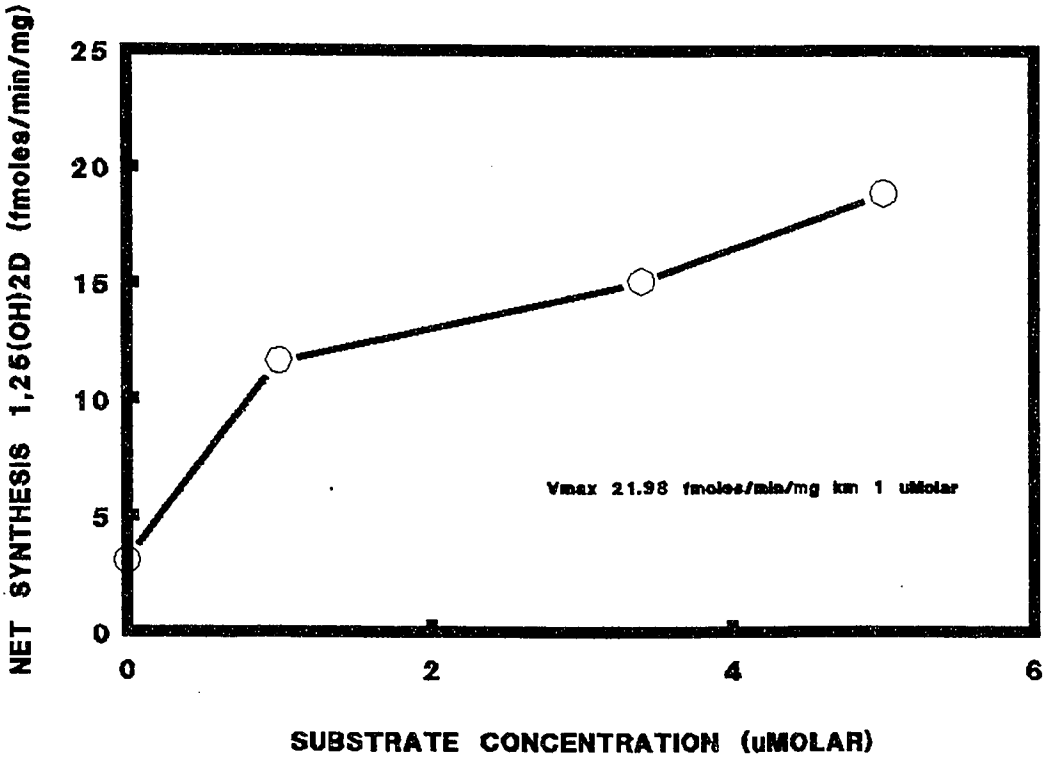
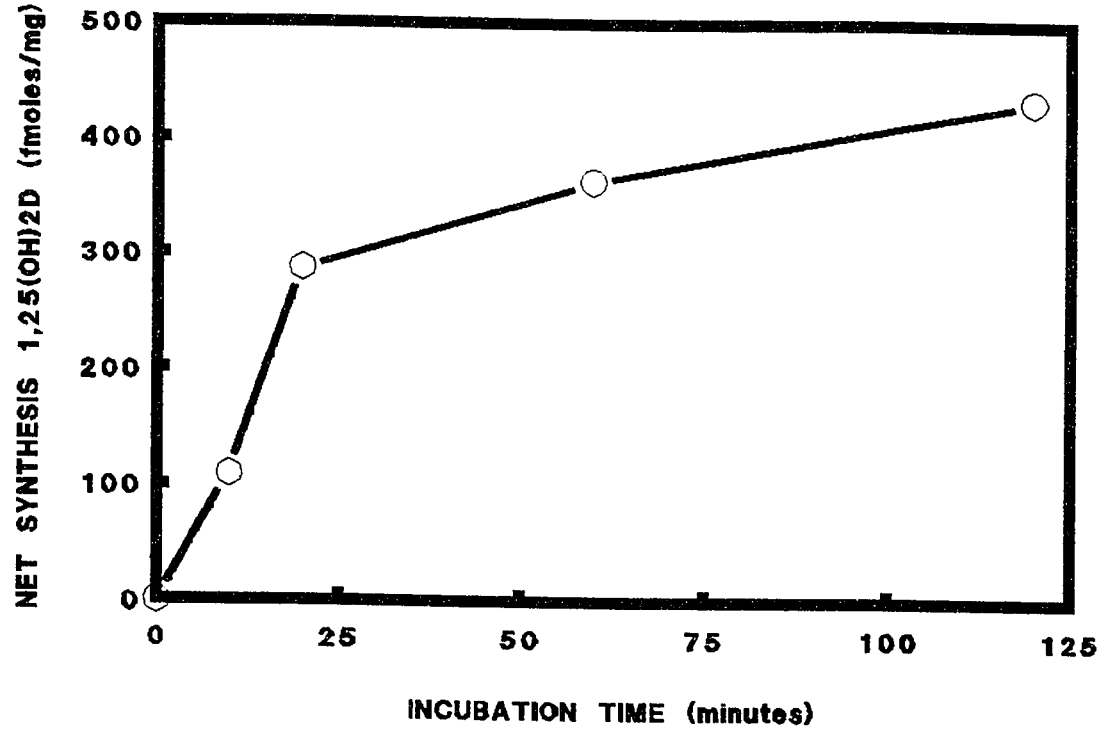

NET SYNTHESIS 1,25(OH)2D (fmoles/min/mg)
25
20
15
10
5
0
0
2
4
6
Vmax 21.98 fmoles/min/mg km 1 uMolar
SUBSTRATE CONCENTRATION (uMOLAR)

FIGURE 2.24 (top) Time course of 1αhydroxylase activity in proximal renal tubules from vitamin D deficient rats.
Effect of incubation time on net $1,25(OH)_2D_3$ synthesis by renal tubules isolated from rats fed low dietary Ca. Data are mean of duplicate determinations from three separate experiments. Synthesis rates were linear for up to 25-30 minutes.

FIGURE 2.25 (bottom) Time course of 1αhydroxylase activity in proximal renal tubules from rats fed low Ca.
Effect of incubation time on net $1,25(OH)_2D_3$ synthesis by renal tubules isolated from rats fed vitamin D deficient diets. Data are mean of duplicate determinations from two separate experiments. Synthesis rates were linear for at least 30 minutes.


NET SYNTHESIS 1,25(OH)2D (fmoles/mg)
500
400
300
200
100
0
0
25
50
75
100
125
INCUBATION TIME (minutes)

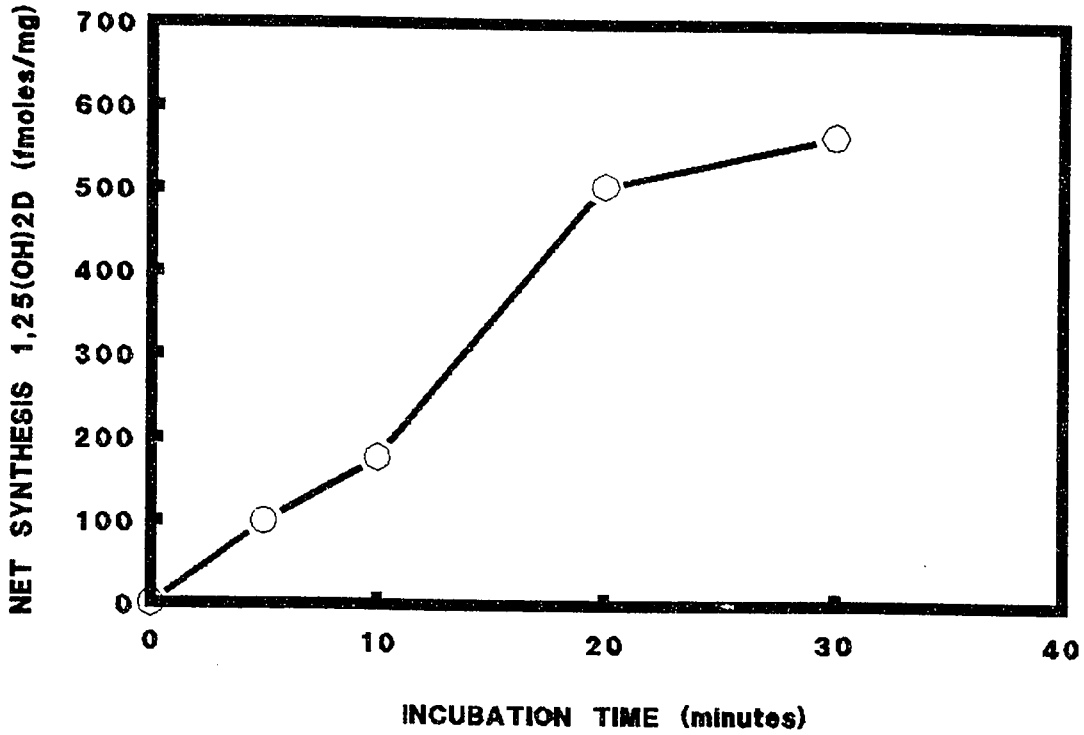
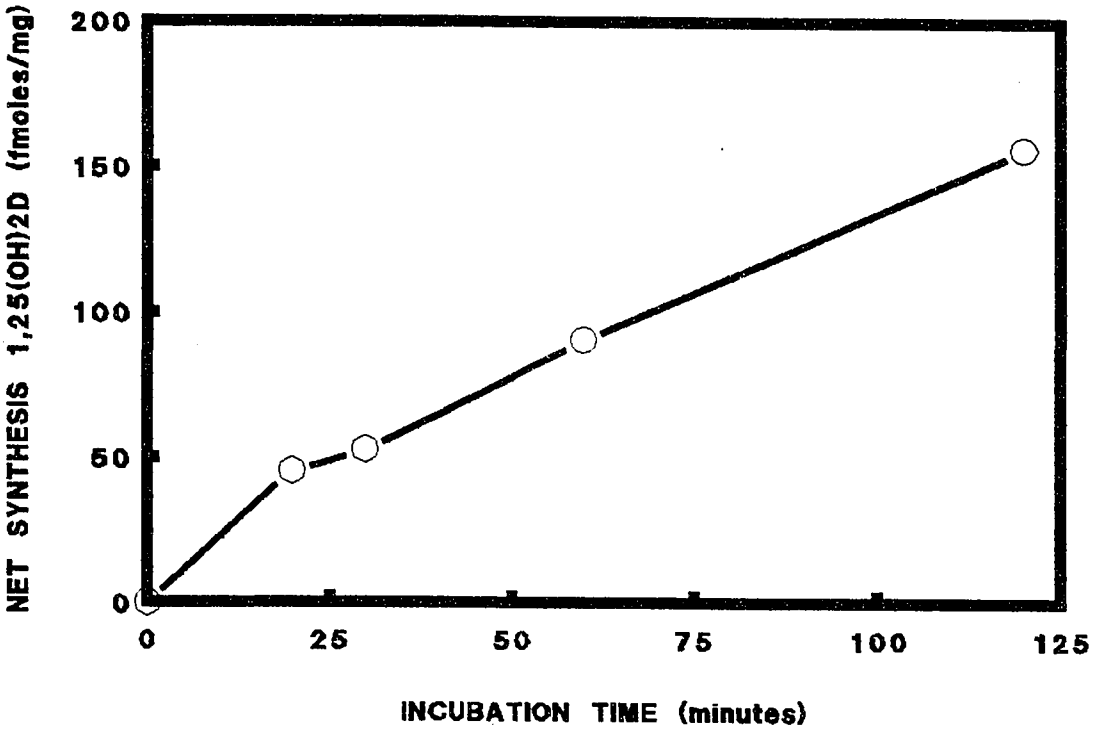

NET SYNTHESIS 1,25(OH)2D (fmoles/mg)
700
600
500
400
300
200
100
0
0
10
20
30
40
INCUBATION TIME (minutes)

FIGURE 2.26 (top) Time course of 1αhydroxylase activity in proximal renal tubules from rats fed control diets.
Effect of incubation time on net $1,25(OH)_2D_3$ synthesis by renal tubules isolated from rats fed adequate dietary Ca. Data are mean of duplicate determinations from three separate experiments. Synthesis rates were linear for at least 2 hours.

FIGURE 2.27 (bottom) Effect of tubule protein on 1αhydroxylase activity in renal tubules from rats fed control diets.
Effect of increasing tubule protein on net $1,25(OH)_2D_3$ synthesis by renal tubules isolated from rats fed adequate dietary Ca. Data are mean of duplicate determinations from one experiment. Synthesis rates were linear up to and including 10 mg tubule protein.


NET SYNTHESIS 1,25(OH)2D (fmoles/mg)
200
150
100
50
0
0
25
50
75
100
125
INCUBATION TIME (minutes)

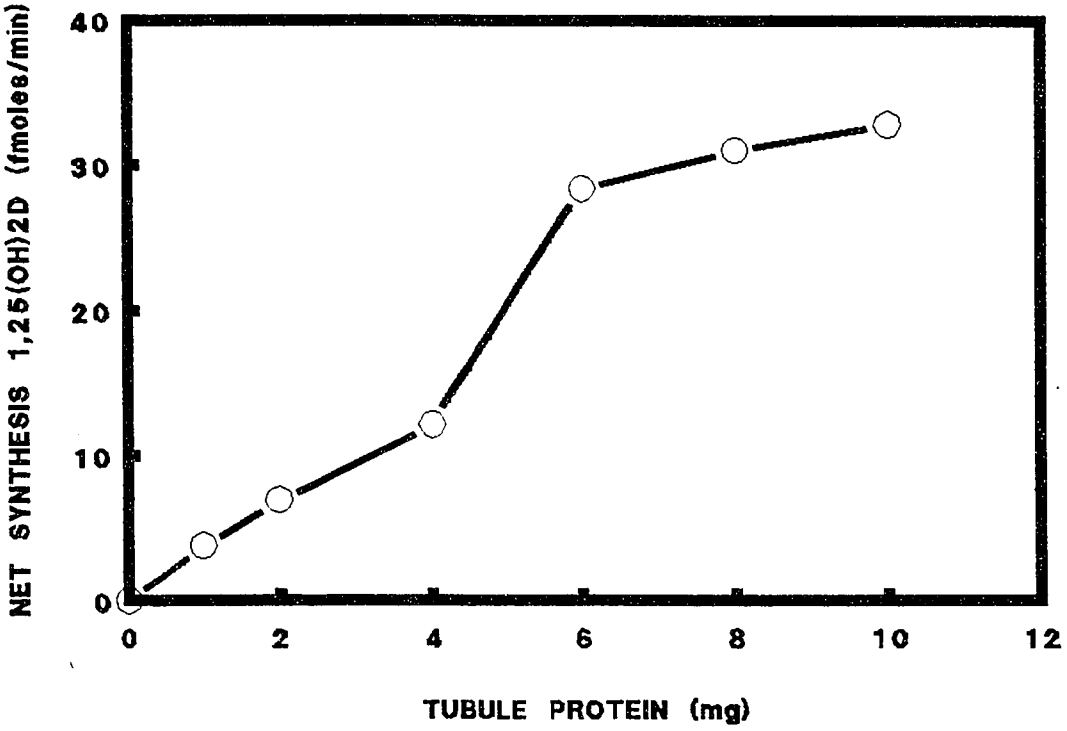

NET SYNTHESIS 1,25(OH)2D (fmoles/min)
40
30
20
10
0
0
2
4
6
8
10
12
TUBULE PROTEIN (mg)

FIGURE 2.28 Correlation between circulating $1,25(OH)_2D_3$ and 1αhydroxylase activity in renal tubules.
Correlation between circulating $1,25(OH)_2D_3$ measured in serum or plasma of individual rats and in vitro 1αhydroxylase activity assayed in paired renal proximal tubule preparations from the same animals. Data are mean of duplicate determinations. A significant ($p<0.003$) correlation was observed between in vitro net $1,25(OH)_2D_3$ synthesis and circulating $1,25(OH)_2D_3$.

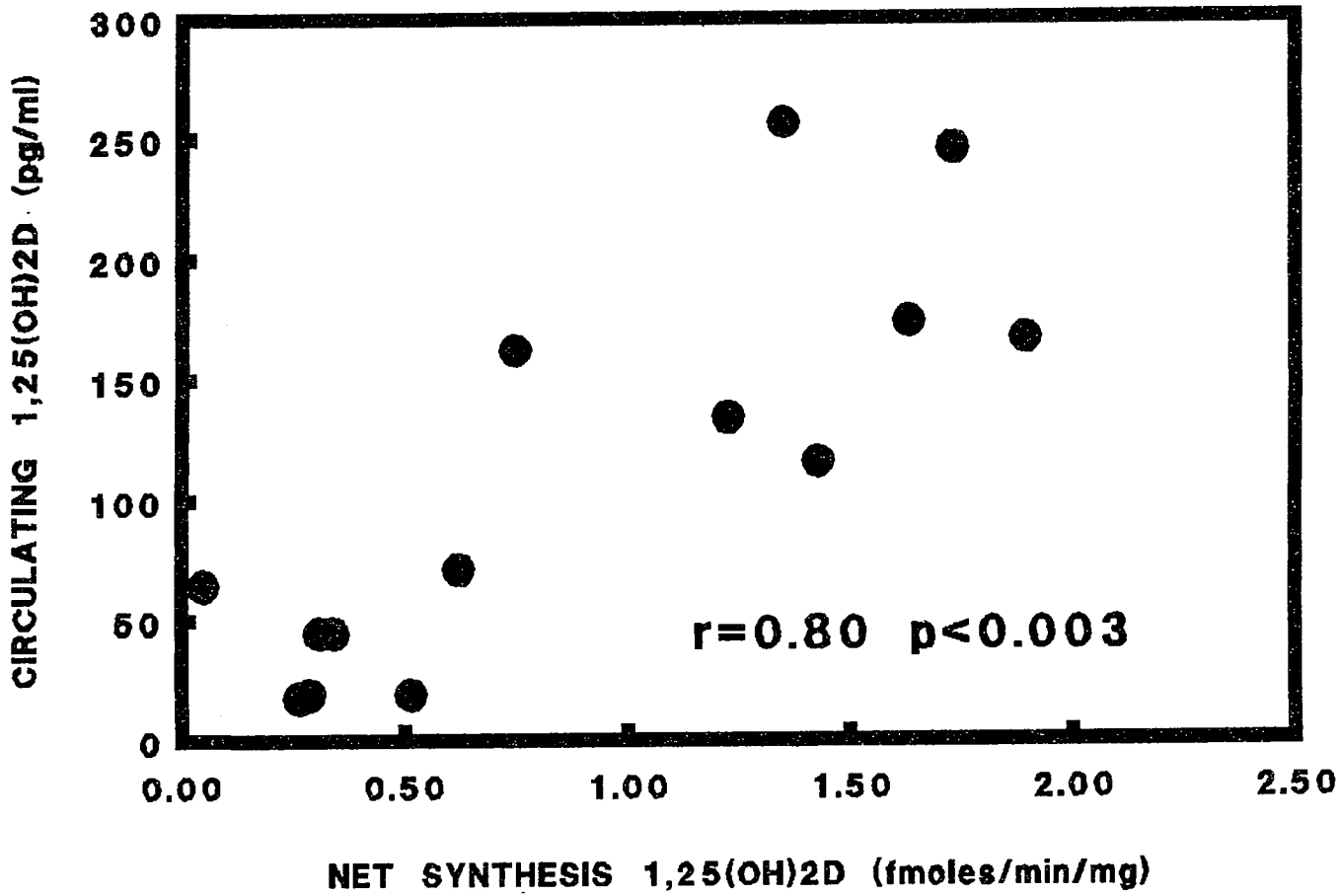

300
250
200
150
100
50
0
CIRCULATING 1,25(OH)2D (pg/ml)
r=0.80 p<0.003
0.00
0.50
1.00
1.50
2.00
2.50
NET SYNTHESIS 1,25(OH)2D (fmoles/min/mg)

FIGURE 2.29 HPLC purification of $1,25(OH)_2D_3$ synthesized by renal proximal tubules from rats fed low Ca on Zorbax-SIL 5 µm using hexane:isopropanol:methanol, 91:7:2.

Purification of $1,25(OH)_2D_3$ synthesized by proximal tubules isolated from rats fed low dietary Ca. Product was pooled from 24 separate enzyme preparations and vitamin D metabolites were extracted by acetonitrile, C18 affinity and silica adsorption chromatography and applied to a straight phase silica bed HPLC 5um column and eluted with hexane:isopropanol:methanol (91:7:2) at a flow rate of 2 ml/min.

a) HPLC trace of HPLC standard $1,25(OH)_2D_3$ (50nMoles)

b) HPLC trace of synthesized $1,25(OH)_2D_3$ spiked with 50nMoles $1,25(OH)_2D_3$ HPLC standard

c) HPLC trace of synthesized $1,25(OH)_2D_3$

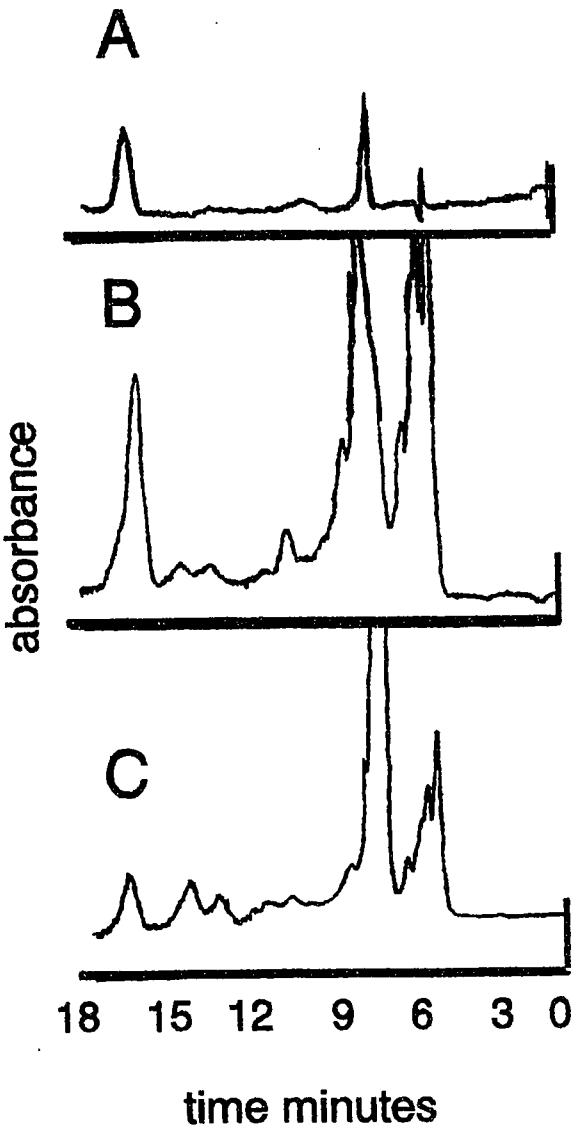

A
B
C
absorbance
18 15 12 9 6 3 0
time minutes

FIGURE 2.30 UV scan of $1,25(OH)_2D_3$ synthesized by renal proximal tubules from rats fed low Ca.

UV scan of $1,25(OH)_2D_3$ synthesized by proximal tubules isolated from rats fed low dietary Ca and purified by C18 affinity and silica adsorption chromatography and straight phase silica HPLC chromatography with hexane:isopropanol:methanol (91:7:2). Product which co-migrated with authentic $1,25(OH)_2D_3$ was collected, dried under a stream of nitrogen, resuspended in UV grade diethyl ether and scanned. Quantitation was achieved using the extinction coefficient 19,400. Maximum absorbance was observed at 265nm which is characteristic of a vitamin D metabolite. No increase in absorbance was observed at 315nm typical of 19-nor-10-oxo-25-hydroxvitamin D_3 (Tran and Henry, 1988).

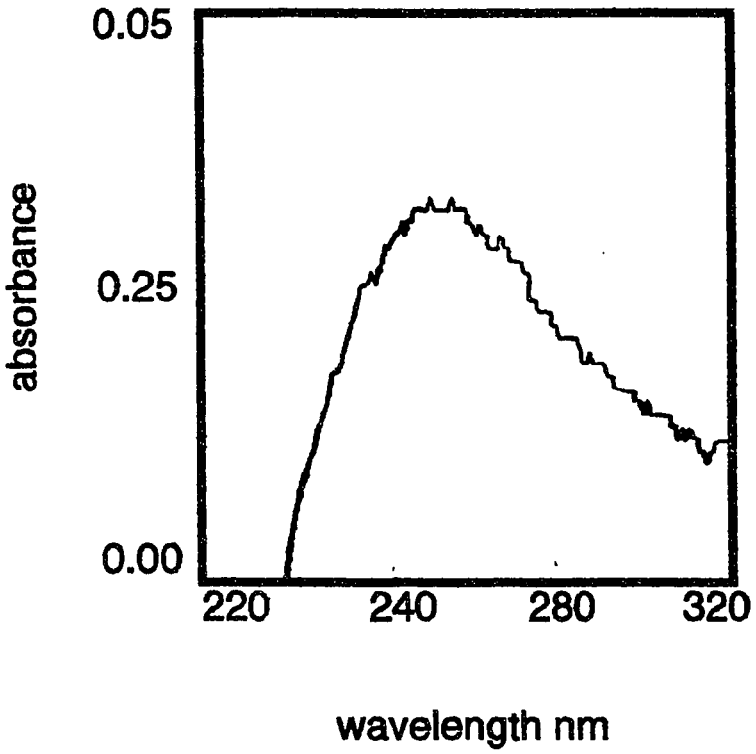

0.05
0.25
0.00
absorbance
220
240
280
320
wavelength nm

FIGURE 2.31 Adenylate cyclase synthesis in response to agonists in primary cultures of rat renal tubules.
Adenylate cyclase activity measured in primary cultures of proximal tubules prepared from kidneys from weanling rats in response to $1x10^{-7}$ M rat n1-24 PTH, 10 μM forskolin and 10 munits/ml vasopressin. Data are mean ± SEM of triplicate dishes from one representative experiment. The data demonstrates adenylate cyclase activity and the presence of intact PTH and vasopressin receptors in primary cultures enriched for proximal tubules.

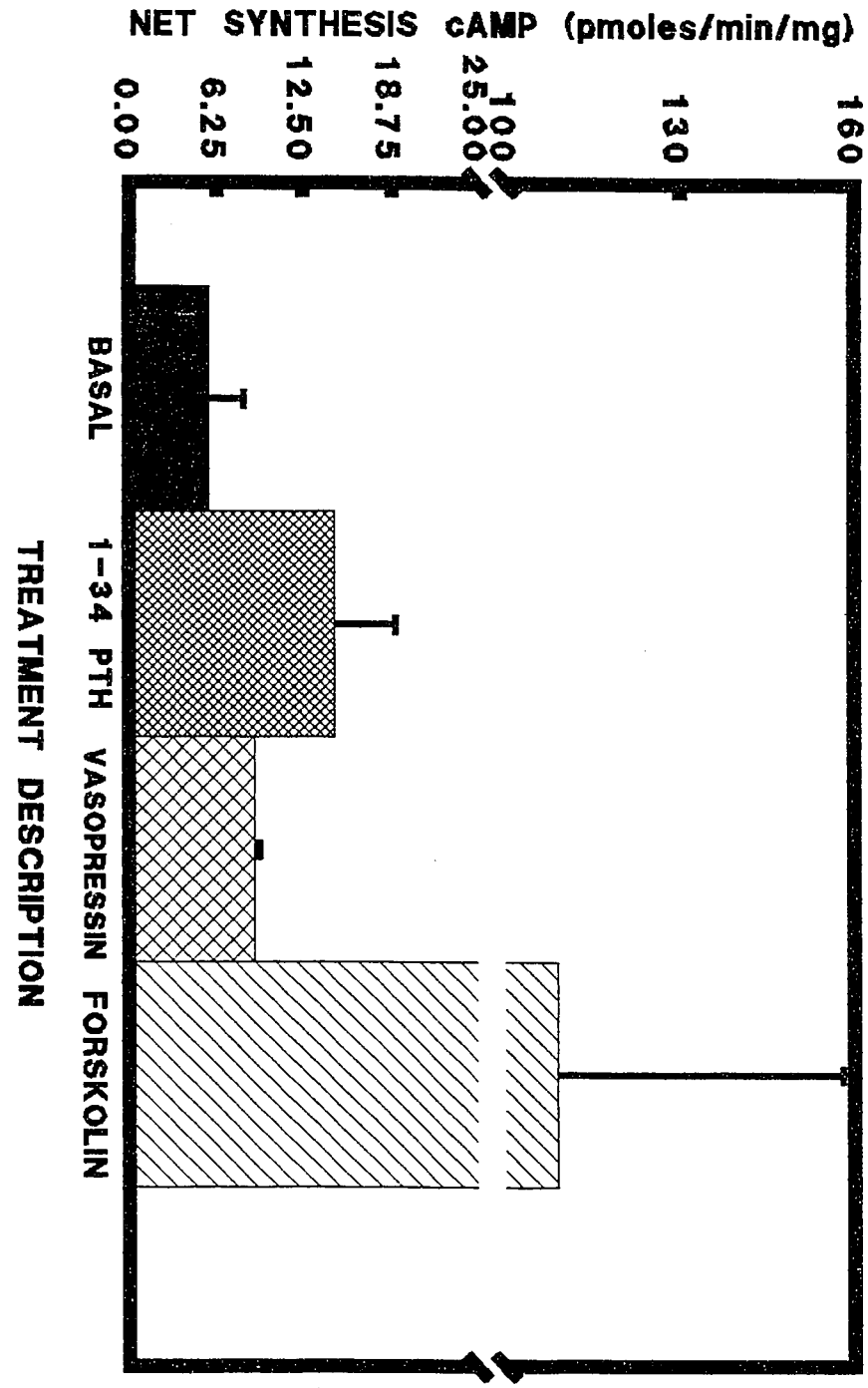
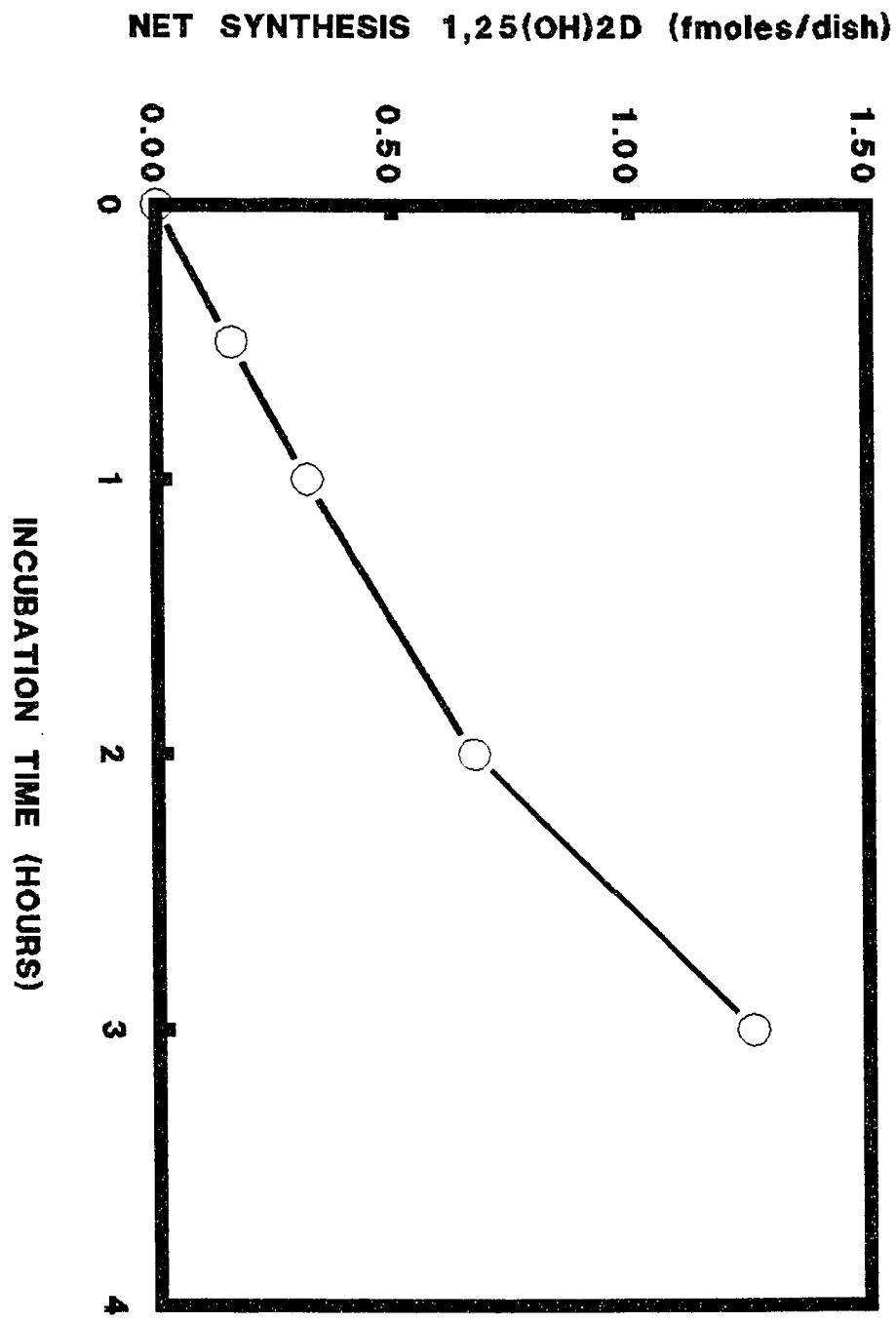

NET SYNTHESIS cAMP (pmoles/min/mg)
0.00
6.25
12.50
18.75
25.00
100
130
160
BASAL
1-34 PTH
VASOPRESSIN
FORSKOLIN
TREATMENT DESCRIPTION

FIGURE 2.32 Time course of 1αhydroxylase activity in primary cultures of rat renal tubules.
Effect of incubation time on net $1,25(OH)_2D_3$ synthesis by primary cultures enriched for proximal tubules. Data are mean of duplicate dishes from one representative experiment.


NET SYNTHESIS 1,25(OH)2D (fmoles/dish)
0.00
0.50
1.00
1.50
0
1
2
3
4
INCUBATION TIME (HOURS)

APPENDIX 2

Curriculum vitae

CURRICULUM VITAE

Valerie M. Weaver
105 Sweetland Ave Apt 2
Ottawa, Ontario
CANADA
K1N 7V1

EDUCATION

1986 - Present	University of Ottawa, Ottawa, Ontario PhD. Candidate DOCTORAL THESIS "Effect of Dietary Minerals and Diabetes on Renal Vitamin D Hydroxylation and Calcium Homeostasis"
1985 - 1986	University of Ottawa, Ottawa, Ontario B.Sc. (Honors) Biochemistry
1983 - 1985	University of Waterloo, Waterloo, Ontario B.Sc. General Science
1975 - 1978	Algonquin College of Applied Arts and Technology 3 Year Biochemical Technology

TECHNICAL WORK EXPERIENCE

1987 - 1991	Teaching Assistant Medical Biochemistry, Physical Biochemistry Lab, 2nd year Biochemistry Lab, Metabolism Lab.
1986 - 1987	Consultant Radiopharmaceuticals Atomic Energy of Canada - Dr. Evans (Vice Pres.) Industrial Products/Food Irradiation Atomic Energy of Canada - B. Wilson (Gen Mg)
Summers 1985-6	Technical Market Development Officer Atomic Energy of Canada - C.M. David
1980 - 1984	Atomic Energy of Canada, Radiochemical Co. Research/Development Asst Radiopharmaceuticals
1978 - 1980	Atomic Energy of Canada, Research Co. Chalk River. Research Asst. Biology Branch

1978	Environment Canada, Toxicology. Biochemical Technologist

SCHOLARSHIPS/FELLOWSHIPS

1992	NSERC - Fellowship
1988 - 1991	Medical Research Council Scholarship
1988 - Present	University of Ottawa Supplemental Graduate Scholarship
1988	NSERC Scholarship - Declined
1987 - 1988	Ontario Graduate Scholarship
1986	University of Ottawa Entrance Scholarship

AWARDS

1990	University of Ottawa Travel Award
1988	AIN Graduate Student Research Award - Proctor and Gamble, FASEB Conference - American Institute of Nutrition
1987	Mary Eccelstone Research Award - Wyeth - CFBS Conference -Canadian Society of Nutritional Sciences
1986	University of Ottawa Gold Medal (Highest academic standing science)
1986	Society of Chemical Industry Prize (Highest academic standing science)
1986	Alumni and Faculty Plaque Biochemistry (Highest academic standing biochemistry)

PUBLICATIONS

Weaver V.M. and Marcotte M.L. Food Irradiation and Consumer Education - The Role of Food and Health Professionals Int. Jrl. App. Radiation and Isotopes (September 1987).

Welsh J.E. and Weaver V. Adaptation to low dietary calcium in magnesium deficient rats. J. Nutr. 118: 729-734 (1988).

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Stone L.A., Weaver V.M., Bruns M.E., Christakos S., and Welsh J.E. Vitamin D Receptors and Compensatory Tissue Growth in Spontaneously Diabetic BB rats. Annals of Nutrition amd Metabolism. 35:196-202 (1991).

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Weaver, V.M., Franks, D.J. and Welsh, J.E. Activation of protein kinase C modulates dihydroxycholecalciferol synthesis in rat renal tubules. in: Norman, A.W. et al., editors, Proceedings of the Eighth Workshop on Vitamin D, W. DeGruyter & Co., Berlin, IN PRESS, (1991)

Weaver, V.M., Franks, D.J. and Welsh, J.E. Activation of protein kinase C modulates $25(OH)D_3$ hydroxylation in isolated rat renal tubules. Cellular Signalling, 4, May 1992.

IN PREPARATION

Weaver V.M. and Welsh J.E. 1,25 Dihydroxycholecalciferol Supplementation and the Hypocalcemia of Magnesium Deficiency

Weaver V.M. and Welsh J.E. Effect of in vivo and in vitro insulin on 25 hydroxycholecalciferol metabolism

Weaver V.M., Franks D. and Welsh J.E. Mineral and Peptide Regulation of Vitamin D Metabolism in Diabetes

Weaver V.M., and Welsh J.E. Role of PKC in impaired 1,25(OH)2D3 Production During Diabetes

ABSTRACTS

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Weaver V.M. and Welsh J.E. 1,25 dihydroxycholecalciferol metabolism and the genesis of hypocalcemia of magnesium deficiency Fed. Proc. 48 (1989).

Welsh J.E., Pulfer L. and Weaver V.M. Effects of streptozotocin diabetes on 1,25 dihydroxycholecalciferol metabolism Fed. Proc. 48 (1989).

Weaver V.M. and Welsh J.E. Effect of diabetes on 25(0H)D 1αHydroxylase activity in isolated renal tubules Fed. Proc. 49 (1990).

Weaver V.M. and Welsh J.E. 1,25 dihydroxycholecalciferol supplementation and the hypocalcemia of magnesium deficiency CFBS 33 (1990).

Weaver V.M., Franks D. and Welsh J.E. Protein Kinase C Modulation of 1,25 dihydroxycholecalciferol synthesis FEBS 20 (1990).

Weaver V.M. and Welsh J.E. Role of Insulin in Impaired 1,25(OH)2D3 Production During Diabetes Eighth Workshop on Vitamin D (1991).

Weaver V.M., Franks D. and Welsh J.E. Activation of Protein Kinase C Modulates Dihydroxycholecalciferol Synthesis in Rat Renal Proximal Tubules Eighth Workship on Vitamin D (1991).

Weaver V.M., Gagnon A.M. and Welsh J.E. Vitamin D Metabolism in Response to Mineral Restriction in Diabetes CFBS 34 (1991).