ROLE OF VITAMIN D IN ARTERIAL CALCIFICATION

A thesis presented

by

S. RAJASREE

to

The Division of Cellular and Molecular Cardiology
in partial fulfilment of the requirements
for the Degree of

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCES AND TECHNOLOGY
TRIVANDRUM

APRIL 1999

CERTIFICATE

I, S. Rajasree hereby certify that I had personally carried out the work depicted in the thesis entitled "Role of Vitamin D in arterial calcification" except where external help sought and acknowledged.

S. RAJASREE

Date: 20 April 1999

Professor C C KARTHA, MD
Head of the Division

Division of Cellular and Molecular Cardiology
Sree Chitra Tirunal Institute for Medical
Sciences and Technology
Trivandrum

CERTIFICATE

This is to certify that Smt. S. Rajasree in the Division of Cellular and Molecular Cardiology of this Institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph. D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The work relating to her thesis entitled "ROLE OF VITAMIN D IN ARTERIAL CALCIFICATION" was carried out under my direct supervision.

C C Kartha
(Guide)
20 April 99

The thesis

entitled

ROLE OF VITAMIN D IN ARTERIAL CALCIFICATION

Submitted

by

S. RAJASREE

for

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

TRIVANDRUM

evaluated and approved

by

Professor C C Kartha

(Guide)

Prof. C. G. Bahuleyan

Names of thesis examiners

Prof. K S Ratnakar

To my beloved parents .....

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

Many are the names that flash across my mind who have made valuable contributions for making this venture a successful one.

Words are inadequate to express my deepest gratitude and indebtedness to my guide Professor C C Kartha, for the direction and motivation he has provided in ample measure all these years towards the completion of this task. It has been an honour and previlege for me to have worked with him. I appreciate him for giving me the freedom of thought right from the conception of this project.

I am grateful to Professor M S Valiathan (our previous Director) presently Vice Chancellor, Manipal University of higher education, who was instrumental in my joining this institute and taking up this particular project, and who is even currently taking keen interest in the progress of my work.

I sincerely thank Professor KG Balakrishnan, Professor Jaganmohan Tharakan, Dr. V Ramankutty and Dr. K Sreenivasan, members of the doctoral advisory committee for their constructive criticisms and suggestions.

I also thank Dr.V Ramankutty whose timely suggestions were of great help to complete the task, Dr. Sankara Sarma for statistical analysis of the data, Dr. Annie John for helping me with electron microscopic studies and Dr. John T Eapen for his help in using atomic absorption spectrophotometer.

I am indebted to Dr. Rajpal former DM student in Cardiology for carrying out fluoroscopic examination of patients, ward sisters who were in charge at that time for their utmost co-operation and goodwill in providing the relevant samples, all the patients who have donated blood samples required for the study and Dr. Girija, Department of Cardiology, Medical College, Trivandrum, for providing blood samples from patients with acute myocardial infarction.

I am thankful to Dr. R Renuka Nair, Dr. K Shivakumar , Dr. Mira Mohanty and Dr TV Kumari who were always there to help me out of the problems when ever I approached them, and Dr. PS Appukuttan, Division of Biochemistry for extending the laboratory facility.

Mrs. K Remani, Mrs. Sulekha and Mrs. Sreelatha helped me to prepare histopathological slides and Dr. Arthur Lal, Dr. Uma Shankar and Mr. Manoj to carry out the animal experiments successfully. I am grateful to them.

My colleagues Mrs. Preetha Nair, Ms. R Preetha and Mr. C Kumar provided me moral support and companionship.

Dr.C.S.P.Iyer Division of Oceanography, Dr.Suresh Das and Mr. Deleepkumar, Division of Photochemistry, Regional Research Laboratory, CSIR, Trivandrum , allowed me to use their laboratory facility and I am thankful to them.

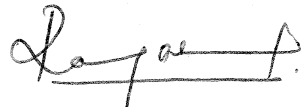
I am indebted to Dr.N Raghuramalu, NIN ,Hyderabad for providing training in radio ligand assay of vitamin D, Dr Nandini Rangaraj and Dr. Sreedhar Rao, Centre for Cellular and Molecular Biology, Hyderabad for giving training in radio receptor assay, to Dr. Uskokovic, Hoffman La Roche, USA, for sending me gift samples of 25 hydroxyvitamin D_3 and 1,25-Dihydroxyvitamin D_3 standards, necessary for the accomplishment of my work.

I am grateful to Professor Hywel Davies, Switzerland for his keen interest and constructive criticism in this study. I am obliged to him for providing me with interesting literature relevant to this work.

I also acknowledge Indian Council of Medical Research, New Delhi, for the financial support extended during the course of the work.

I am indebted to Dr. K Mohan Das, our Director, Dr. V V Radhakrishnan Dean, Dr. R Shivakumar, Head, BMT Wing and Mr. AV George, Registrar for extending institutional facility for my work.

I am thankful to all my family members especially my mother, my brother, M. Harikrishnan, my husband, VC. Vijayakumar, and my daughter, R Gauthami for their supportive attitude for sustaining my effort for the completion of this work.



S. Rajasre

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SYNOPSIS

SYNOPSIS

Arterial calcification has received increased attention in recent years. Till recently the prevailing view was that arterial calcification is merely a passive process that occurs only in end stage atherosclerosis and that it simply signifies dying cells. Current opinion is that arterial calcification is an organized regulated process with cellular and molecular mechanisms similar to organized bone formation.

An important regulator of bone calcification and potentially of arterial calcification is the hormone 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$,) the most active form of vitamin D. Vitamin D is formed from the precursor 7-dehydrocholesterol, through ultraviolet activation in the skin and it is speculated that perhaps the oxidized form of cholesterol may mimic some of the molecular mechanisms of vitamin D. It is also known that vitamin D ingested in the diet is carried in the circulation on lipoproteins. Thus the entry of lipoproteins into the arterial wall may also facilitate entry and accumulation of the promineralization factor vitamin D.

Several lines of evidence point to the possible relationship of vitamin D with cardiovascular diseases. Jim Moon observed that the epidemic of ischemic heart disease in North America directly paralleled the increased use of vitamin D and the decreased availability of dietary magnesium, two major factors that act synergistically to induce

calcification of arteries and kidneys. Increased vitamin D intake is associated with hypercalcemia, acute myocardial infarction and urolithiasis. Linden suspected that the known hypercholesterolemic effect of vitamin D might be the mechanism. Another clue for a causal link between arteriosclerosis and vitamin D is the association between osteoporosis and arterial calcification. Davies in a provocative article has drawn attention to the early events in transplant arteriopathy and argues that mitogens such as vitamin D are the initiating factors in atherosclerosis. According to him lipids are possibly complicating rather than causative factors in atherosclerosis. Watson et al., however, reported an inverse correlation of 1,25-dihydroxyvitamin D_3 with the extent of vascular calcification in patients with coronary artery disease.

Scragg et al., also found an inverse association between plasma 25- hydroxyvitamin D_3 and myocardial infarction in a community based case-control study. Schmidt and co-workers observed no difference in serum levels of 25- hydroxyviatmin D_3 between patients with myocardial infarction and controls.

There are also experimental data that indicate a causative role for vitamin D in arterial calcification and atherosclerosis. Administration of massive doses of vitamin D induces arteriosclerotic lesions in a number of animal species. In animals fed a hypercholesterolemic diet, given vitamin D and exposed to nicotine, atherosclerotic lesions have been observed.

A review of epidemiological and experimental studies on the association of vitamin D with cardiovascular diseases reveals that the available evidence for a causal

association is suggestive, yet sparse. In clinical studies, sample sizes were not large enough, controls were inappropriate, or measurements of vitamin D levels in serum were not done or confounding variables were not appropriately adjusted for. There has not been any effort to correlate vitamin D levels with major risk factors in coronary artery disease or for subgroup analysis to relate vitamin D levels with acute myocardial infarction, chronic coronary artery disease and coronary artery calcification. Population based surveys, compared to clinical studies have revealed entirely contradictory findings on the relationship of vitamin D status and myocardial infarction. Epidemiological surveys have not been performed in a tropical environment where there is a possibility that high level of solar exposure would lead to elevated serum vitamin D levels.

In experimental animals, lesions of both atherosclerotic nature and those resembling Monckeberg's arteriosclerosis have been noted. In animal studies attempts have not been made to relate serum 25-hydroxyvitamin D_3 levels to the morphologic nature of the lesions and to lipid profiles in serum and lipid content in the arterial wall.

This thesis contains the results of investigations performed to fill up some of the above lacunae. Two models of arterial calcification have been chosen:

(1) atherosclerotic coronary artery disease and coronary artery calcification in humans and (ii) aortic calcification induced in animals by administration of vitamin D.

OBJECTIVES

1. To assess the vitamin D status in patients with coronary artery disease
2. To observe whether coronary artery calcification in patients with coronary artery disease correlates with serum levels of 25-hydroxyvitamin D_3
3. To observe whether there is any association of serum levels of 25- hydroxyvitamin D_3 with major risk factors for coronary artery disease
4. To define the morphological features of aortic lesions in animals with elevated serum vitamin D levels
5. To observe whether increased serum 25-OH-D_3 levels correlate with serum lipid profiles in animals with aortic calcification
6. To assess whether aortic calcification is related to 1,25-dihydroxyvitamin D_3 receptor status and receptor mediated calcium uptake in aortic medial smooth muscle cells

MATERIALS AND METHODS

The study consisted of two parts.

Part A: Case Control study

The study population consisted of 143 men with coronary artery disease (cases) and 70 male control subjects.

Fifty-five of the patients were those admitted to Medical College, Trivandrum with chest pain and diagnosed to have acute myocardial infarction based on electrocardiographic criteria and typical changes in serum enzyme levels. Eighty-eight cases were patients with coronary artery disease admitted at Sree Chitra Tirunal Institute for Medical Sciences and Technology for evaluation by coronary angiography and diagnosed to have coronary artery disease. Presence or absence of coronary artery calcification was determined by fluoroscopy.

Biochemical analysis

About 8 ml of venous blood was drawn after a 12 hour fast from each subject and collected in sterile glass container and incubated at 37 ^{0}C for 2 hours. Following clot retraction, samples were centrifuged at 2000 rpm for 15 minutes. Serum was separated and stored at 4 ^{0}C until analysis.

Serum 25-hydroxycholecalciferol (25-OH-D_3) levels were assayed by competitive protein binding radio ligand assay described by Chen et al. Levels of calcium and magnesium in the serum was determined by atomic absorption spectrophotometry.

Inorganic phosphate was estimated by following the method of Fiske and Subbarrow. Serum levels of total cholesterol, triglyceride and high-density lipoproteins were determined by enzymatic assays using assay kits (Boehringer Mannheim).

Part B: Experimental study on rabbits

Twenty healthy female New Zealand rabbits were selected and randomized into two groups.

Group I comprised of 14 animals which were given vitamin D_3. Ten thousand IU crystalline cholecalciferol (vitamin D_3) dissolved in 1 ml of cottonseed oil was injected intramuscularly to each animal twice a week for one month.

Group II had six animals which served as controls. These animals were given injections of 1 ml of plain oil at an interval of three days for one month. All the animals were fed a commercial rabbit diet. Water was given ad libitum.

At the end of one month, animals were killed and blood and tissue were collected.

Biochemical estimations

Aorta was collected at sacrifice for analysis of total lipids, calcium, magnesium, 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) receptor, and ^{45} Ca uptake. Total lipids in the aorta of the animals were determined according to the method of Bligh and Dyer. Determinations of calcium and magnesium levels in aortas were done using an atomic absorption spectrophotometer. Serum was collected at sacrifice for estimating the levels

of 25-hydroxyvitamin D_3, calcium, magnesium, total cholesterol, triglyceride and high-density lipoprotein.

Histopathological studies

Samples of aortas fixed in 10% neutral buffered formalin were processed for paraffin embedding. Serial sections were stained with haematoxylin and eosin, Van Gieson's stain for elastic fibers and von Kossa stain for calcium.

Statistical analysis

Student's 't' test was performed for comparing the mean values of each parameter between cases and controls, as well as for comparison between subgroups. The difference was considered statistically significant when $P< 0.05$. Pearson's method was used for determining the correlation coefficients in cases to assess the degree of association between 25-hydroxyvitamin D_3 and other independent variables. The association with other known risk factors was tested using Chi Square method. Multivariate analysis was done for obtaining the relative risk with respect to 25-hydroxyvitamin D_3 level and other known risk factors for coronary artery disease. The median level of 25-hydroxyvitamin D_3 was taken as the cut off value. The cut off values taken for LDL and HDL levels were 160 mg% and 35 mg% respectively.

RESULTS

Serum levels of 25-OH-D_3 were significantly higher in patients with angiographic evidence of coronary artery disease and acute myocardial infarction when compared with controls. Serum 25-hydroxyvitamin D_3 levels correlated with calcium and magnesium levels, but not with serum levels of cholesterol and low density lipoproteins. Patients with angiographic evidence of coronary artery disease had higher levels of low density lipoprotein, triglyceride and lower levels of high-density lipoprotein when compared to the controls. Cases had higher number of smokers and hypertensives. There was no significant difference in the number of diabetics and those with a family history of coronary artery disease between cases and controls.

In sub group analysis there was no difference in serum 25-OH-D_3 levels between patients who had coronary calcification and those who did not (n=39). In multivariate analysis odds ratio for serum 25-hydroxyvitamin D_3 was 4.16 ($P < 0.005$).

Four of the 14 animals which received vitamin D_3 injections had extensive, gritty, whitish lesions with adherent thrombi in both abdominal and thoracic aortas. In another four animals aortas were dilated and intimal ulcers were also seen. Aorta was normal in appearance in all of the control animals.

Histological abnormalities were seen in the aortas of 8 test animals. Fragmentation of internal elastic lamina, focal loss of elastic fibers and fragmentation and degeneration of elastic fibers in the media were seen in all the eight animals with gross lesions. In four of the test animals there was extensive calcification in the media (von Kossa positive).

Calcified areas were also positive for the presence of iron. None of the animals had lesions resembling atherosclerosis. Inflammatory infiltrates were also absent.

Serum levels of 25-OH-D_3 were seen increasing from week 1 to week 4, in animals which received vitamin D_3. When compared with control animals, the animals in group I had elevated levels of serum 25-OH-D_3. Serum 25- hydroxyvitamin D_3 did not correlate with serum levels of cholesterol, triglyceride and high-density lipoprotein. Serum 25-hydroxyvitamin D_3 levels were higher in rabbits with aortic calcification when compared with the levels in rabbits without calcification in the aorta. Lipid levels in aorta were not significantly different between test and control animals. In animals with calcification of the aorta, there was significant elevation of 1,25-dihydroxyvitamin D_3 receptor as well as increased ^{45} Ca uptake in aortic medial smooth muscle cells. Calcium levels in the aorta correlated with 1,25-dihydroxyvitamin D_3 receptor as well as ^{45} Ca uptake.

CHAPTER 1
INTRODUCTION

CHAPTER 1

INTRODUCTION

Two common lesions in arteries are Monckeberg's arteriosclerosis and atherosclerosis. Monckeberg' arteriosclerosis is characterized by loss of elastic tissue in the media of the arterial wall and is a common feature of old age. In atherosclerosis there is intimal thickening from fatty deposits and smooth muscle cell proliferation in elastic arteries and large and medium sized muscular arteries. Both these conditions can lead to ischemia of many vital organs and are responsible for several clinical syndromes, morbidity and mortality. Calcification of the arterial wall is characteristic of both atherosclerosis and arteriosclerosis.

Arterial calcification has received increased attention in recent years. Till recently the accepted view was that arterial calcification is merely a passive process that occurs only in end-stage atherosclerosis and that it simply signifies dying cells. Current opinion (Doherty TM and Detrano RC 1994) is that arterial calcification is an organized regulated process with cellular and molecular mechanisms which are similar to those of osteogenesis.

An important regulator of bone calcification and potentially of arterial calcification is the hormone 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2 D_3$), the most active form of vitamin D. Vitamin D is formed from the precursor 7-dehydrocholesterol through ultraviolet activation in the skin. It is speculated that perhaps the oxidised form of cholesterol may mimic some of the molecular actions of vitamin D. It is also known that vitamin D ingested in the diet is carried in the circulation on lipoproteins. Thus the entry of lipoproteins into the wall of the artery may also facilitate entry and accumulation of the promineralization factor vitamin D.

Several lines of evidence point to the possible relationship of vitamin D with cardiovascular diseases. Moon, Brandy and Davison (1992) observed that the epidemic of ischemic heart disease in North America directly paralleled the increased use of vitamin D and the decreased availability of dietary magnesium, two major factors that act synergistically to induce calcification of arteries and kidneys. Increased vitamin D intake is associated with hypercalcemia, acute myocardial infarction and urolithiasis. Linden (1974) suspected that the known hypercholesterolemic effect of vitamin D might be the mechanism. Another clue for a causal link between arteriosclerosis and vitamin D is the association between osteoporosis and arterial calcification (Moon J, Brandy B and Davison AJ 1992).Davies (1989) in a provocative article has drawn attention to the early events in transplant

against a causal association. In clinical studies, either sample sizes are not large enough, controls are inappropriate, or measurements of vitamin D levels in serum have not been done or confounding variables have not been appropriately adjusted for. There has not been any effort to correlate vitamin D levels with major risk factors in coronary artery disease or for subgroup analysis to relate vitamin D levels with acute myocardial infarction, chronic coronary artery disease and coronary artery calcification.

Epidemiological surveys to relate vitamin D status and coronary artery disease have not been performed in a tropical environment where high levels of solar exposure could lead to elevated serum vitamin D levels. In experimental animals, attempts have not been made to relate serum vitamin D levels to the morphological nature of the lesions, to lipid profiles in serum and lipid content in the arterial wall.

This thesis contains the results of investigations performed to fill up some of the above lacunae. Two models of arterial calcification have been chosen:

(1) atherosclerotic coronary artery disease and coronary artery calcification in humans and (2) aortic calcification induced in animals by administering vitamin D.

Specific objectives of the study were

1. measure 25-hydroxyvitamin D_3 levels in serum of patients with coronary artery disease
2. observe whether serum levels of 25-hydroxyvitamin D_3 correlate with coronary artery calcification in patients with coronary artery disease
3. analyse whether serum 25-hydroxyvitamin D_3 levels correlate with any of the major risk factors for coronary artery disease
4. define the morphological features of lesions in aorta of animals periodically given unphysiological doses of vitamin D
5. examine the relationship of serum 25-hydroxyvitamin D_3 levels with serum lipid profiles in animals given large doses of vitamin D
6. assess 1,25-dihydroxyvitamin D_3 receptor status and calcium uptake in medial smooth muscle cells of aorta of animals given excessive vitamin D and
7. relate 1,25-dihydroxyvitamin D_3 receptor status and calcium uptake in medial smooth muscle cells to morphological lesions in aorta of animals given excessive vitamin D.

CHAPTER II
REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

For life to be maintained, the composition and properties of the local fluid environment of the body's cell must remain constant within certain limits. This is ensured by homeostatic control mechanisms, which depend on adequate circulating systems which surround and permeate the tissues providing a continuous perfusion of body fluids. The circulatory system consists of heart, elastic and muscular arteries, arterioles, capillaries, veins and lymphatics.

STRUCTURE OF ARTERIES

Normal muscular and elastic arteries have three morphologically distinct layers viz., the intima, the media and the adventitia. Structure of an artery is shown in figure 1 A.

The intima or innermost layer, consists of a luminal side lined by a single continuous layer of endothelial cells and bounded peripherally by a fenestrated sheet of elastic fibers, the internal elastic lamina. Between these boundaries are

various components of extracellular connective tissue matrix, and in children an occasional smooth muscle cell (SMC). With increasing age in man, intimal smooth muscle and extracellular matrix components accumulate slowly and generally at a uniform rate, except in certain areas where nodular accumulations called intimal cushions develop.

Arteries conventionally are divided into two main types according to the most prominent tissue in their media.

1. Muscular artery

The media or middle layer of the muscular artery consists entirely of diagonally oriented SMCs, surrounded by variable amounts of collagen, small elastic fibers and proteoglycan (mucopolysaccharides). The SMCs spiral through the vessel wall and in most instances, attach to one another by specific junctional complexes.

The adventitia or outermost layer of the muscular artery consists principally of recognizable fibroblasts intermixed with SMCs loosely arranged between bundles of collagen and surrounded by proteoglycan. It is usually separated from the media by a discontinuous sheet of elastic tissue, the external elastic lamina. The structure of a muscular artery is shown in figure 1B.

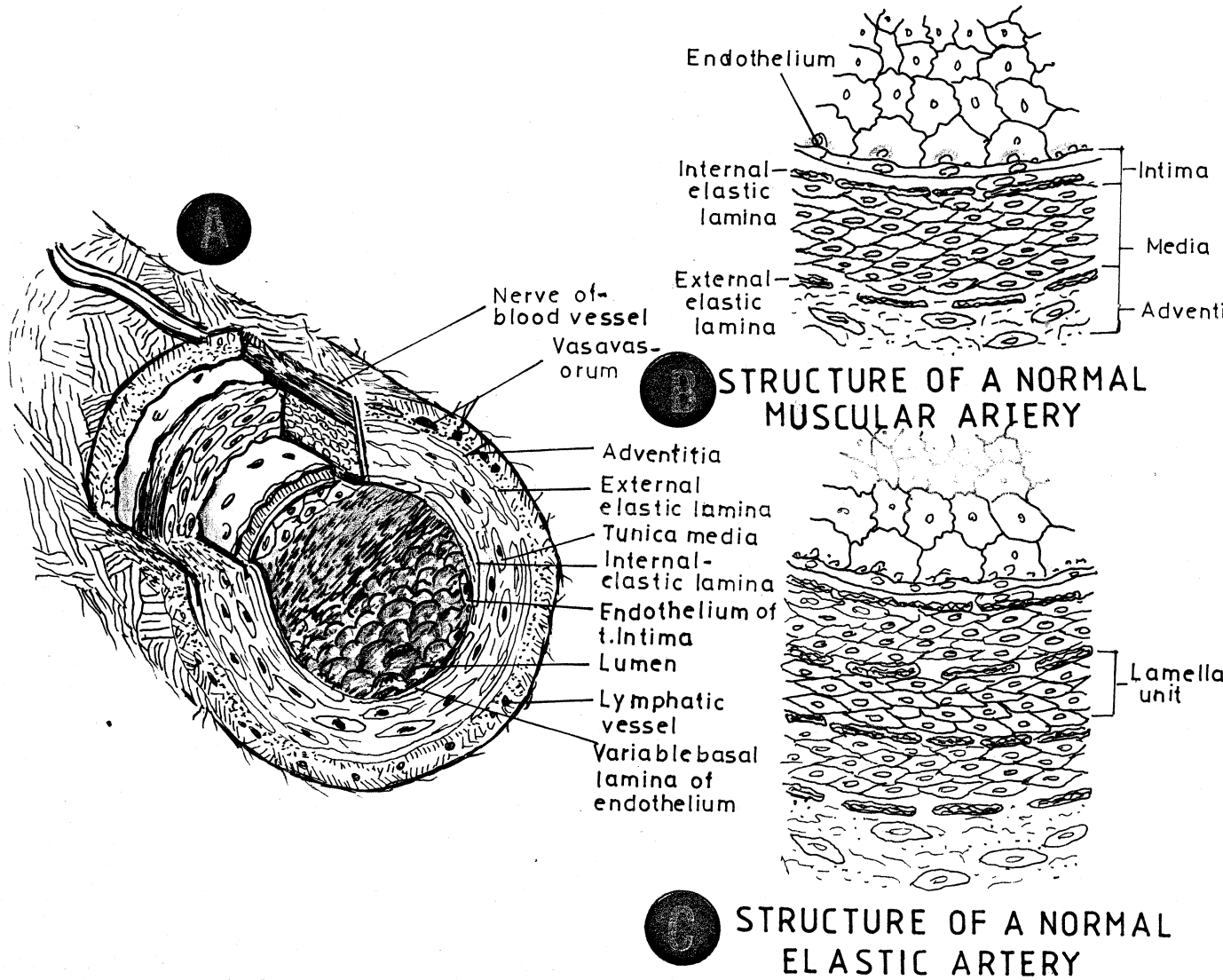
2. Elastic artery

The media contains numerous layers of longitudinally oriented elastic laminae and a significant amount of smooth muscle per unit area than muscular arteries. The thickness of medial elastic laminae varies between vessels. As a general rule, there is an indirect relationship between the size of an artery and the width of its media. Bundles of collagen are common in the media of parts of aorta in the thorax, but their location varies between different levels of the vessel. In the arch and upper thoracic portions of the aorta, collagen is aggregated in the inner media, while collagen is seen mainly in the outer media in the middle and lower levels of the thoracic aorta. Collagen bundles are uncommon, or are not found in the media of the abdominal portions of the aorta.

The elastic artery has a collagenous adventitia. The thickness of the adventitia of elastic arteries varies markedly between vessels. Vasa vasorum are found in all parts of the adventitia of elastic arteries. Their size diminishes from the outer to the inner parts of the layer. The boundary between the media and the adventitia of elastic arteries is marked in some of the smaller vessels by well-developed external elastic lamina. The structure of an elastic artery is shown in figure 1C.

Arteries are susceptible to various forms of injury, resulting in degenerative and inflammatory responses leading to repair and remodeling and

Figure 1
STRUCTURE OF ARTERIES



a spectrum of pathological lesions. There are two common types of lesions in arteries and both the lesions can lead to ischemia of many vital organs and are responsible for several clinical syndromes, morbidity and mortality.

(a) Monckeberg's arteriosclerosis is a common feature of old age and is characterized by loss of elastic tissue in the media and calcification resulting in hardening of the arterial wall.

(b) Atherosclerosis is another chronic disease of elastic arteries and of large and medium sized muscular arteries. There is intimal thickening from accumulation of foam cells, extracellular lipid, smooth muscle cell proliferation and extracellular matrix synthesis.

Calcification of the arterial wall is a feature of both atherosclerosis and arteriosclerosis.

PATHOLOGICAL FEATURES OF ATHEROSCLEROSIS

A striking feature of atherosclerotic vascular disease is its focal topography. Atherosclerotic lesions do not occur randomly. They are found at specific lesion prone sites that differ structurally and functionally from non-lesion prone sites. These lesion prone areas are distinguished by increased permeability of the endothelium to plasma proteins such as albumin, fibrinogen and low-density lipoprotein (LDL) cholesterol. (Ross R and Glomset JA 1976 ; Gotlieb AI and Havenith MG 1991).

The fundamental features of atherosclerosis are:

1. focal nature and characteristic distribution of lesions in blood vessels
2. predominant intimal manifestations
3. heterogenous nature of the plaque constituents and
4. the presence of both proliferative and degenerative processes in the lesion.

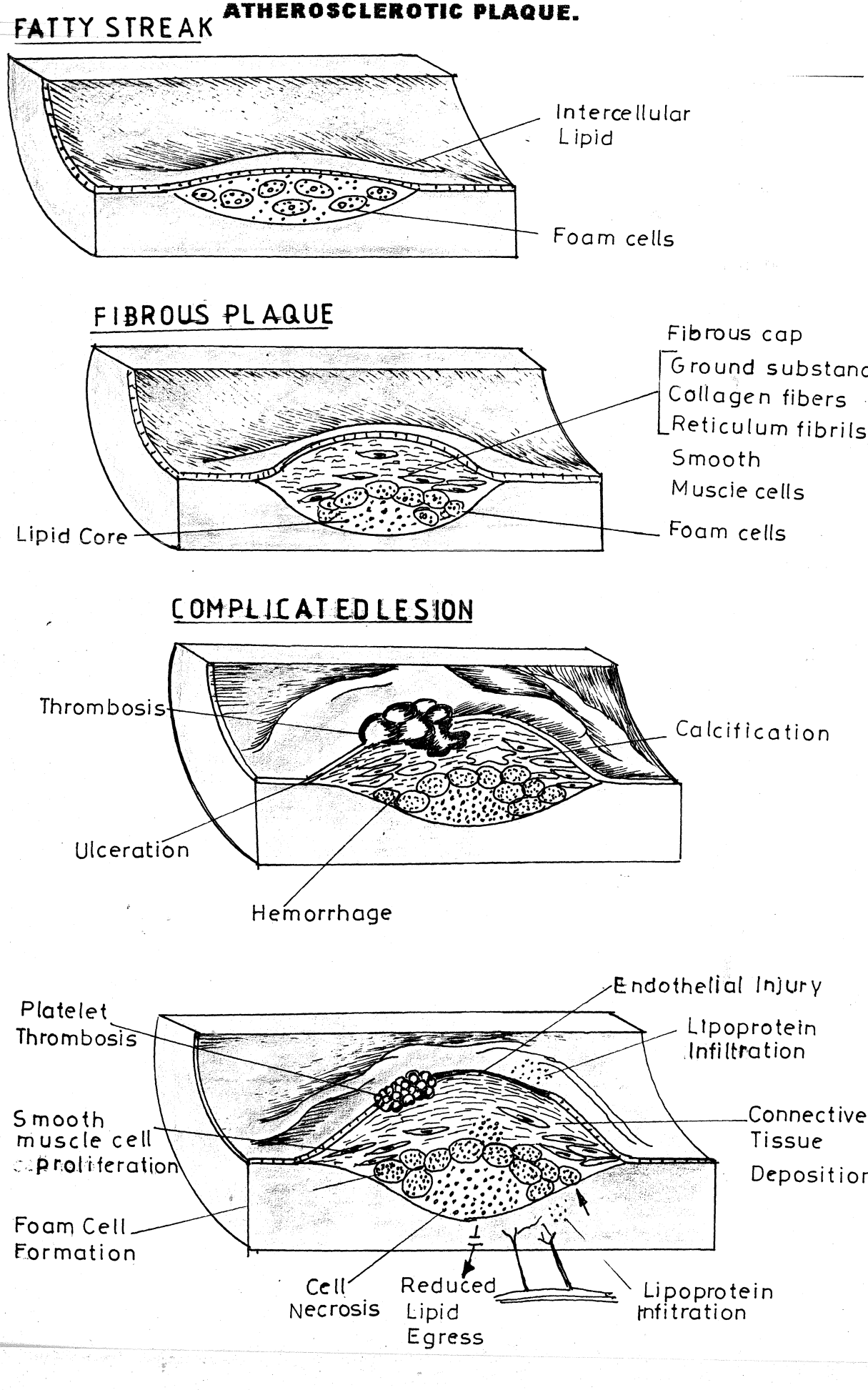
Advances in techniques like electron microscopy and immuno histochemistry contributed to excellent studies on morphological lesions in atherosclerosis. These studies formed the basis for various theories on atherogenesis.

The three distinct intimal atherosclerotic lesions that are commonly encountered are the fatty streak, the gelatinous lesion and the raised fibrolipid plaque (Figure 2). There is no agreement as to what constitutes the early lesion and also whether what are called early lesions, inevitably develop into plaques. A diffuse intimal thickening (DIT) appears to be a result of growth and ageing in the arterial system and is probably a reaction to hemodynamic stress. Focal intimal cushions with similar morphology of DIT are seen at or near points of arterial branching and may represent sites of predilection for the development of atherosclerosis or form part of the atherosclerotic process itself. Controversy clouds the relationship between fatty streaks and the raised fibrolipid plaque. At least, three varieties of fatty streaks have been described. One of them which occurs in young adults belonging to population groups with a high background

Figure 2

STAGES OF ATHEROSCLEROSIS

PATHOLOGIC PROCESSES LEADING TO THE DEVELOPMENT OF AN ATHEROSCLEROTIC PLAQUE.



level of atherosclerosis is suggested to be a progressive lesion and a precursor of fibrolipid plaque. This lesion differs from the juvenile fatty streak and those seen in the elderly, in all population groups. The lipid in juvenile fatty streaks is in the form of extracellular accumulation. There is also an increase in extracellular components of connective tissue in the fatty streaks. The fatty streak is also cellular; the predominant cells being the mononuclear cells, which are blood derived monocytes. Cells containing variable number of lipid droplets and ultrastructurally identifiable as smooth muscle cells are also present in deeper layers.

The data on the frequency, topography and age distribution of gelatinous lesions are insufficient to assess their role as a precursor of raised fibrolipid plaque.

The fibrolipid plaque is the archetypal lesion of atherosclerosis and exists in a number of morphological forms. The lesion is more common in the abdominal aorta and around the mouth of intercostal and lumbar arteries and essentially consists of a yellow lipid rich basal pool covered by a connective tissue cap. The plaque results from a building up of smooth muscle cells, macrophages, cell debrii, extra cellular matrix, cholesterol and cholesteryl esters

underneath the endothelial inner layer of arteries. Ulceration, thrombosis, hemorrhage and calcification are superimposed. The most important complication in its natural history is thrombosis, which leads to clinical events. The major event preceding thrombotic occlusion appears to be either a split in the connective tissue cap (fissuring) or intimal hemorrhage. (Ross R 1986 ; Amati GD and Silver MD 1991; Ross R 1993). Recently, it is recognized that a thin cap and cap inflammation predispose to plaque rupture (Fuster V 1997)

PATHOLOGICAL FEATURES OF MONCKEBERG'S ARTERIOSCLEROSIS

Monckeberg' medial calcification or Monckeberg's sclerosis is a form of medial calcification usually associated with aging, predominantly found in the limb arteries. The condition is unrelated to atheromatous calcification, although both are found mainly in middle aged and elderly people. Macroscopically the arteries in Mockeberg's calcification are rigid and brittle but not stenotic unless atheroma is also present. Microscopically, there are dense bands and islands of calcification in the media between and in the two elastic laminae. Occasionally ossification may be seen. The medial smooth muscle usually is extensively replaced by pale staining acellular, hyalinized fibrous tissue. A dystrophic form of basophilic calcification is observed within the altered media. Since the process involves only the media, it does not cause luminal narrowing. Luminal

narrowing, if present, is usually because of coexistent intimal atherosclerosis (Ross A 1991).

PATHOLOGICAL FEATURES OF ARTERIAL CALCIFICATION

A common feature of atherosclerosis and Monckeberg's arteriosclerosis is calcification of the arterial wall. There is increasing interest in the pathogenic mechanism of arterial calcification.

For two centuries, coronary arterial calcification was considered to be the very essence of coronary sclerosis. Virchow (1863) noted that vascular calcification was similar to bone formation and described atherosclerotic coronary lesion as an ossification and not mere calcification.

Arterial calcification and its role in atherogenesis were neglected in later years when it was realized that cholesterol and lipoproteins are more important in atherogenesis. With the development of angiographic techniques, arterial calcification was more easily recognized but was considered a manifestation of the later stages of the degenerative process of atherosclerosis. Thus atherosclerotic calcification is commonly regarded as a passive process of adsorption or precipitation, merely a secondary effect of advanced atherosclerotic degenerative process.

Atherosclerotic calcification begins as early as the second decade of life immediately after the formation of the fatty streak . Calcific deposits are however, found more frequently and in greater amounts in elderly individuals and in more complex lesions. Hydroxyapatite, the predominant crystalline form of calcium deposits (Schmid K et al., 1980) is formed primarily in vesicles that pinch off from arterial wall cells and the process is analogous to the way matrix vesicles pinch off from chondrocytes in the developing bone (Anderson HC 1983, 1988 and 1989 and Tanimura A , McGregor DH and Anderson HC 1983, 1986 a and 1986 b).

One of the most curious aspects of arterial calcification is its intimate association with atherosclerosis. The reason for this connection is completely unknown. High resolution imaging studies using digital subtraction cinefluoroscopy (DSC) ,and ultrafast computed tomography (Agatston AS et al., 1990; Tanenbaun SR, Kondor GT and Vaselik KE 1989; Breen JFet al., 1992) have revealed that calcification appears in atherosclerotic lesions much earlier than what was previously believed (Stary HE 1990). Contrary to the earlier view that arterial calcification signifies dying cells, Doherty and Detrano (1994) hypothesized that arterial calcification is an organized, regulated process with cellular and molecular mechanisms similar to organized formation of bone. The mineral in atherosclerotic calcification is hydroxyapatite, the same mineral as in

bone. Cartilage, endochondral ossification, bone and even haemopoietic tissue have been observed in the atherosclerotic arteries of experimental animals.

Three possible reasons are postulated for formation of bone in the arterial wall:

(1) Artery wall cells are not terminally differentiated and retain the potential for differentiation into other mesenchymal cells such as chondroblasts and osteoblasts

(2) A sub population of cells in the artery wall has osteoblastic potential and

(3) Osteoblastic cells perhaps migrate into the arterial wall accompanying angiogenic sprouts or by chemotaxis of circulating immature cells of the bone marrow (Bostrom K et al., 1995).

MOLECULAR BASIS FOR ARTERIAL CALCIFICATION

Fitzpatrick et al., (1994) used *in situ* hybridization to identify mRNA of matrix proteins associated with mineralization in coronary artery specimens. Specifically they identified a cell attachment protein (osteopontin), a protein associated with calcium (osteonectin) and a gamma carboxylated protein that regulates mineralization (osteocalcin).

Osteopontin is a phosphorylated glycoprotein, regulated by local cytokines with known involvement in the formation and calcification of bone.

Immunohistochemistry of the specimens examined by Fitzpatrick and colleagues (1994) showed positive staining for osteopontin throughout the plaque, and intense, highly specific staining for osteopontin in the outer margins of all diseased segments at each calcification front. Two other studies also revealed that osteopontin can be seen in tissues of atherosclerosis but it appears to be present only in sites of concomitant coronary atherosclerotic disease (Ikeda J et al., 1993; Ingram RT et al., 1993). Hirotta et al., (1993) demonstrated with Northern blotting that osteopontin mRNA expression is related to severity of atherosclerosis. On the other hand, osteonectin mRNA expression decreases with development of atherosclerosis. Shanahan and coworkers (1994) and Ikeda et al., (1993) independently reported that the predominant cell types in the areas associated with this ectopic bone protein expression are macrophage derived foam cells, though some smooth muscle cells can also be identified.

Giachelli and associates (1993) using immunochemistry and *in situ* hybridization showed that medial SMCs in uninjured arteries contain very low levels of osteopontin and its mRNA. Injury to either the adult rat aorta or carotid artery however, initiated time dependent increase in both osteopontin protein and mRNA within the arterial SMC, implying a role for osteopontin in the proliferative and migratory phases of repair after arterial injury. They also observed in confluent vascular SMCs *in vitro* that basic fibroblast growth factor,

transforming growth factor and angiotensin II, all proteins implicated in the arterial response to injury, elevated osteopontin expression. Bostrom and coworkers (1993 and 1995) examined calcified human atherosclerotic lesions using *in situ* hybridization and found expression of bone morphogenic protein type 2 (BMP-2) a potent osteogenic differentiation factor in calcified sections. Their results support the idea that atherosclerotic calcification is closely related to osteogenesis.

In bone, BMP-2 is produced by osteoblasts and stored in the bone matrix. Seven BMPs are now known and all except BMP-1 are members of the transforming growth factor beta superfamily. BMP-2 induces heterotopic bone formation at the site of injection in skeletal muscle *in vivo* (Khouri RK , Koudsi B and Reed H 1991). The receptor for BMP-2 appears to be a serine-threonine-kinase and some of the downstream effectors may be adhesion molecules (Edelman GM and Jones PS 1993). The transcriptional mechanisms remain unknown. Bone morphogenic proteins participate in the epithelial - mesenchymal interaction not only in developing cartilage, teeth and bone, but also in the formation of other organs including heart (Lyons KM, Petton RN and Hogan BLM 1990). It is believed that BMPs released by epithelial cells, induce recruitment and condensation of local mesenchyme to form underlying structures such as arterial media and cardiac septae and valves. Recognition of

the role of BMPs in encoding tissue identity during embryonic development has led to the hypothesis that factors in atherosclerosis may unmask repressed developmental programs for osteogenesis.

Localization of calcification in the artery does not readily identify the cells in the arterial wall which produces BMP-2 and the bone mineral. Calcification occurs within the media, at the medial-intimal interface or within the neointima of plaque. In normal development certain bones are formed by endochondral ossifcation. In this process, embryonic mesenchymal cells aggregate to form cartilage. Later micro vessels invade and degrade the cartilaginous matrix that eventually calcifies (Rooney P and Kumar S 1993). The osteoprogenitor cells carried in with angiogenic microvessels resemble microvascular pericytes, which are believed to have osteoblastic potential (Brighton CT et al.,1992). One possibility is that pericytes entering the medial layer of atherosclerotic arteries along with vasa vasorum from the adventitia are responsible for arterial calcification.

The role of mineralization in the pathogenesis and fate of the coronary plaque is unknown. Although coronary calcification has in the past been regarded as a passive process of adsorption or precipitation, evidence reviewed here suggests that this may not be the case (Doherty TM and Detrano RC 1994; Watson KE and Demer LL 1996). If it is true that

coronary calcification is an organized, regulated process, then to what end is this organization and regulation directed ? Does calcification serve some functional role ?

Lewis et al., (1996) speculated that coronary arterial calcification may protect threatened myocardium by strengthening weakened atherosclerotic plaque prone to rupture. Calcified lesions and hypocellular fibrotic lesions are much more stiff than cellular lesions. Biochemical data suggest that calcified areas are unlikely to be associated with plaque rupture. If a plaque develops a heavily calcified cap, it is about five times harder than a cellular lesion or normal vessel wall and very resistant to rupture. This may in part explain the high frequency of calcification in old population. Extensive calcification may have survival value. Evidence for the relative stability of calcified lesion has been obtained using intravascular ultrasound. Coronary calcification might perhaps represent an attempt by the arterial wall to stabilize and strengthen itself, thereby minimizing the risk for plaque rupture.

The suspicion that calcium is involved in the atherogenic process is long standing, and has been reinforced by the finding in recent years that calcium antagonists and chelating agents can impede the process of atherogenesis in experimental animals (Fleckenstein G et al., 1994). In man there is some evidence that calcium antagonists retard the progress of atherosclerosis.

VITAMIN D AND ARTERIAL CALCIFICATION

An important regulator of bone calcification and potentially of atherosclerotic calcification is the hormone $1,25(OH)_2D_3$, the most active form of vitamin D. Vitamin D is formed from the precursor 7-dehydrocholesterol through ultraviolet activation in the skin and it is speculated that perhaps oxidized form of cholesterol may mimic some of the molecular actions of vitamin D. It is also known that vitamin D ingested in the diet is carried in the circulation on lipoproteins. Thus, entry of lipoproteins into the artery wall may also facilitate entry and accumulation of the promineralization factor, vitamin D.

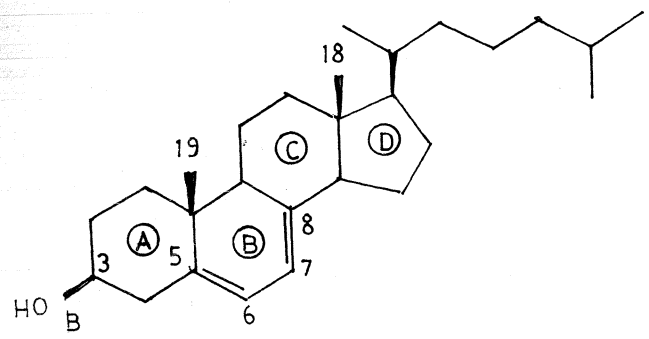
VITAMIN D

The term Vitamin D refers to a group of secosteroids with antirachitic properties possessing in common a conjugated triene system of double bonds, of which cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) are the best known examples.

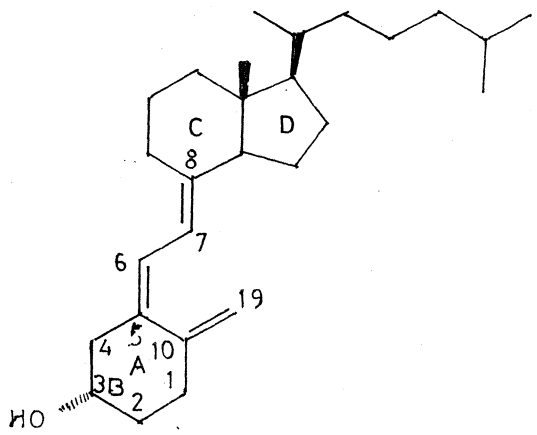
History, Structure and Function

Sir Edward Mellanby in 1919 first identified that vitamin D present in cod liver oil could cure rickets in children. Vitamin D was chemically characterized only in 1932 when Windaus et al.,(1932) determined the structure

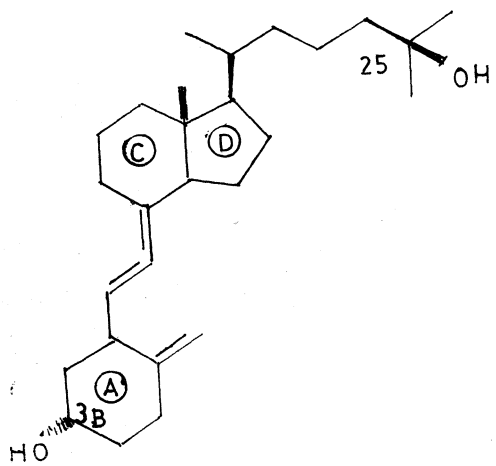
Figure 3
STRUCTURE OF VITAMIN D METABOLITES



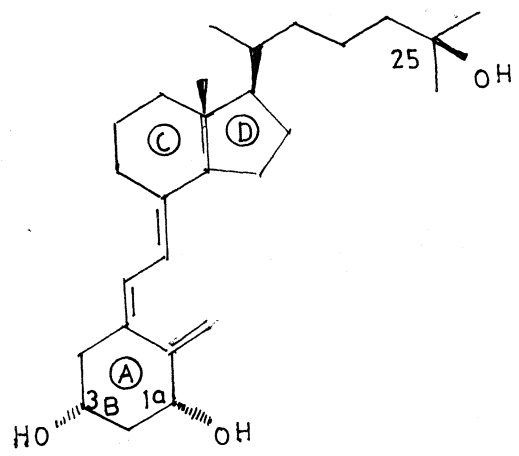
7 - Dehydrocholesterol



Cholecalciferol (vitamin D_3)



25-Hydroxy cholecalciferol



1a,25-Dihydroxy-cholecalciferol

of antirachitic factors that resulted after UV irradiation of 7–dehydrocholesterol. Crowfoot and Dunitz (1948) carried out X-ray crystallographic analysis of the vitamin D molecule. Their results led to the identification of the open ring structure of the vitamin and emphasized the seconature of vitamin D.

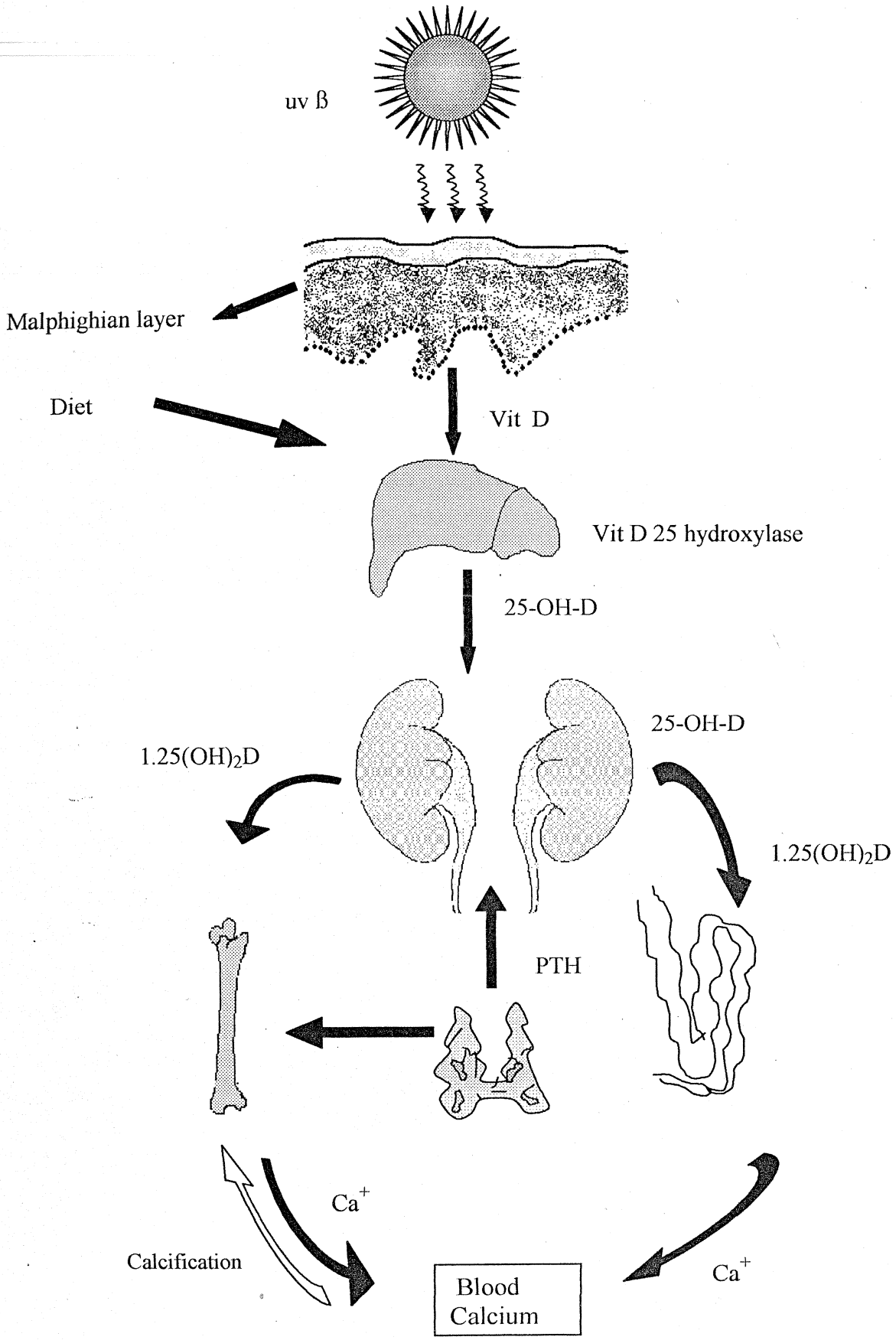
Structure of vitamin D

The chemical structure of vitamin D_3 or cholecalciferol (Figure 3) is closely related to that of its provitamin 7-dehydrocholesterol, produced by photochemical reaction. Structurally it is related to the four-ring nucleus of other steroids. The important role of ultraviolet irradiation in the production of vitamin D was recognized as early as 1924 (Hess AF and Weinstock M 1924 and 1925 ; Hess AF, Weinstock M and Heiman FO 1925). This was the key insight that ultimately permitted Windaus and his collaborators (1932) to carry out the chemical characterization of vitamin D.

Sunlight with wavelength between 290 nm and 315 nm photolyses 7-dehydrocholesterol present in the skin to pro vitamin D_3. Pro vitamin D_3 a thermally labile compound undergoes internal isomerization of its double bond to form the thermodynamically stable vitamin D_3, which is then translocated from the epidermis to the dermal capillary bed. In the skin, malpighian layer contains the maximum concentrations of the vitamin.

Figure 4

METABOLIC PATH WAYS OF VITAMIN -D



Metabolism

The metabolism of vitamin D is shown in figure 4. Vitamin D_3 is synthesized in the skin from 7-dehydrocholesterol in a reaction catalyzed by ultraviolet light (Holick MF 1981). Additionally, vitamin D_3 from dietary sources is taken up into the blood stream from the intestine. Vitamin D_2, which behaves metabolically like vitamin D_3, is provided only by dietary sources. Absorption takes place in jejunum and ileum. The transport of vitamin D metabolites in the blood is achieved mostly through non-covalent binding to vitamin D binding protein. The liver takes up as much as 60-80% of vitamin D. Further processing occurs in the liver which is the major site of hydroxylation of vitamin D_3 at carbon 25 (Ponchon G and DeLuca HF 1969; Olson EB Jr. et al., 1976). Hydroxylation by the enzyme 25-hydroxylase takesplace in the endoplasmic reticulum and requires NADPH, magnesium, molecular O_2 and a cytoplasmic factor (Madhok TC and DeLuca HF 1979). The enzyme requires cytochrome P450. Circulating concentration of 25-OH-D_3 is considered to be a reflection of the vitamin D status of an individual.

The second important site of transformation of vitamin D is the kidney. 25-OH-D_3 is transported on a specific globulin to the kidney, where it undergoes further hydroxylation. The enzyme 25-OH-D_3 hydroxylase introduces a hydroxyl group at the alpha position of carbon 1 of the A ring by the activity of the 1 alpha

Figure 5

Molecular Mechanism of Calcitriol (1,25 dihydroxy Vitamin D3)

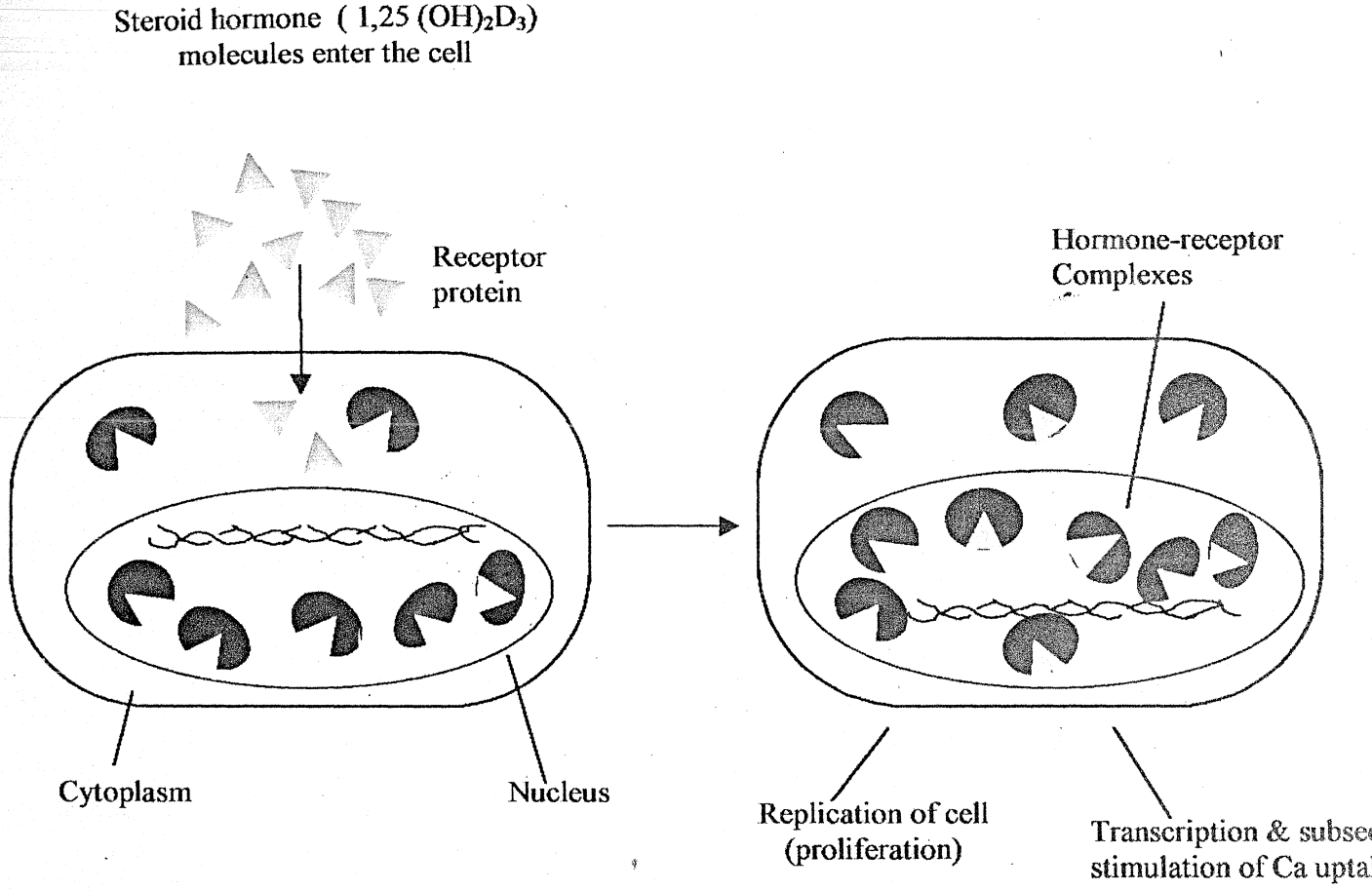
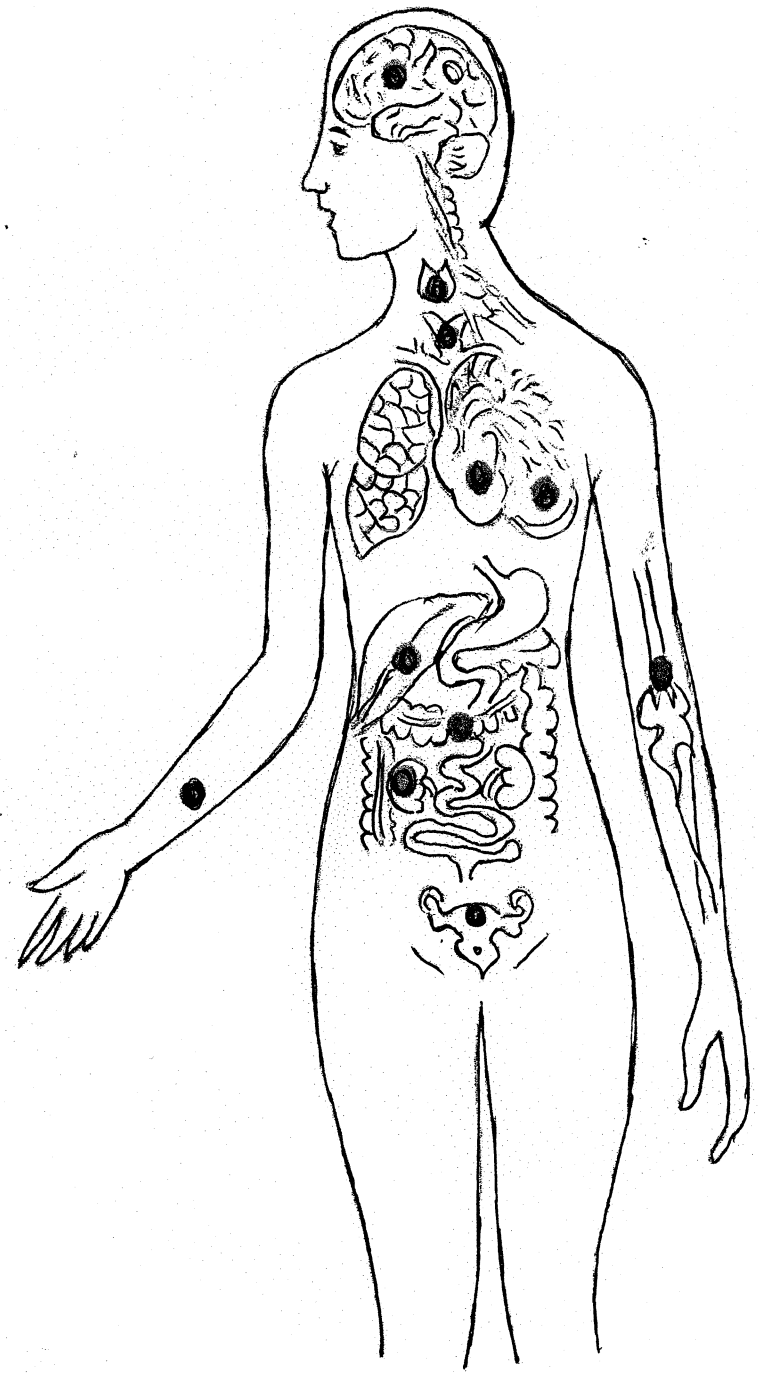


Figure 6
LOCATION OF RECEPTORS FOR $1,25(OH)_2D_3$



hydroxylase which occurs in kidney tissues specifically in the mitochondria. This reaction yields the most active potent metabolite 1 alpha 25-dihydroxyvitamin D_3 ($1,25(OH)_2$ D_3) which enables intestinal transport of calcium and phosphate as well as mobilization of bone calcium (Fraser DR and Kodicek E 1970 ; Holick MF et al., 1971). Since Vitamin D is essential for calcium absorption it may also play a role in the prevention of osteoporosis by ensuring adequate calcium.

Molecular action of 1,25-dihydroxyvitmin D_3

Calcitriol, $1,25(OH)_2D_3$, the most active metabolite of vitamin D because of its lipid solubility and low molecular weight, passes readily through the cell membrane into the interior of the cell, where there are specific receptors to which it attaches. The receptor sterol complex then migrates into the nucleus of the cell, interacts with DNA, and can alter the transcriptional activity of the gene (Alberts B et al., 1983). The molecular action of $1,25(OH)_2$ D_3 in a cell is schematically presented in figure 5.

In the classical target tissue, $1,25(OH)_2D_3$ receptor binding and nuclear association results in transcription of new mRNA species (Emtage JS, Lawson DEM and Kodicek E 1973; Hausler MR and Mc Cain T 1977; Walters MR, Hunziker W and Norman AW 1980; Franceschi RT, Simpson RV and Deluca HF 1981). The induced proteins usually include one or more calcium binding proteins (Wasserman RH and Taylor AW 1960; Siebert P, Hunziker W and

Norman AW 1982; Norman AW, Roth J and Orch L 1982). Recently, receptors have been identified for $1,25(OH)_2D_3$ in a wide variety of tissues including the arterial wall (Koh E, Monmoto S and Fukuo K et al., 1988). Locations of $1,25(OH)_2D_3$ receptors in the human body are shown in figure 6 .

The intriguing possibility that $1,25(OH)_2D_3$ may affect calcium function within cardiac cells is suggested by the discovery of intracellular $1,25(OH)_2D_3$ receptor in the heart (Stumpf WE, Sar M and De Luca HF 1981; Walters MR, Cunea DL and Jamison AP 1983). The presence of these receptors in cardiac muscle may prove to be of clinical significance .

The presence of specific vitamin D receptor (VDR) has been documented in cultured bovine aortic endothelial cells (Merke J et al.,1989). Their data revealed that endothelial cells are a traget site for both $1,25(OH)_2D_3$ production and receptor mediated actions such as increased proliferation and activation of protein kinase. Authors commented that endothelial cells have the molecular machinery to react to $1,25(OH)_2D_3$. Because injury to the endothelial lining is a key facet of the current theory on the pathogenesis of atheroscelrosis, a negative or positive influence of $1,25(OH)_2 D_3$ in the reendothelialization process could be of great significance in determining the incidence of atherosclerotic plaque formation.

Regulation of vitamin D metabolism

The first level of regulation is thought to occur in the skin in the malphigian layer either by the formation of melanin pigmentation or by other sterols such as tachysterols and luministerols (Holick MF et al.,1980). The next level of regulation is in the kidney (Fraser DR 1980; Hentry HL and Norman AW 1984). It seems that kidneys alter the secretion of 1 alpha hydroxylase enzyme in an appropriate manner so as to maintain the calcium level in the circulation. The intra mitochondrial accumulation of both calcium and phosphate depresses the activity of 1 alpha hydroxylase enzyme thereby decreasing the synthesis of $1,25(OH)_2D_3$. Parathyroid hormone (PTH), sensitive to calcium levels in blood, regulates Vitamin D metabolism. $1,25(OH)_2$ D_3 like other steroid hormone regulates its own metabolism through feed back mechanisms (Fraser DR 1980; Hentry HL and Norman AW 1984). In deficiency states the activity of 25-OH-D_3 1 alpha hydroxylase activity is markedly increased. In contrast, with excess in levels there is a marked inhibition of 1 alpha hydroxylase activity and concomitant enhancement of 24-hydroxylase activity. It is likely that $1,25(OH)_2D_3$ may initiate the synthesis of mRNA for the 24-hydroxylase enzyme.

METABOLIC IMBALANCE

Vitamin D is good in right dose and dangerous in over dose.

Deficiency states

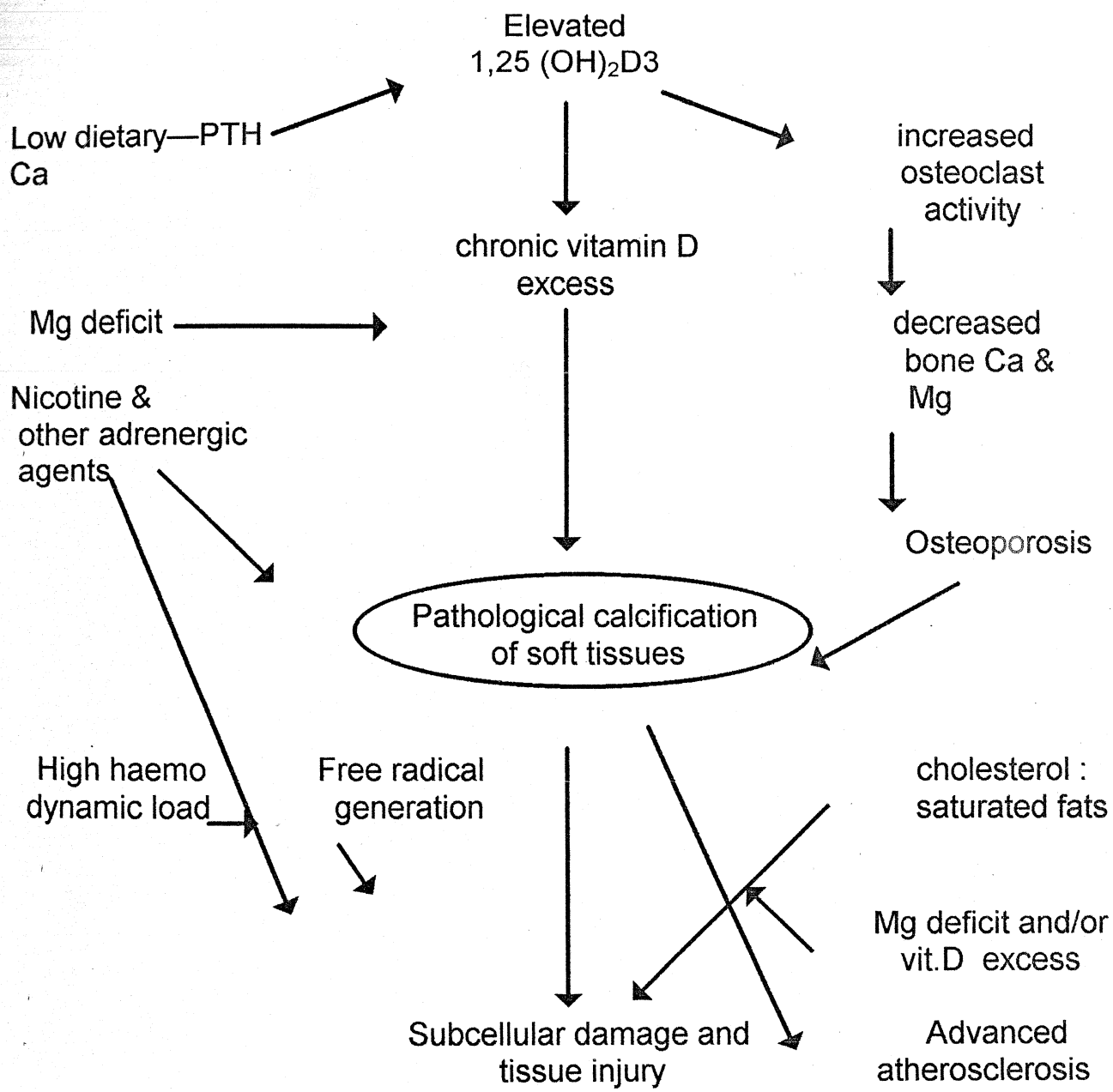
Many biochemical and physiological imbalances occur in the deficient state which if not corrected result in rickets in growing children and osteomalacia in adults. Rickets occur when the newly synthesized organic matrix fails to mineralize resulting in soft bones. Biochemical changes include low plasma levels of calcium and inorganic phosphate with concomitant high plasma levels of alkaline phosphatase. In early deficiency a deficit in absorption of calcium occurs. This often leads to secondary hyperparathyroidism in which PTH is secreted in response to low plasma calcium concentration.

Toxicity, its manifestation and mechanisms

Like other fat-soluble vitamins, an overdose of vitamin D results in systemic toxicity, largely because this class of secosteroid is stored in adipose tissue and is slowly released into the circulation. Vitamin D ingested in excess results in hypercalcemia, which is caused by excessive absorption of calcium by the intestine and enhanced bone resorption. The symptoms of this intoxication include polydypsia, polyuria, irritability, lassitude, poor weight gain,

Figure 7

Deleterious effect of vitamin D excess in cellular and metabolic pathways.



diarrhoea etc. Biochemical features are hypercalcemia and elevated levels of serum creatinine (Seelig MS 1969). In hypercalcemia, vascular smooth muscle may contract abnormally leading to hypertension. It also leads to calcium phosphate precipitation within renal tubule and to the formation of stone in the lower urinary tract, calcification of the media of the artery, myocardium etc. Chronic hypervitaminosis D results in an arterial lesion that includes both degeneration of SMCs and coronary arterial abnormality. Though $1,25(OH)_2$ D_3 is thought to be the active metabolite for calcium enhancement, it is also presumed that hypercalcemia results from a pharmacological action of 25-OH-D_3 on intestinal vitamin D receptor.

Clinical and physiological manifestations of vitamin D toxicity in laboratory animals, especially the rabbit (Hass GM et al., 1958), rat (Bajwa GS, Morrison LM and Ershoff BH 1971) or monkey (Peng SK et al., 1979) are similar to those seen in humans overdosed with this vitamin. The major effect is hypercalcemia, with extensive calcification in extraskeletal tissues, particularly blood vessels and the kidneys. The nature of the toxic metabolite in hypervitaminosis D is still a controversy. Many workers opine that vitamin D itself in normal doses lacks biological activity, and that the syntheses of 25-OH-D_3 and $1,25(OH)_2$ D_3 are carefully regulated.

Vitamin D is transported on a specific plasma binding globulin, while upto 40% is transported by lipoproteins. During hypervitaminosis D these binding sites

may become saturated with an increase in the free pool of vitamin D allowing it to gain access to cell membranes.

Deleterious effects of 1,25-dihydroxy vitamin D excess.

Deleterious effects of 1,25-dihydroxyvitamin D excess is schematically presented in figure 7.

Vitamin D may get incorporated into cellular membranes and alter the membrane permeability to calcium. An increased calcium influx into cells would increase calcium sequestration by mitochondria and the endoplasmic reticulum resulting in the formation of calcium deposits and damage to the organelles (Trump BJ Berezesky IK and Osornio-Vargas AR 1981). An elevation in cytplasmic calcium levels may perturb many biochemical reactions regulated by calcium. The altered metabolism and organelle damage may result in cell necrosis and formation of large calcium deposits, features commonly observed in hypervitaminosis D (Holmes RP and Kummerow FA 1983).

POSSIBLE ROLE OF VITAMIN D TOXICITY IN THE PATHOGENESIS OF CARDIOVASCULAR DISEASES.

Clinical and epidemiological observations

Several lines of evidences point to a possible relationship between vitamin D toxicity and cardiovascular diseases.

Moon, Brandy and Davison in 1992 drew attention to the epidemic of ischemic heart disease (IHD) in North America which directly paralleled with the increased use of vitamin D.

In 1917 the curative effect of cod liver oil in rickets was documented. Six years later, the import of fish liver oil in the United States of America (USA) rose from 500,000 gallons to 2.5 million gallons. Next year onwards the use of UV - irradiated milk was introduced in the USA. During the years 1948 to 1972 the manufacture of vitamin D rose from 35 lb to 14,000 lb. During the latter part of this period, vitamin D_2 was added to baked foods, breakfast cereals, milk, poultry products, baby foods and non-alcoholic beverages. Vitamin D tablets containing 200 IU were commonly sold in drug stores at that time. Along with the excessive delivery of vitamin D, there was also a continually declining intake of magnesium thanks to the use of magnesium deficient artificial fertilizers and many processing procedures that decreased magnesium content in foods. Moon, Brandy and Davison (1992) concluded that the epidemic of IHD in North America directly paralleled both the increasing use of vitamin D and the decreasing availability of dietary magnesium.

Infantile hypercalcemia is associated with markedly elevated blood levels of vitamin D, elevated blood cholesterol and susceptibility to coronary artery disease (CAD). Excessive fortification of milk with vitamin D in England during the years 1952-1957 correlated with high incidence of idiopathic hypercalcemia

of infancy (Lightwood R et al., 1956 ; Lightwood R and Stapleton T 1957). The disease was also common in Germany where supplementation with high dosage of vitamin D was common.

In 1960 Food Drug Act considered legislation to limit the amounts of vitamin D delivered to the USA. In 1972 the regulation went into effect and the maximum permissible level of vitamin D in foodstuffs was fixed as 400 IU.

From 1970 onwards death rate from IHD in the USA began to decline. Moon, Brandy and Davison (1992) observed that a decreasing incidence in death from IHD in North America coincided with decreased delivery of vitamin D.

Epidemiological studies have correlated moderately high vitamin D intake with the pathogenesis of cardiovascular diseases. Linden (1974) examined in Tromso, Norway the data of patients with myocardial infarction and found a correlation between moderately high intake of vitamin D with increased incidence of myocardial infarction, renal calcinosis and urolithiasis. He concluded that, where intake of natural foods rich in vitamin D is common, there is increased incidence of hypercholesterolemia and susceptibility to sudden death from IHD. Arteriotoxicity of vitamin D has been known for many years. Linden emphasized the involvement of vitamin D in coronary disease and suspected that the known hypercholesterolemic effect of vitamin D might be the mechanism. A causal link between atherosclerosis and vitamin D was proposed

also because of the association between osteoporosis and arterial calcification (Moon J, Brandy B and Davidon AJ 1992). Vitamin D, possibly removes calcium from the bones and puts it into the arteries.

Taussig (1966) commented that the inborn variation in a man's ability to metabolize vitamin D may be responsible for the valvular changes seen in aortic stenotic lesions in selected group of children. The increase in blood cholesterol often seen in hypervitaminosis D further suggests that such variation in susceptibility to vitamin D may possibly be related to vascular lesions associated with abnormal cholesterol metabolism .

Friedman and Roberts (1966) suggested that excess vitamin D during gestation may be responsible for the syndrome of craniofacial abnormality, supravalvular aortic stenosis and infantile hypercalcemia.

Excess vitamin D can also lead to magnesium deficiency. Magnesium deficiency has been implicated in damage to the cardiovascular system, skeletal tissues and kidneys. Seelig (1969) proposed a causal relationship among vitamin D excess, magnesium deficiency and lesions of the cardiovascular system and kidneys. According to Seelig there are possible variations in the range of individual reactivity to vitamin D. Those who are hypersensitive to vitamin D could be highly susceptible to toxicity even with moderately high intake of the vitamin. Combined with magnesium inadequacy in infancy, it may have a role in the genesis of early lesions of cardiovascular diseases.

Davies H (1989) in a provocative article has focussed on the early events in atherosclerosis that occur in coronary arteries of transplanted hearts. Coronary arterial intima following injury, undergoes a sequence of changes before the appearance of lipids. The first stage is intimal hyperplasia and disruption of the internal elastic laminae. Then the medial smooth muscle cells migrate into the intima. Lipid incursion occurs later. The lesion seen in transplant arteriopathy is indistinguishable morphologically from that which is found in classical atherosclerosis. The early intimal and medial changes may be immune engendered in the arteries of transplanted hearts. Davies argues that a search for the cause of atherosclerotic diseases should focus on the prelipid stages. The lipids are possibly complicating rather than causative factors. Mitogens such as vitamin D may also catalyse the aberration of the normal mechanisms controlling cellular proliferation (Davies H 1989)

There are several studies, which contradict the view that vitamin D is a causative factor for atherosclerotic heart disease. Schmidt et al. (1977) observed in his study that there is no difference in serum 25-OH-D_3 levels between patients with myocardial infarction (MI) and controls. Scragg and co-workers (1990) found an inverse association between plasma 25-hydroxyvitamin D_3 and myocardial myocardial infarction in a community based case-control study. One hundred and seventy nine patients with MI who presented to hospital within 12 hours of the onset of symptoms were individually matched with controls

in their analysis. Patients with MI were found to have significantly lower mean 25-OH-D_3 levels than controls. Watson et al., (1997) reported an inverse correlation of 1,25$(OH)_2D_3$ with the extent of vascular calcification in patients with coronary artery disease. On the other hand Arad et al., (1998) did not find any correlation between serum levels of calcium 1,25$(OH)_2D_3$ or PTH to coronary calcification or to the extent of coronary stenosis.

Experimental evidences

There are experimental data that indicate a causative role for vitamin D in atherosclerosis. Several studies have been conducted in animals by oral feeding or intravenous injection of vitamin D metabolites and observing the pathological changes in the cardiovascular system. Dehanger and Donath showed that vitamin D significantly alters the formation of atheroma in rabbits. High doses of vitamin D changes the concentration of both cholesterol and total lipids in blood and induces hyperphosphatemia, factors which favour the formation of atheroma.

Investigations in rats by Einsensten and Groff (1957) indicate effects similar to those observed in rabbits. Massive doses produce a marked increase in serum calcium and seromucoid levels. There is increased calcareous deposition in the heart, kidneys and blood vessels together with accumulation of polysaccharides at those sites. The calcium deposits appear to occur in a matrix containing acid mucopolysaccharides.

According to Einsensten and Groff (1957) the rise in the level of serum calcium with an increase in serum seromucoid and deposition of calcium in tissues suggest that administration of massive doses of vitamin D results in release of bone minerals and organic matrix into the circulation with subsequent deposition in soft tissues.

Hass et al., (1958) induced in rabbits medial calcification with secondary mesenchymal reactions in aorta by feeding or injection of high doses of vitamin D for 4 weeks. Friedman and Roberts (1966) demonstrated transplacental passage of excess of vitamin D in pregnant rabbits. At birth the offsprings had significantly higher serum calcium levels than controls. Aortic valve abnormalities similar to supravalvular aortic stenosis in humans were noted in the offsprings of rabbits given high doses of vitamin D.

Extensive atherosclerotic lesions of the aorta and coronary artery were seen by Bajwa, Morrison and Ershoff (1971) in 100% of the rats on vitamin D supplemented diet after 6 weeks. Lesions were mostly in the media and consisted of degeneration, calcification, plaque formation, metachromatosis and lysis and fragmentation of the elastic lamina. Some of the surviving animals exhibited subintimal mesenchymal cell proliferation. In several animals there was rupture of the medial plaque through the intima. Intimal calcification extended into the entire wall of the artery.

Toda , Leszcznski and Kummerow (1983) used swine and rabbits to study the possible role of vitamin D_3 in the initiation and development of atherosclerosis. In swine, sequential aortic changes were examined with the aid of electron microscopy. In rabbits metabolic consequences of hypervitaminosis D were observed. Fibromuscular intimal thickening was seen in the ascending and thoracic aorta of the swine fed 62,500 IU of vitamin D_3 / kg of diet for three months and 3 months after vitamin D_3 withdrawal, atherosclerotic lesions were found. In rabbits, pronounced aortic smooth muscle cell necrosis developed after forced feeding of 10,000 IU vitamin D_3 daily. Serum analysis indicated that blood calcium did not differ from that of animals fed corn oil alone, but the level of serum 25-OH-D_3 measured by high pressure liquid chromatography (HPLC) was 30 times more than that in the control animals. These data suggest that cholecalciferol (the oxidized sterol with vitamin D_3 activity) affects the integrity of the arterial wall.

The effect of moderate magnesium deficiency on coronary arteries of swine fed various levels of vitamin D_3 has been investigated by Ito, Cho, and Kummerow (1990) using light and electron microscopy. Subnormal magnesium intake appears to intensify vitamin D_3 induced intimal lesions in arteries. The degree of cell degeneration and intimal thickening which was induced by high vitamin D intake was as great in swine whose diet was low in magnesium and

moderately high in vitamin D as it was in those on twice as much vitamin D. Also, the degree of arterial calcification was intensified by inadequate magnesium intake at the two higher vitamin D intakes. Suboptimal dietary magnesium in combination with an excess of vitamin D, seems to have an additive effect in the initiation of ultrastructural changes in the coronary arteries. The number of foam cells iincreases with increasing serum levels of vitamin D_3.

1,25$(OH)_2$ D_3 receptor and hypervitaminosis D

An event resulting in an upregulation or increased density of vitamin D receptor (VDR) in the target cells is likely to result in hyper responsiveness to 1,25$(OH)_2D_3$. Hughes et al. (1976) have suggested that in vitamin D toxicity, target tissues are responding to high plasma concentration of 25-OH-D_3 . When concentration is high, 25-OH-D_3 can bind to VDR and mimic 1,25$(OH)_2D_3$ to stimulate bone calcium resorption, and to increase intestinal calcium transport. Other functions attributable to 1,25$(OH)_2D_3$ are also possibly affected. In chronic hypervitaminosis D, 1,25$(OH)_2D_3$ concentration is relatively unaffected, but plasma calcium is elevated and toxicity may occur. Smooth muscle cell (SMC) necrosis can be caused by enhanced membrane permeability to calcium following 25-hydroxyvitamin D_3 incorporation into SMC membranes.

Beckman and coworkers (1990) have demonstrated that VDR up regulation occurs in vitamin D excess. They showed that administration of high amounts of vitamin D_3 to rats resulted in hypercalcemia, increase in plasma 25-OH-D_3 concentration and significant upregulation of intestinal VDR concentration.

Greater target tissue VDR concentration is associated with increased responsiveness to biological stimuli. The observed increase in intestinal VDR could lead to enhanced responsiveness of intestinal cells to vitamin D metabolites. VDR upregulation may therefore be an unrecognized significant pathological factor involved in the manifestation of hypervitaminosis D.

Excess concentrations of 25-OH-D_3 and its metabolites in target tissue may inhibit completely the normal path of 1,25$(OH)_2$ D_3 degradation by saturating the degradating enzyme system. As a result of enzyme inhibition the half-life of 1,25$(OH)_2D_3$ would be prolonged leading to increased VDR occupancy and VDR synthesis. VDR upregulation may play a significant role by enhancing target cell responsiveness in the pathogenesis of hypervitaminosis D (Beckman MJ et al., 1990). It is unclear whether the increased tissue responsiveness associated with vitamin D toxicity results from accumulation of 1,25$(OH)_2$ D_3 metabolite or 25-OH-D_3 and its metabolites, in target tissues. A detailed examination of VDR occupancy in vitamin D toxicity might provide some insight. Koh et al., (1988) have demonstrated not only the presence of 1,25 $(OH)_2$ D_3 receptor in rat

coronary artery smooth muscle cells but also dose dependent enhancement of proliferation and change in shape of the SMCs when exposed to $1,25(OH)_2D_3$. Tsutomu and Hiroyuki (1988) have examined using cultured vascular smooth muscle cells derived from rat aorta, whether $1,25(OH)_2 D_3$ directly affects cellular calcium uptake. He demonstrated that $1,25(OH)_2D_3$ stimulates calcium uptake in vascular smooth muscle cells. The effect is dose dependent at physiological concentrations and specific for the sterol. Their results suggest that stimulation of calcium uptake by $1,25(OH)_2 D_3$ is mediated through binding of the vitamin to its receptor. A similar effect has been reported in primary cultures of aortic myocytes isolated from spontaneously hypertensive rats (Bukoski RD, Xue H and Mc Carron DA 1987). Imai et al., (1970) suggested that certain oxidized lipids are the possible cause for arterial SMC necrosis. Oxidized lipids include both oxidized polyunsaturated fattyacids and sterols. Cholecalciferol can also be regarded as one type of oxidized sterol derived from cholesterol.

RELEVANCE OF VITAMIN D TOXICITY IN A TROPICAL ENVIRONMENT

It is accepted that the major source of vitamin D is skin exposed to the UV B rays contained in sunlight. Given the global differences in UV radiation and in dietary sources of vitamin D, disparities in vitamin D status can be expected among populations of different regions of the world. One undeniable feature of

the equatorial region is that there is much sunlight and the intensity of UV radiation is high and continuous. Exposure of skin for 5-8 hours spread over 11 weeks can raise vitamin D levels in serum from 10 ng/ ml to 100 ng / ml (Davie M and Lawson DEM 1980). Repeated total body exposures causing mild erythema raise plasma 25-OH-D_3 concentration as much as long term ingestion of 10,000 IU of vitamin D per day does (Holick MF1992). Average circulating levels of 25-OH-D_3 is 64 ng / ml in life guards with a minimum of four weeks of prolonged exposure to sunlight (Haddad JG and Chyu KJ 1971). Exposure to tropical sunlight during a vacation of one week raises plasma 25-OH-D_3 levels by 25 ng /ml in foreign tourists to tropical countries.

There are also other possible sources of vitamin D in the tropics. Vitamin D status of cow's milk depends on exposure to sun. In winter the vitamin D levels in cow's milk are reported to be 4 IU / quarts where as in summer it is 40 IU / quarts.

Plants too are good sources of vitamin D. *Cestrum diurnum* and *Solanum malacoxylon* have calcinogenic properties (Boland RL 1986; Wasserman RH et al., 1975) and cattle feeding on these plants are at risk of vitamin D toxicity i.e., development of calcinotic disease (Wasserman RH and Nobel TA 1980; Kwatra MS et al., 1974). Vitamin D activity in *Cestrum diurnum* naturalized at Hyderabad was found to be 3 times higher than the activity reported from the West (Prema

TP and Raghuramulu N 1993). Authors comment that the difference is because of the equatorial climate. Drying foodstuffs in sunlight make them rich in vitamin D. Provitamin D_3 is widely distributed among phanerogams and cryptogams. *Alfalfa*s exposed to UV light are good sources for vitamin D_2 and D_3. Planktons in tropical environment (collected from Hussain Sagar Lake, Hyderabad) are rich in vitamin D and the vitamin D levels are high when compared to western data. (Sunita R and Reghuramulu N 1994). Fish liver contains high concentration of vitamin D. The levels differ according to geographical climate.

There are no documented cases of vitamin D intoxication because of excessive exposure to sunlight. Instances of toxicity from diet because of excess fortification have been reported. Jacobus et al., (1992) reported occurrence of eight cases of vitamin D intoxication that appear to have been caused by intake of milk excessively fortified with vitamin D.

The present study was initiated to explore the possible relationship between hypervitaminosis D and arterial calcification as well as the relationship between vitamin D status of an individual and the risk for atherosclerotic heart disease.

METHODS AVAILABLE FOR ESTIMATING VITAMIN D LEVELS

There are several methods described for the assay of vitamin D.

1. Biological determination

a. Official assays

1. Rat Line Test : Mc Collumn et al., (1922) described the rat line test. The term official in reference to the assay indicates that the reproducibility and accuracy of the assay when conducted according to the procedures described by the U.S. Pharmacopoeia or the Association of official agricultural chemists is such that the results can be accepted legally (AOAC 1960).

The line test for vitamin D is conducted in recently weaned, rachitic rats. Weanling rats are fed the classic rachitogenic ration for 19-25 days. After the development of florid rickets, a 7 day assay period ensues. During this time 9 groups of 7 to 10 animals receive in their diet either graded series of known amounts of cholecalciferol or the unknown test sample. One week is adequate to demonstrate the "curative effect" of dietary vitamin D. At this time animals are killed, the radii and ulnae are dissected, cleaned of adhering muscle and fat tissue, sliced longitudinally and placed in a solution of silver nitrate. The basis of the assay is the deposition of silver in areas of bone where new calcium has recently been deposited. These regions turn black upon exposure to light. The black line across the cartilage plate of unknown samples is visually scored in

relation to the intensity or width of lines generated by the vitamin D_3 standards. The line test assay is capable of detecting 1-12 I.U of vitamin D.

2. AOAC chick test

Principle :- The Association of Official Agricultural Chemists gives an official description of the AOAC chick assay (AOAC 1960). The principal use of AOAC chick test is to assess quantities of vitamin D_3 as distinct from that of D_2 and it is vital for the accurate determination of the cholecalciferol levels of poultry feeds.

Method - The assay is conducted by placing groups of 20 newly hatched chicks in the specified vitamin D deficient diet containing added levels of D_3 or test substance. After 3 weeks of feeding, the percent bone ash of dried lipid free tibias is determined. A rachitic bird typically has 25-27 % bone ash, whereas a vitamin D supplemented bird has 40-45% bone ash. The disadvantage is that the assay is of 3 weeks duration and involves processing of the bones and hence time consuming. The assay is also expensive and is not frequently employed.

B. Other assays

1. Stimulation of intestinal calcium absorption.

The most extensively studied action of vitamin D is its potent ability to effect the stimulation of calcium absorption across the small intestine. Two basic types of assays are available; those employing techniques *in vivo* and others employing manipulation of the intestine *in vitro*.

There is a time lag of approximately 36 to 48 hours prior to the development of maximal response to vitamin D. The test dose or standard dose of vitamin D is administered to the animal 24-48 hour prior to assessment of calcium transporting ability of the small intestine.

The technique *in vivo* for quantitating calcium absorption in the chick involves utilization of rachitic chicks that have been raised on a test diet containing low levels of available calcium (Hibberd KA and Noman AW 1969). In the event that a simultaneous assay *in vivo* of mobilization of bone calcium is to be carried out, the birds 3 days before the assay must be switched to a rachitogenic diet-containing zero added calcium.

The chick then receives one dose of the test compound in a vehicle. Twenty-four or 48 hours later (more appropriate for vitamin D), the chicks are anesthetized and a small incision is made on the abdomen of each bird. The duodenal loop is lifted out, injected with 0.2 ml of a solution containing 4 mg

$^{45}Ca^{2+}$ as the chloride and approximately 6 x 10^{6} dpm $^{45}Ca^{2+}$.The loop is then returned to the peritoneal cavity and the opening closed with a wound clip. Thirty minutes after receiving the ^{45} Ca, the chicks are killed by decapitation and blood is collected. Determination of ^{45} Ca is conducted in a liquid scintillation counter. Total serum calcium concentration (for bone calcium mobilization evaluation) can be determined by atomic absorption spectrometry.

It is also possible to carry out the assessment of vitamin D activity in terms of stimulation of intestinal calcium transport by using preparations of intestinal loops that have been everted and incubated with solutions of radioactive calcium under *in vitro* conditions. This technique has been employed largely in studies in mammals rather than birds. A vitamin D standard or test compound is given either orally or intraperitoneally 24- 48 hours in advance of the assay. At the time of assay the animals are killed, upper small intestine is dissected free, and a 10 cm length of duodenum is removed from the animal and turned inside out. The everted intestine is then washed in ice-cold Kerb's Ringer solution, and a gut sac with the mucosal surface on the outside and the serosal surface on the inside is prepared from each segment by taping off with suture, each end of the intestinal segment. The inside of the sac is filled with 0.4-0.6 ml of the standard medium of Kreb's Ringer- bicarbonate containing radioactive ^{45} Ca. These sacs are then placed in an incubation flask containing 2-5ml of the standard

incubation medium that contains the radioactive calcium. The gas phase is normally replaced with 95% O_2 , 5% Co_2, and the flask is incubated for a standard period of time, usually 2-3 hours. At the end of the incubation, equal aliquots are taken from the inside (serosal) and outside (mucosal) media and are transferred to liquid scintillation vials for determination of radioactivity content. The data are expressed as a ratio of concentration of ^{45} Ca in counts per minute/ milliliter medium i.e., inside/ outside. The assay is capable of detecting 10-100 IU (25-250 ng) of vitamin D_3.

In comparison to the chick *in vivo* assay, this technique suffers from the fact that it is considerably more time consuming because of the tedious nature of preparing the everted intestinal sacs and the duration of the incubation period.

2. Mobilization of bone calcium

It is also possible to simultaneously determine the vitamin D mediated elevation of serum calcium if the chicks employed in the assay have been raised on a diet devoid of calcium for a period at least 3 days prior to the time of the assay. When a compound with vitamin D activity is administered there is a highly characteristic elevation of serum calcium, which is proportionate to the dose of the steroid administered. Since there is no dietary calcium available under the conditions of

the assay, the only source of calcium for elevation of serum calcium during 24-48 hour test period is bone. The serum calcium level of a rachitic 24 day old chick is usually 45-50 mg per 100 ml of serum. Within 24-48 hours after administration of 5 - 500 IU of vitamin D_3, the serum calcium levels can rise by 4-5 mg per 100 ml of serum and up to values of 9.5-10 mg Ca per 100 ml.

The advantage is that the assay measures different aspects of the animal's response to vitamin D. If the intestinal absorption of ^{45}Ca has been conducted simultaneously, only a negligible portion of the 4 mg of Ca placed in the duodenum is transferred to the blood in the 30 minute test period (Hibberd KA and Norman AW 1969).

3. Stimulation of growth

It is possible to develop an assay for vitamin D activity in the chick utilizing the growth promoting properties of the steroid. One day old hatching chicks are placed on a rachitogenic diet and given standard amounts of vitamin D_3 or the test compounds three times a week. Groups of birds are weighed periodically and their weights are plotted versus age. In the absence of vitamin D, the rate of growth essentially plateaus by the fourth week, but it can be seen that 5-10 IU per day or 50 IU of vitamin D_3 per week is sufficient to maintain a maximal growth rate of the chick (Hibberd KA and Norman AW 1969).

The disadvantage is that a 21day period is required for precise determination of the rate of growth. However, the assay has a sensitivity comparable to that of the rat line test or the 21 day prophylactic assay of vitamin D activity in the chick.

4. Immunoassay for calcium binding protein

The vitamin D dependent calcium binding protein (CaBP) is not present in the intestinal mucosa of vitamin D deficient chicks and is synthesized only in response to the administration of a compound containing vitamin D activity. It is possible to use the presence of CaBP as an end point in a vitamin D bioassay. The assay is highly sensitive having a linear range of 1-50 ug of CaBP, which results from the administration of 1-200 IU of vitamin D_3. A principal advantage of the immunoassay for calcium binding protein is that in the absence of vitamin D there is a zero baseline, i.e., no calcium binding protein is present (Christakos S and Norman AW 1979).

5. Chemical determination

Vitamin D has a highly characteristic ultraviolet absorption spectra. The initial techniques available for quantitating vitamin D were based on the measurement of the ultraviolet absorption at 264 nm. At this wavelength the molar extinction coefficient of vitamin D_2 and D_3 is 18,200. Thus a solution with 2.1 ug /ml of vitamin D will have an absorbency of 0.10 at 264 nm in a cuvette with 1.0 cm light path.

The disadvantage of the method is that the sample must be scrupulously purified prior to the assay to remove potential UV absorbing contaminants.

6. Colorimetric method

This method is based upon the measurement of the salmon-pink color that develops when vitamin D interacts with antimony trichloride ($SbCl_3$) in ethylene dichloride. The absorbency of the pink solution at 500 nm is proportional to the concentration of vitamin D. Prior to color development and depending on the sample matrix to be analyzed, vitamin D needs to be purified from interfering substances.

The principal disadvantage of the procedure is obviously the laborious multistep nature of the assay and the necessity for careful timing of the reaction, because the time required for the appearance of the maximal intensity of color is very short (AOAC 1975).

7. Gas chromatography

Gas chromatography should have strong potential for the development of assays capable of detecting physiological quantities of vitamin D. Because of problems concerning isomerization of vitamin D compounds that occurs when the sample is vaporized for incubation into the gas chromatography column,

these procedures are however, yet to be widely adopted for the analytical determination of vitamin D. Gas chromatography separation processes inherently have an exceedingly high resolving capability thanks to the high number of theoretical plates present in a typical column. Value of gas chromatography to an analytical determination of vitamin D however cannot be assessed simply on the basis of resolving power (Sklan D, Budowski P and Katz M 1973).

8. High pressure liquid chromatography

Liquid chromatography is a separation method in which a mixture of components is resolved into its constituent parts by passage through a chromatographic column. It is carried out by passing the mobile phase containing the mixture of the components through the stationary phase, which consists of a column packed with solid particles. Physical and chemical forces acting between the solutes and the two phases are responsible for the retention of the solutes on the chromatographic column. It is the difference in the magnitude of these forces that determines the resolution and hence separation of the individual solutes.

Alternatively, the separation may be considered to be determined by the distribution of the solutes between the two phases.

In HPLC, element from the solvent reservoir is filtered, pressurized and pumped through the chromatographic column. A mixture of solutes injected at the top of the column is separated into components on travelling down the column and the individual solutes are monitored by the UV detector and peak recorded on a chart recorder (Eisman JA, Shepard RM and DeLuca HF 1977).

9. Competitive protein binding radio ligand assay.

The assay is essentially an equilibrium saturation analysis reaction which involves binding of labeled ^{3}H 25-OH-D_3 with vitamin D binding protein and competition of standard unlabeled 25-OH-D_3 for the same limited number of binding sites on the binding protein resulting in a decrease in the amount of labeled 25-OH-D_3 bound as a function of increasing concentration of standard unlabeled 25-OH-D_3. The free and bound moieties are separated by addition of dextran coated charcoal which sequesters only the unbound 25-OH-D_3. There are several advantages for this method. Many samples can be estimated at a time, sample volume required for the assay is comparatively less and extraction and preparation of the sample is very easy. The assay is more specific for each metabolite. (Haddad JG and Chyu KJ 1971; Chen TC, Adrian K T and Holick MF 1990).

CHAPTER III
MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The study has two parts. Part A comprises of a case–control study in patients with ischemic heart disease and Part B consists of experimental studies in animals.

PART A - CASE CONTROL STUDY

1. Design

Study population consisted of 143 men with coronary artery disease (cases) and 70 male control subjects.

Cases: Fifty five of the patients were those admitted to Medical College, Trivandrum with chest pain and diagnosed to have acute myocardial infarction based on electrocardiographic criteria and typical changes in serum enzyme levels. Eighty eight cases were patients with coronary artery disease admitted at Sree Chitra Tirunal (SCT) Institute for Medical Sciences and Technology, Trivandrum for evaluation by coronary angiography and diagnosed to have

coronary artery disease. Presence or absence of coronary artery calcification was determined by fluoroscopy.

Controls: Seventy age group matched men registered at the SCT institute with neurological problems or chest diseases were selected as control subjects. Individuals with a diagnosis of any malignant disease, granulomatous disease, renal failure or hyperparathyroidism were not included. Those patients who were taking vitamin D supplements or drugs such as phenobarbitone, known to affect serum vitamin D levels, were also excluded.

Risk factor evaluation

Family history of coronary heart disease was considered pertinent if a first degree relative had suffered a myocardial infarction or had died suddenly before the age of 65 years. Smoking history was assessed by asking each subjects about their smoking habits during the previous year. Diabetes or hypertension was considered present if a subject had received dietary or medical therapy or both for these disorders.

coronary artery disease. Presence or absence of coronary artery calcification was determined by fluoroscopy.

Controls: Seventy age group matched men registered at the SCT institute with neurological problems or chest diseases were selected as control subjects. Individuals with a diagnosis of any malignant disease, granulomatous disease, renal failure or hyperparathyroidism were not included. Those patients who were taking vitamin D supplements or drugs such as phenobarbitone, known to affect serum vitamin D levels, were also excluded.

Risk factor evaluation

Family history of coronary heart disease was considered pertinent if a first degree relative had suffered a myocardial infarction or had died suddenly before the age of 65 years. Smoking history was assessed by asking each subjects about their smoking habits during the previous year. Diabetes or hypertension was considered present if a subject had received dietary or medical therapy or both for these disorders.

BIOCHEMICAL INVESTIGATIONS

a. Collection of serum

Fasting blood samples, about 8 ml, from both cases and controls was collected in a sterile glass test tube and incubated at 37^0 C for 2 hours. Following clot retraction, samples were centrifuged at 2000 rpm for 15 minutes. Serum was separated and stored at – 20^0 C till analysis.

b. Determination of serum levels of 25-hydroxyvitamin D_3 by competitive protein binding radio ligand assay

Protein binding radio ligand assay described by Chen et al., (1990) was employed for determining the serum 25-hydroxyvitamin D_3 levels.

b.1. Principle

The assay is essentially an equilibrium saturation analysis reaction which involves binding of labeled (^{3} H) 25-OH-D_3 with vitamin D binding protein and competition of standard unlabeled 25-OH-D_3 for the same limited number of binding sites on the binding protein resulting in a decrease in the amount of labeled 25-OH-D_3 bound as a function of increasing concentration of standard

unlabeled 25-OH-D_3 bound. The free and bound moieties are separated by addition of dextran coated charcoal which sequesters only the unbound 25-OH-D_3.

b.ii. Preparation of reference 25-OH-D_3 standard

Crystalline 25-hydroxycholecalciferol (25-OH-D_3) was obtained as a gift from M. Uskokovic, Hoffman La Roche, USA. Its purity was checked by UV absorption spectrophotometry. About 5 μg was dissolved in 2 ml of absolute ethanol and absorption spectra from 220 nm to 300 nm were taken using a double beam Shimadzu Spectrophotometer (Figure 8). The concentration of standard was calculated from the absorbance at 265 nm. The concentration of standard was calculated using the equation given below and it confirmed the purity of 25 hydroxycholecalciferol used.

A = ECL where A = Absorbance C = concentration

E = Molar extinction coefficient , L =Path length

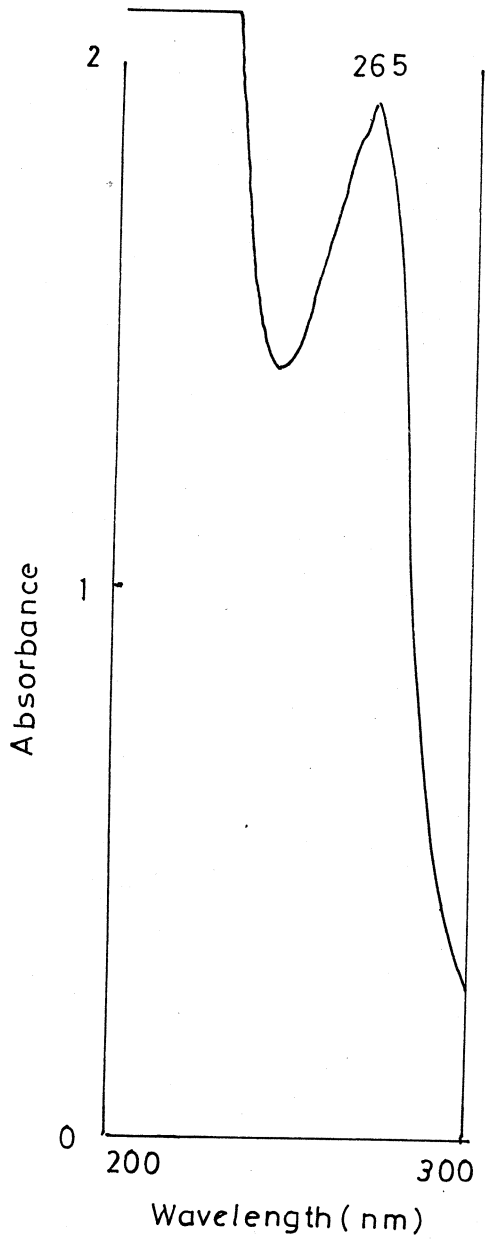
C = A/E = Mol / L

Concentration = OD at 265 nm / 18200 = X gm Mol / L

$$\text{Concentration in (ug/ml)} = \frac{\text{X gm Mol}}{\text{L}} \times 400 \text{ (Mol weight of 25-OH-}D_3\text{)}$$

Figure 8

Absorption specrum of 25-hydroxyvitamin D_3



An aliquot of the standard was taken and diluted with absolute ethanol until a concentration of 10 µg/ ml was obtained. This solution was then further diluted to yield a working standard with concentration ranging from 0.5 ng to 8 ng per ml. Both concentrated and diluted standards were stored under N_2 gas, in the dark at – 20 ^{0} C.

b. iii. Preparation of radio labeled 25-OH-D_3

Radio labeled 25-OH-D_3 (25 –hydroxy [23,24(N)-^{3}H]) was procured from Amersham, United Kingdom. Stock solution of approximately 5 µci /mmol was prepared and stored at –20 ^{0} C. Tracer solution was prepared in absolute ethanol and the concentration was adjusted to 5000 cpm in 10 µl.

b.iv. Preparation of sodium barbitone buffer

Barbital acetate stock solution was prepared by dissolving 9.71 gm sodium acetate and 15.74 gm sodium barbitone in one liter of distilled deionized water. Assay buffer was prepared by adding 400 mg bovine serum albumin (BSA) to 20 ml stock buffer and bringing the final volume to 400 ml with distilled deionized water. Sodium azide (0.1 gm) was added and the pH was adjusted to 8.6

b.v. Preparation of serum vitamin D binding protein

Serum obtained from normal rat was diluted 100 fold with assay buffer without BSA. Aliquots (0.5 ml) was stored at – 20 ^{0} C. The solution was further diluted for use in the assay to a concentration which will yield 30 % to 40 % specific binding of ^{3}H 25-OH-D_3 (radio labeled) in the absence of added cold 25-OH-D_3 the reference standard. The titer determination of binding protein was carried out using the procedures described by Chen et al. (1990).

b. vi. Preparation of dextran coated charcoal

A total of 0.3 gm dextran T-70 and 3gm activated charcoal were mixed with 100 ml of assay buffer without BSA and gently stirred in magnetic stirrer for one hour. The pH was adjusted to 8.6 and the suspension was stored at 4 ^{0}C. The suspension was stirred gently for 15 minutes prior to use in the assay.

b. vii. The assay of 25-OH-D_3 in serum samples

Serum sample (200 μl) was mixed with 0.8 ml of absolute ethanol in a test tube and mixed using a vortex mixer. After keeping in ice for 20 minutes, the tubes were centrifuged at 3000 rpm for 20 minutes. The supernatant was separated.

Four sets of test tubes were taken and 50 µl of supernatant was transferred into test tubes in duplicates (set 1). In another set of tubes (set 2), 50 µl of 25-OH-D_3 (10µg /ml) was added to determine the non-specific binding of the tracer. To prepare the standard graph, pure 25-OH-D_3 at varying concentrations (0.5 to 8 ng /ml) was taken in another set of tubes (set 3). Fifty microliter of 100% ethanol in set 4 test tubes served as blanks. Serum vitamin D binding protein was added in all three sets of test tubes except the blanks. Subsequently, the tracer solution (10 µl) was added in all the sets including the blanks. All the tubes were incubated overnight at – 20 ^{0} C. The next day, 100 µl of cold dextran coated charcoal was added in all tubes, except blank, and mixed. Tubes were allowed to stand for 20 minutes at room temperature and centrifuged at 3000 rpm for 15 minutes. This will sequester the unbound 25-OH-D_3. The supernatant from each tube was transferred into scintillation vials and 5 ml of scintillation cocktail was added to each vial. The cocktail was prepared using 880 ml of dioxane, 20 ml of ethylene glycol, 100 ml of methanol, 60 gm of naphthalene, 4 gm of PPO and 0.2 gm of POPOP. Then, each vial was counted in a liquid scintillation counter (Wallac 1409). Flow chart for the assay of 25-OH-D_3 in serum samples is given in figure 9.

Figure 9

FLOW CHART FOR COMPETITIVE PROTEIN BINDING RADIO LIGAND ASSAY FOR SERUM 25-OH D_3

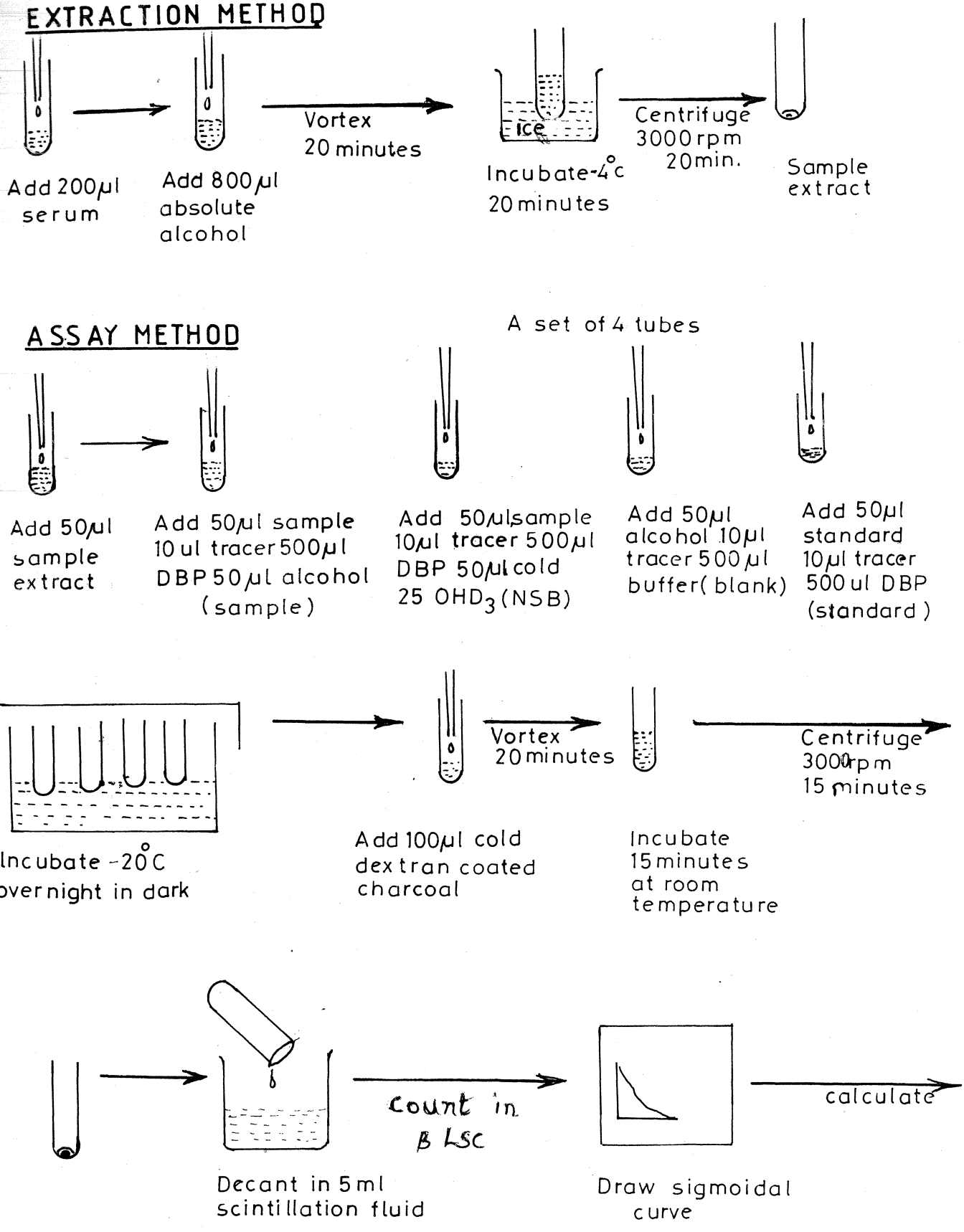
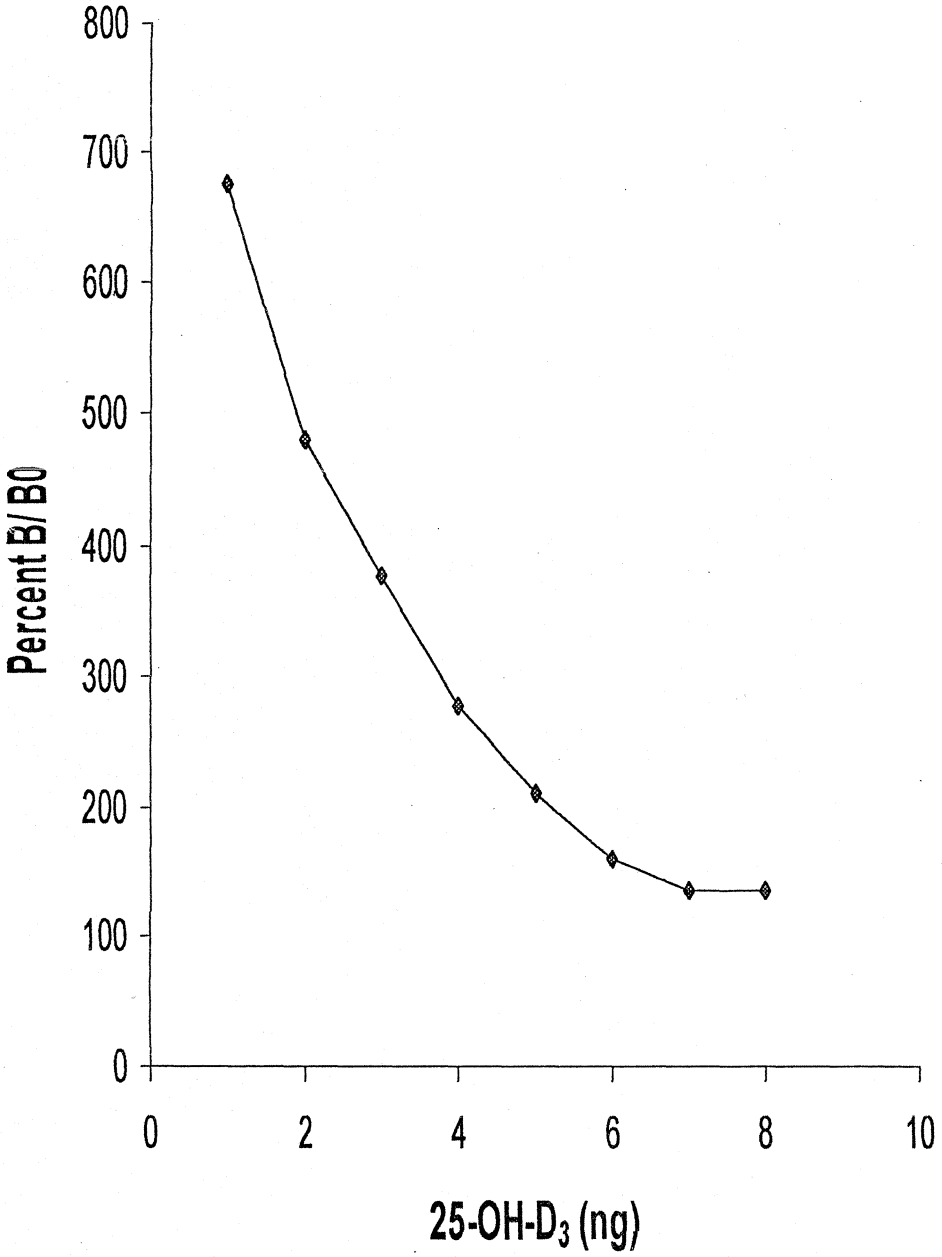


Figure 10
Standard curve for competitive protein binding radio ligand assay of 25-OH-D_3



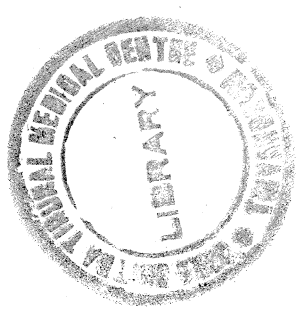
After obtaining the counts, the counts from blanks (set 4) was subtracted from those from Sets 1 and 3. The standard graph consisting of percentage binding versus different standard concentrations was prepared using the counts obtained from Set 3 tubes (Figure 10). The concentration of 25-OH-D_3 in the serum samples was determined from the readings obtained from the graph using the formula given below.

$$\% \text{ Binding} = \frac{\text{Cpm of standard or unknown}}{\text{Cpm of blank tubes} - \text{Cpm of non specific binding}}$$

The final result was expressed in ng /ml. In order to express the values in nmol / l, the figure obtained was multiplied by 2.5.

C. Estimation of serum calcium and magnesium

Calcium and magnesium in the serum was determined by atomic absorption spectrophotometry using an Atomic Absorption Spectrophotometer (International Laboratory IL 551) following the procedure described by Gimblet GG, Marney AF and Bonsnes RW 1967).



Principle: The technique of atomic absorption spectroscopy relies essentially on the absorption of light by freely dissociated atom produced by passing the sample through an oxyacetylene flame(air : acetylene 40: 60). The sample in liquid form is reduced to a spray of fine droplets and introduced into the flame, when high chemical reactions destroy any organic matter in the sample and dissociates inorganic compounds into free atoms. Since atoms of each elements absorb light at a characteristic wavelength the concentration of an element is determined by measuring its absorption at a given wave length (422.7 for Ca atoms and 285.2 for Mg atoms).

The serum (100 μl) was diluted to 50 times and taken into a test tube with 0.1 % lanthanum chloride solution in it. It was centrifuged at 3500 rpm for 10 minutes at room temperature to effect clear separation and the supernatant was collected. Aspirating the solution with a thin slotted air-acetylene burner the absorbance was recorded. Calcium and magnesium standards were procured from Sigma Chemicals, USA. Standard stock solutions in different concentrations were prepared by diluting standards with deionised water. Standard graphs were plotted and concentration of sample was calculated. Standard graphs for calcium and magnesium are given in figures 11 and 12 . Calcium and magnesium concentrations in the serum were expressed in mg /dl.

Figure 11
Standard graph for Calcium

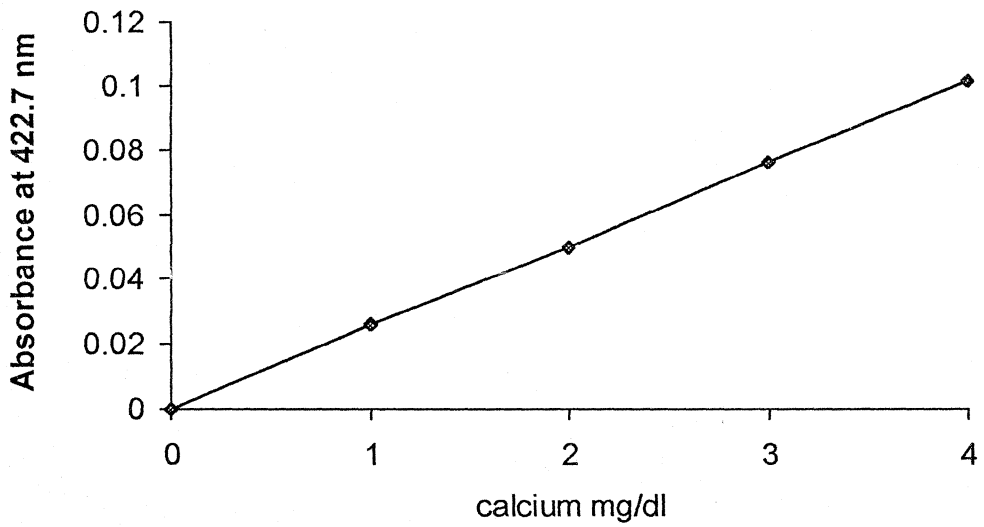
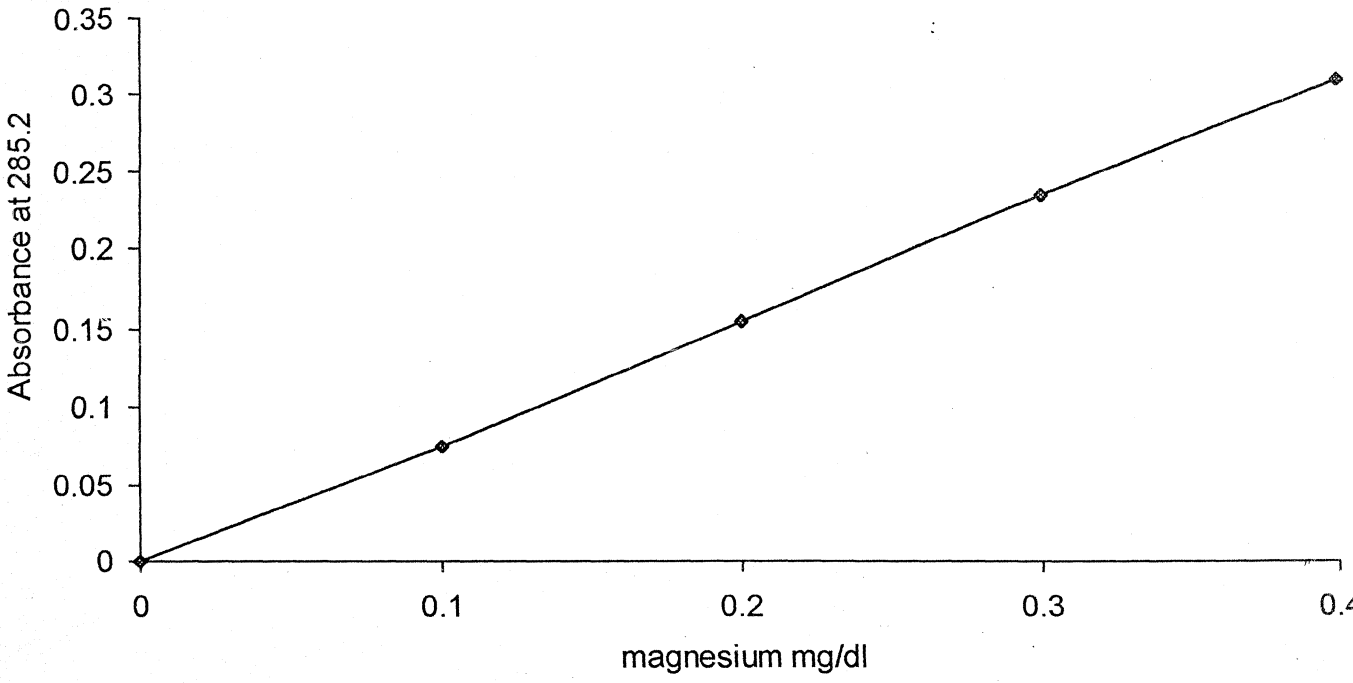


Figure 12
Standard graph for magnesium



d. Determination of inorganic phosphate

Inorganic phosphate was determined by the method of Fiske and Subbarow (1925).

d.i. Principle: Inorganic phosphate couples with molybdic acid to form a yellow phospho-molybdate that can be reduced to give a blue colour which is directly proportional to the amount of inorganic phosphate present. Double distilled water (totally free from phosphorous) was used for the procedure even for cleaning the tubes.

d. ii. Preparation of reagents

Standard phosphorous solution was prepared by dissolving 340 mg of potassium dihydrogen phosphate (KH_2PO_4) in 100 ml of water and making it upto 250 ml using distilled water. This solution contained 1 milli mole of phosphorous per ml. Sulphuric acid (H_2SO_4) solution was made by adding concentrated sulphuric acid (200 ml) to 520 ml of distilled water.

Ammonium molybdate (25 gm) was dissolved in 200 ml of distilled water and to this 500 ml of concentrated sulphuric acid was added. Thus molybdate solution was made. The final volume was made upto one liter using distilled water and thoroughly mixed. The solution was stored in a brown bottle.

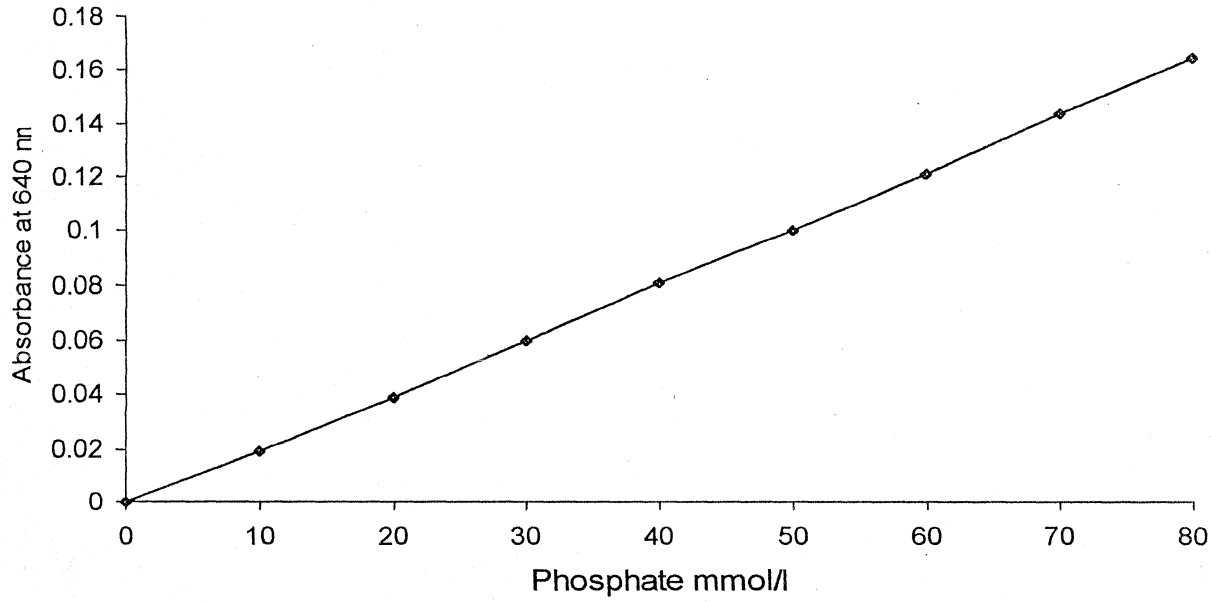
Amino naphtha sulphonic acid (ANSA) reagent was prepared as follows: Ten grams of sodium bisulphite ($NaHSO_3$) was dissolved in 200 ml of distilled water, mixed and filtered. Twenty grams of sodium sulphite (Na_2SO_3) was dissolved in 100 ml of distilled water, filtered and stored. Amino naphtha sulphonic acid powder (500 gm) was added to 195 ml of sodium bisulphite solution and mixed. To this, 5 ml of sodium sulphite solution was added and mixed thoroughly. If the resultant solution was not clear, 0.5 ml of sodium sulphite solution was added and mixed. The ANSA reagent was stored in a brown bottle in cold conditions. The solution was stable for about a month.

Ten percent trichloro acetic acid was prepared by dissolving 10 grams of trichloro acetic acid in 100 ml of distilled water.

d.iii. The assay of inorganic phosphate in serum

From the standard phosphorous solution, aliquots were transferred to different test tubes to give 10 μg, 20 μg, 30 ug, 40 μg, and 50 μg phosphorous solutions and the volume was made up to one ml with double distilled water. One ml of diluted serum sample was transferred to different test tubes. In blank tubes only 1 ml of distilled water was taken. To the tubes 0.4 ml of 10 % TCA, 0.4 ml of molybdate solution, 0.2 ml of ANSA reagent and 4 ml of double distilled water

Figure 13
Standard graph for inorganic phosphate



were added and mixed well. All the test tubes were kept for 5 minutes and the absorbencies of the standards as well as samples were measured at 640 nm using a Shimadzu spectrophotometer. Standard graph was drawn (Figure 13) and the concentration of the samples were calculated.

e. Estimation of lipid profiles

e. i. Total cholesterol

Principle: The enzymatic procedure involves cleavage of the cholesterol esters by cholesterol esterase and oxidation of the free cholesterol by cholesterol oxidase. Monotest cholesterol kits (high performance CHOD PAP method) from Boehringer Mannheim was used. 100 μl serum was treated with 1 ml of reagent and kept for 15 minutes at room temperature. The absorbency of the sample was measured against reagent blank within one hour at 500 nm in a UV visible spectrophotometer (Shimadzu). Concentration of the sample was calculated by comparing with that of standard precimat cholesterol (concentration 200 ng/ dl).

e. ii . High density lipoprotein (HDL) cholesterol

Principle: The cholesterol contained in the isolated HDL fractions was quantitated by the use of an enzymatic reagent, a procedure based on the stoichiometric production of H_2O_2 from cholesterol and subsequent calorimetric reaction in which 4 aminophnazone and sulphonated 2,4 dichlorophenol are present as the chromogenic system.

For isolation of HDL, 0.2 ml of serum and 0.2 ml of polyethylene glycol (PEG 6000-8000) solution were mixed together. After 10 minutes of incubation at 20-25 ^{0} C, the mixture was centrifuged at 2000 rpm for 10 minutes. The supernatant. was collected. PEG precipitates LDL and VLDL . 100 gm/ ml PEG 6000 in a buffered solution with pH 10 was used. Glycine sodium hydroxide buffer was prepared by weighing 1.50 gm glycine and dissolving it in distilled water. The pH was adjusted to 10 with 1 N NaOH and the volume made up to 100 ml. Polyethylene glycol buffer was prepared by dissolving one gram of PEG 6000 in glycine sodium hydroxide buffer and making up the volume to 5 ml. This solution was used for the isolation of HDL.

e. iii. The Assay of HDL

Fifty ml of supernatant was pipetted into a test tube and one ml of cholesterol reagent was added and mixed in a vortex mixer. The sample was incubated at room temperature for 15 minutes. The absorbency of the sample was measured at 500 nm against reagent blank within one hour.

e. iv. Triglycerides

Principle: Triglycerides are enzymatically hydrolysed and the liberated glycerol is subsequently determined by calorimetry.

A reagent strip was immersed in one bottle of buffer solution and the bottle contents was stirred for approximately 10 seconds. Buffer solution was allowed to stand for 5 minutes and stirred once again for approximately 10 seconds. The reagent strip was then discarded. For estimating serum triglyceride levels, 10 μl of serum was taken in a test tube, and 1 ml of reagent (triglyceride reagent Monotest kit, Boehringer Mannheim) added. The mixture was kept for 10 minutes for incubation. Absobency was read at a wavelength of 500 nm in a Shimadzu UV visible spectrophotometer and concentration determined against a blank reagent.

PART II – EXPERIMENTAL STUDY

Twenty healthy New Zealand rabbits were used. Female rabbits, aged 6 months were only available in our animal house and hence they were chosen for the study after de- worming. Animals were grouped into two. Group I comprised of 6 animals and were designated as controls. Group II comprised of 14 animals and were labeled as test animals. Both groups were fed a commercial diet purchased from Kamadhenu Agencies, Bangalore. Water was given *ad libitum*.

After one week , 2-3 ml of blood were taken from the ear lobes of all the animals for base line investigations. Serum was separated and stored at – 20 ^{0} C till analysis. Animals in the test group were injected 10,000 IU of cholecalciferol intramuscularly twice a week. The material for injection was prepared by dissolving crystalline vitamin D obtained from Sigma Chemicals, USA in one ml of cottonseed oil. The oil was screened for toxicity by our Toxicology Department and was certified for our purpose. Control animals were given intramuscular injections of one ml of plain cottonseed oil, twice a week. Blood samples were collected every week from all the animals. All animals were monitored regularly and their weight recorded at weekly intervals. At the end of one month, animals were sacrificed and blood and aortas were collected.

Serum was separated from the blood samples and stored at – 20^0 C till analysis. Levels of 25-OH-D_3, calcium, magnesium, total cholesterol, triglyceride and high density lipoprotein (HDL) were determined by the procedures described in Part A.

Aortas collected from the animals were used for both histological and biochemical investigations.

a. BIOCHEMICAL STUDIES

a . i. Total lipids in aorta

Concentrations of total lipids in the aortas of the animals were determined following the method of Bligh and Dyer (Bligh EG and Dyer WJ 1959). In this method, a mixture of chloroform and methanol (1:2 v / v) is used. Tissue (about 500 mg-wet weight) was first ground in a pestle and motor with about 10 ml of distilled water. The homogenized tissue was transferred to a conical flask and 30 ml of chloroform-methanol mixture (1:2 v / v) was added and mixed well. For complete extraction, the mixture was kept overnight at room temperature in the dark. After the incubation, 20 ml of chloroform and 20 ml of water were further added into the flask. The resulting solution was subjected to centrifugation.

Three layers were obtained; a clear lower layer of chloroform containing all the lipids, a coloured aqueous layer of methanol with all water soluble material and a thick pasty interface.

The methanol layer was discarded and the lower layer was carefully collected free of the interface by sucking out with a fine capillary tube and filtering through glass wool. The organic layer extracted was taken in a pre-weighed vial and carefully evaporated by keeping in a warm water bath. The weights of the extracted lipids were recorded. The difference in weights gave the total lipid content of tissue. It was expressed in mg / gm of wet tissue.

a. ii. Determination of Calcium and Magnesium

About 500 mg of aorta was taken and dried in an oven till constant weight. About 50 mg of dried sample was taken in a digestion flask and one ml of concentrated nitric acid (HNO_3) was added. The mixture was kept at room temperature overnight. Then the flask was heated in a hot sand bath till near dryness. Then a mixture of perchloric acid and nitric acid (1: 1) was added and reheated till dry. The flask was cooled and 10 ml of 0.5 N HNO_3 was added. This was further diluted and the concentrations of magnesium and calcium was determined using atomic absorption spectrophotometer as described earlier.

a. iii. Determination of 1,25-dihydroxy cholecalciferol receptor levels in aorta

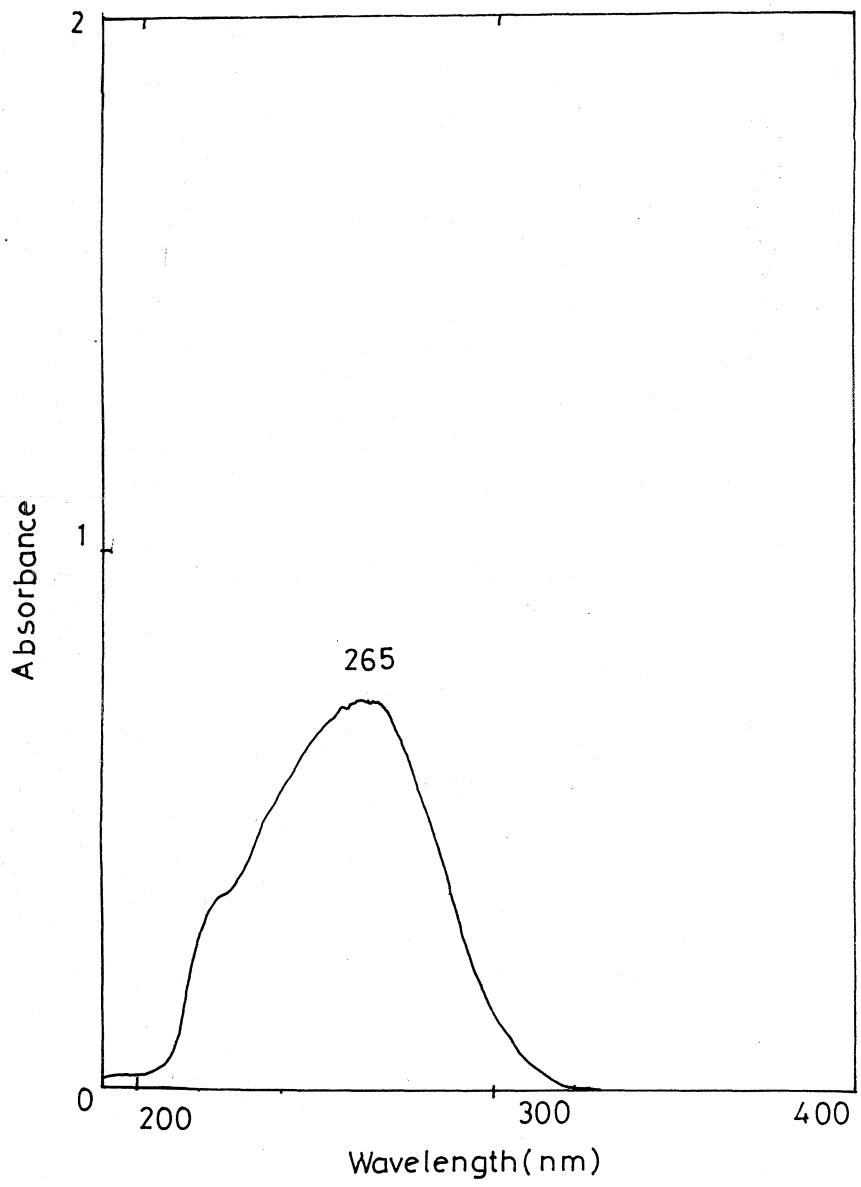
Determination of 1,25-dihydroxy cholecalciferol receptor levels was done following the methods described by Walters MR, Wicker DC and Riggle PC 1986; Koh E et al., 1988 ; Beckman MJ, Horst RH , Reinhart TA and Beitz DC 1990.

Principle: Assay involves the use of a radio labeled steroid over an appropriate concentration range in the presence and absence of an unlabeled competitor. The binding to lower affinity contaminants ie. , non specific binding is eliminated by competition. The free steroid is precipitated using dextran-coated charcoal and the steroid bound to the receptor present in the supernatant is counted in a liquid scintillation counter. Scatchard analysis is applied to this saturation assay to give the number of high affinity binding sites of the steroid and the dissociation constant of the steroid receptor complex.

Preparation of reagents

Sucrose glycerol buffer was prepared by adding 0.25 M sucrose, 1.5 nM magnesium chloride ($MgCl_2$) and 10 nM Hepes in 50 % v / V glycerol and pH adjusted to 7.4. The buffer was kept at 4^0 C throughout the assay.

Figure 14
ABSORPTION SPECTRUM OF $1,25\ (OH)_2D_3$



The radioactive 1, 25$(OH)_2$ D_3 (1 alpha, 25-dihydroxy (26,27- Methyl-[H^3] cholecalciferol) was obtained from Amersham, UK. The specific activity of labeled 1,25$(OH)_2D_3$ was 177 ci / mmole. It was diluted with absolute alcohol to give a concentration of 300 fmoles per 50 μl.

Crystalline 1,25 dihydroxycholecalciferol (1,25$(OH)_2$ D_3) was obtained as a gift from M. Uskokovic, Hoffman La Roche, USA. Its purity was checked by UV absorption spectrophotometry. About 5 μg was dissolved in 2 ml of absolute ethanol and absorption spectra from 220 nm to 300 nm were taken using a double beam Shimadzu Spectrophotometer (Figure 14). The concentration of the standard was calculated from the absorbance at 265 nm. The concentration of standard was calculated using the equation given below and it confirmed the purity of 1,25$(OH)_2D_3$ used.

A = ECL where A = Absorbance C = concentration P = Path length

E = Molar extinction coefficient C = A/ E = Mol/ L

$$\text{Concentration} = \frac{\text{OD at 265 nm}}{18200} \times \text{gm Mol/ L}$$

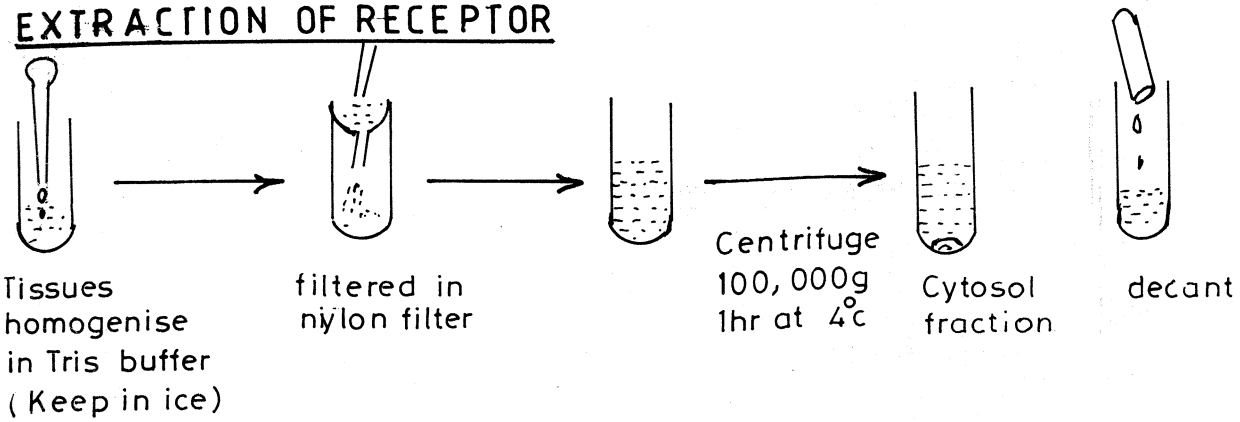
$$\text{Concentration (ug/ ml)} = \frac{\text{X gm Mol}}{\text{L}} \times 416 \text{ (Mol.weight of } 1,25(OH)_2D_3)$$

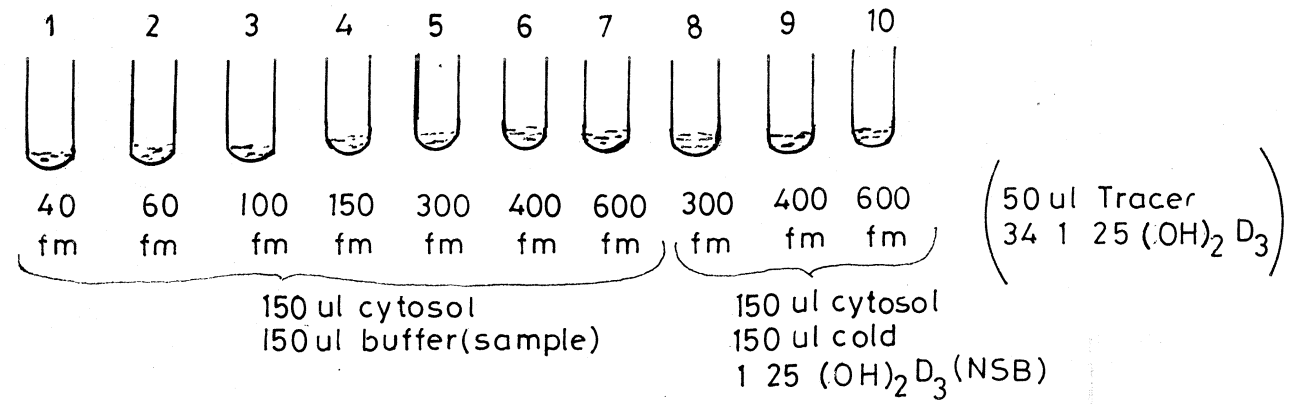
Collected tissue was stored in sucrose glycerol buffer at – 20 ^{0} C. Prior to the assay, tissue was removed from the sucrose glycerol solution and allowed to rehydrate for 15 minutes in isotonic saline. Then the medial layer of the aorta was removed and homogenised in presence of Tris buffer in a homogenizer. Tris buffer was prepared by adding 50 mM Tris, 1.5 mM EDTA, 5.0 mM dithiothrietol (DTT), 300 mM KCl and 300 μM phospho methyl sulphonic acid (PMSF). The pH was adjusted to 7.4 and the buffer was stored at 4 ^{0} C. The homogenate was filtered with a nylon filter and the filtrate was used for further analysis. The tissues were further homogenised and filtered. The filtrates were combined and ultracentrifuged at 100,000 g for one hour and at 4 ^{0} C. The supernatant was decanted and 150 μl of the cytosol in duplicate was used for the assay. A seven point assay was employed in this experiment.

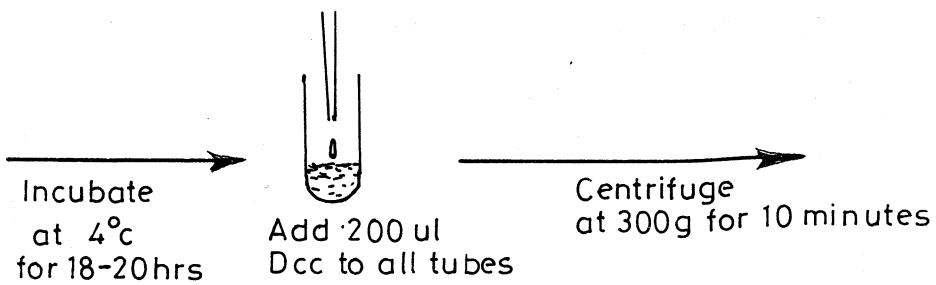
A set of 10 incubation tubes (small vials) labeled 1-10 was used. To vials 1-7 were added increasing concentrations of tracer (20, 30, 50,70,150,200 and 300 fmoles). To the vials 8 –10, 150, 200 and 300 fmoles of the tracer and cold (non radioactive) 1,25$(OH)_2D_3$ reference standard (500 ng / ml) was added to determine non specific binding of the sterol. Tris buffer (150 μl) was added to all the 10 assay tubes. To another set of 10 tubes, 150 μl of supernatant (cytosol), tracer (^{3} H 1,25 - dihydroxycholecalciferol) and cold 1, 25 $(OH)_2D_3$ reference

Figure 15

FLOW CHART FOR RADIO RECEPTOR ASSAY OF $1,25(OH)_2 D_3$ IN VSMC IN AORTA OF RABBITS.







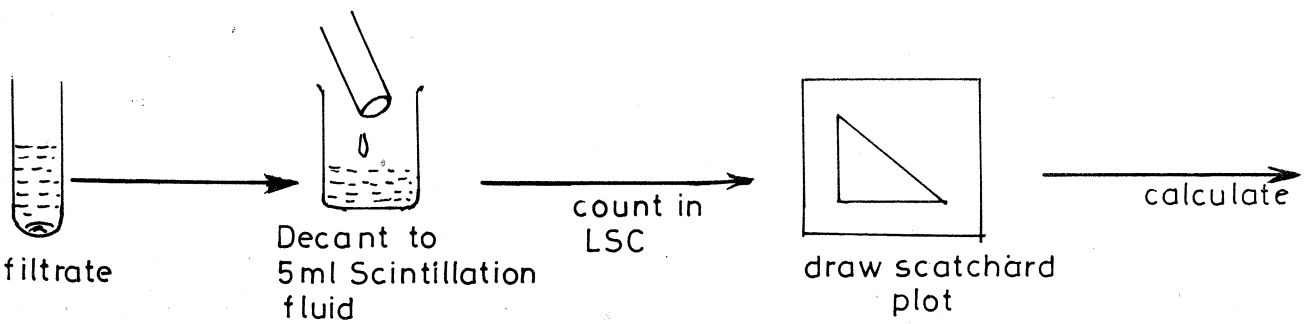
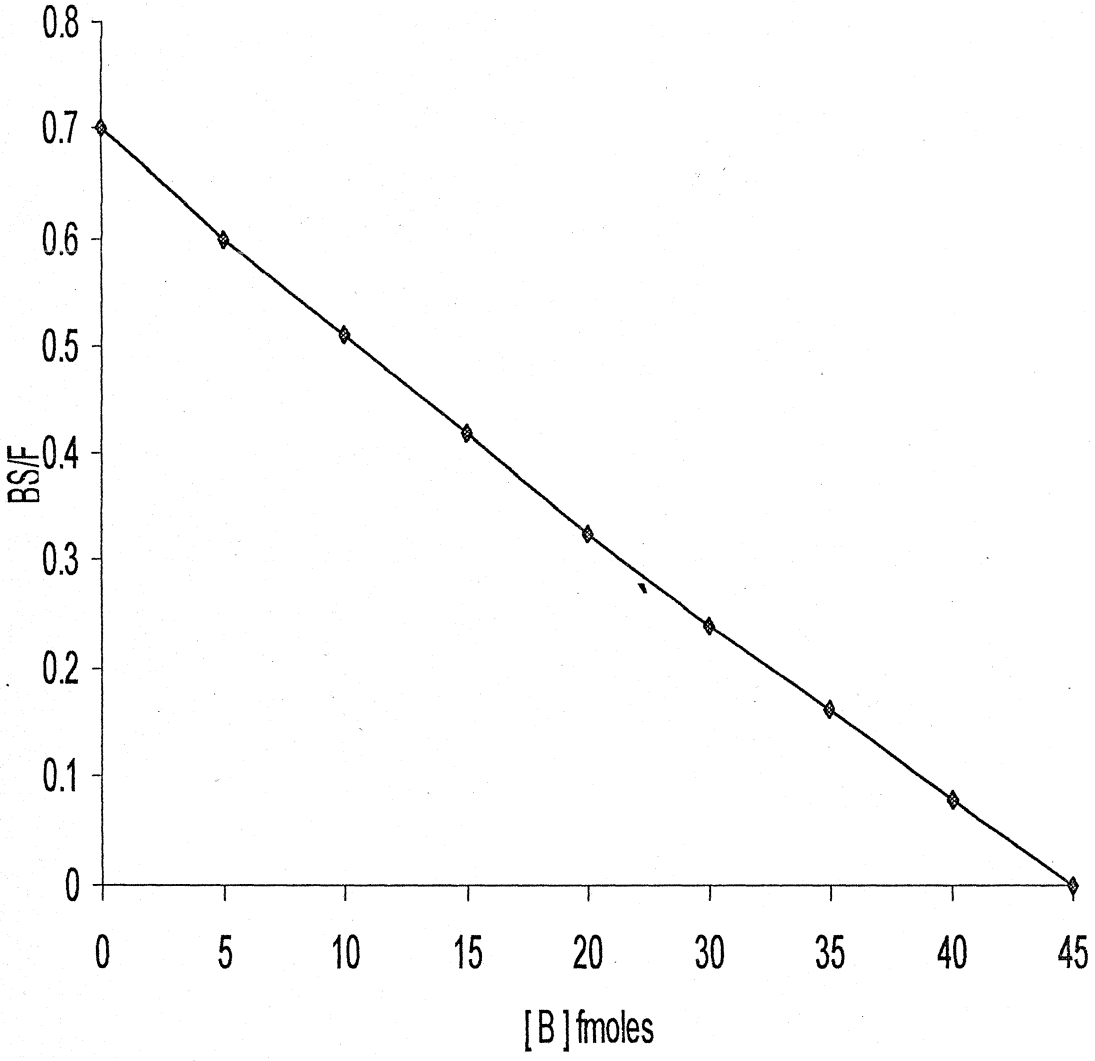


Figure 15 a

Scatchard plot for $1,25(OH)_2D_3$ receptor in the aortas of experimental rabbits



standard were taken. The samples were incubated overnight at 4 ^{0} C in dark. The free sterol was precipitated using dextran-coated charcoal (DCC). DCC was prepared by adding 0.2 gm activated charcoal, 0.025 gm dextran and 10 ml glycerol to 90 ml of tris EDTA buffer. The suspension was stirred in a magnetic stirrer for about one hour and kept at 4 ^{0} C in the dark. The sterol bound to the receptor present in the supernatant was counted in a scintillation counter using aqueous scintillation fluid prepared by adding 880 ml of Dioxan, 20 ml of ethylene glycol, 100 ml of methanol, 60 gm of naphthalene, 4 gm of PPO and 0.2 gm of POPOP.

Scatchard analysis was applied to this saturation assay to give the number of high affinity binding sites of the sterol and the dissociation constant of the sterol receptor complex. Flow chart for the assay is given in figure 15.

Calculations

[M]: concentration of labeled sterol in different tubes in fmoles

TC: total counts (average) SC: counts of sample tubes (average)

%B: Percentage binding (SC/TC) x 100

[B]: Total bound steroid (SC/TC) x [M]

F: Free sterol as measured in tubes 1-7 of assay: [M] – [B]

FNS: Free sterol measured in tubes 8-10 of assay.

For tubes 8-10, $\frac{[B]}{\text{FNS average}} \times F$ was taken

BNS: Non specifically bound steroid = $\frac{[B]}{\text{FNS average} \times F}$

BS: Specifically bound steroid = [B] – BNS

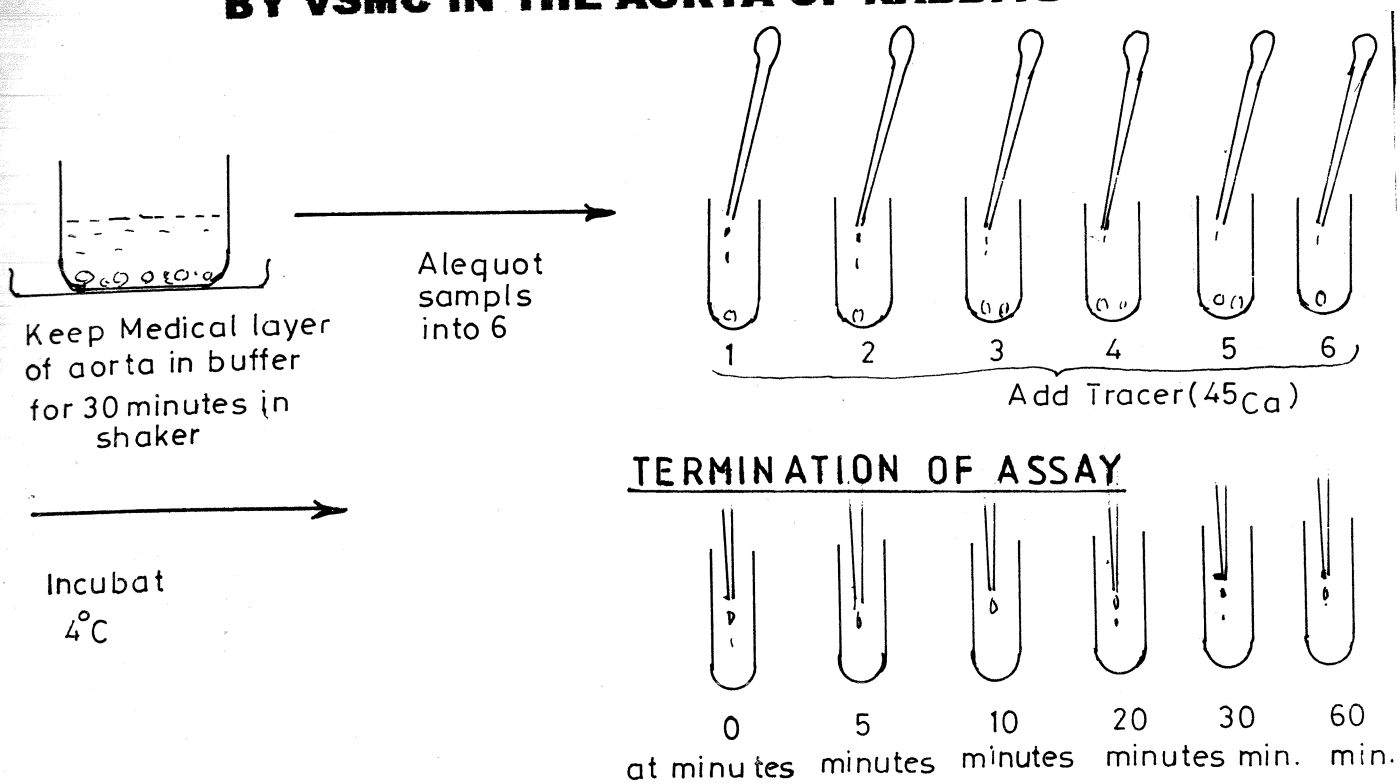
A scatchard plot analysis (BS / F versus [B] will give the number of high affinity binding sites and the dissociation constant of the steroid receptor complex. The results were expressed in fmoles / mg protein.

a.iv. Kinetics of ^{45} Calcium uptake in the medial cells of the aorta

Radioactive Ca was obtained from Bhabha Atomic Research Centre (BARC), Bombay. It was diluted with absolute ethanol so as to obtain a concentration of 0.01 μCi corresponding to 22000 dpm.

Figure 16

FLOW CHART FOR THE ASSAY OF ^{45}Ca UPTAKE BY VSMC IN THE AORTA OF RABBITS



Equilibrium buffer was prepared by mixing 3 mM $Lacl_3$, 137 mM NaCl, 5.2 mM KCl, 0.8 mM $MgCl_2$ and 10 mM Hepes in distilled water, adjusting the pH to 7.1 and making upto 100 ml.

Aorta was taken and medial layer consisting of smooth muscle cells were separated and kept with rocking in equilibrium buffer for 30 minutes. The sample was divided into four aliquots and kept in 4 separate vials. One μCi of tracer (^{45}Ca) added to all the tubes and kept for incubation at 4 ^{0} C. Calcium uptake was terminated by adding lanthanum chloride ($Lacl_3$) buffer at different time points. At `0' time of incubation, tube 1 was removed and calcium uptake terminated by adding 2 ml of $Lacl_3$ buffer. Similarly at 10 minutes, 30 minutes and 60 minutes intervals, tubes were removed and 2 ml of $Lacl_3$ was added. All the tubes were incubated at 4^0 C for 30 minutes. After incubation, tubes were centrifuged and supernatant was discarded. The tissues were washed twice with $LaCl_3$ buffer. The tissue was digested with 1N NaOH (500 μl) by keeping it at room temperature for 30 minutes. The digest was centrifuged, supernatant was decanted and an aliquot of the sample was added to 5 ml of scintillation fluid and counted for radioactivity. A portion of the aliquot was used for protein assay by Lowry's method. Calcium uptake was expressed in cpm / mg protein. Flow chart for the assay is given in figure 16.

Protein assay was carried out with the above solutions. The aliquots (200 μl) of sample in buffer for protein estimation were transferred to separate tubes. To the tubes, 2.5 ml of alkaline copper reagent was added and kept for 15 minutes at room temperature. Following this, 250 μl of Folin's reagent was added and mixed. After incubating for 30 minutes at room temperature, the absorbency was measured at 660 nm using a Shimadzu Spectrophotometer.

b. PATHOLOGICAL STUDIES

Aortas were longitudinally cut open and examined. Those with gross morphological changes were photographed. Aortas were then fixed in 10% buffered formalin and processed subsequently. Samples were dehydrated in alcohol, cleared in chloroform and embedded in paraffin wax. Sections of 8 μm thickness were cut using a microtome and stained by different methods.

b. 1. Haematoxyline and Eosin staining

Haematoxylin powder (3g) was dissolved in 25 ml of absolute alcohol. Iodine (1 gm) was dissolved in 100 ml of absolute alcohol. Saturated ammonium alum was prepared in 1400 ml of distilled water. To saturated ammonium alum

solution, haematoxyline solution and iodine solution were added, mixed and made upto 2 liters with distilled water. The solution was boiled for 3 minutes, cooled and filtered before use.

Eosin was prepared by dissolving one gram of Eosin Y in 20 ml of distilled water and making up to 100 ml with 95 % alcohol. To prepare the working solution one part of the stock solution was diluted with 3 parts of 80 % alcohol. Glacial acetic acid, 0.5 ml , was added to every 100 ml of stain.

Paraffin sections on glass slides were deparaffinised in xylene. Subsequently, slides were passed through 100 % to 50 % alcohol in stages and finally hydrated by keeping it in running water. Following this, the slides were stained with haematoxyline for 2-5 minutes. Then slides were washed and differentiated in 0.1 % acid alcohol (0.1 ml concentrated HCl in 100 % alcohol). After keeping the slides for 15 minutes in running tap water for blueing, the slides were stained with eosin for 1 minute. The slides were dehydrated in graded alcohol (50 % to 100 %), cleared in xylene, mounted using DPX and viewed under an optical microscope.

b.ii. Elastic Van Gieson's staining for elastic fibers

Crystal violet (2.5 gm), basic fuschin (2.5 gm) and dextrose (1 gm) were added to boiling distilled water to dissolve the dyes. To this, resorum (10 gm) was also added. While boiling, aqueous ferric chloride (30% $FeCl_3$) was added slowly and continuously stirred with a glass rod. The solution was cooled and filtered. The deposit was washed with distilled water till the filtrate became colourless and the deposit was azure blue in colour. The filter paper with the residue was scrapped and dissolved in 550 ml of absolute alcohol and 1 ml of concentrated HCl was added to it. To facilitate dissolving, the solution was gently warmed on an electric hot plate. The solution was cooled and filtered. To the filtrate, 10 ml of concentrated HCl was added and the mixture was allowed to stand for 24-48 hours before use. The colour of the solution was dark greenish blue. Working solution (EVG solution) was prepared by diluting 38 ml of the stock solution in 30 ml of 70 %alcohol.

The microtome cut sections were fixed on a clean slide and deparaffinised in xylene. Then the sections were transferred to 100 % and 90 % alcohol in steps. The slides were kept in EVG solution overnight and washed in alcohol. Counter staining was done with Van Gieson's stain (1% acid

fuschin -- 1ml, saturated picric acid – 9ml). Following staining, the slides were dehydrated, cleared and mounted in DPX.

b. iii. von Kossa's stain for calcium

Citrate buffer (0.1 M pH 4.5), disodium hydrogen phosphate (0.2 M), citric acid (0.1 M) and silver nitrate (5 % w / v in distilled water) were prepared and mixed. Deparaffinised sections were kept in 100 %, 90 % and 70% alcohol (for 5 minutes each), washed in tap water and rinsed in distilled water. Silver nitrate stain was poured on top of the slide and kept under UV light for ½ an hour. After staining the slide was rinsed in distilled water, dipped in 70% of alcohol and counter stained with eosin stain for one minute. After rinsing in distilled water the slide was dried, cleaned with spirit, then cleared in xylene and then mounted with DPX.

b. iv. Ultrastructural studies of aorta

Samples of aorta from 3 test animals and 3 control animals were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7. Following fixation in glutaraldehyde the tissues were kept in 1 % osmium tetroxide in 0.1 M phosphate buffer for two hours. After washing the samples with phosphate

buffer, they were dehydrated in different concentrations of acetone (50%, 70%, 90%, and 100%).

Then the tissue was passed through different concentrations of filtered toluene resin (Spurr low viscosity embedding media, Polysciences, Inc) mixture (3:1,1:1,1:3) and finally embedded in pure resin and the blocks were polymerised at 70^{0} C.

Semithin (1 micron) sections were cut using glass knives using an ultra microtome (LKB Broma 2088 Ultratome V) and sections were stained using 1% toluidine blue for light microscopy.

Ultrathin sections (60-80 nm) were cut using a diamond knife and were stained with uranyl acetate and lead citrate (Reynold's 1963). The stained sections were viewed under transmission electron microscope (Hitachi: H-600) at an accelerating voltage of 75 kV. Representative micrographs were taken for further observations.

STATISTICAL ANALYSIS

Student's ` t ' test was performed for comparing the mean values of each parameter between cases and controls, as well as for comparing between subgroups. The difference was considered statistically significant when $P< 0.05$.

Pearson's method was used for determining the correlation coefficients in cases to assess the degree of association between serum levels of 25 hydroxyvitamin D_3 and other independent variables. The association of known risk factors was tested using Chi Square method. Multivariate analysis was done for obtaining the relative risk with respect to 25-OH-D_3 level and other known risk factors for coronary artery disease. The median level of 25-OH-D_3 was taken as the cut off value. The cut off values taken for LDL and HDL levels were 160 mg% and 35 mg% respectively.

In animal experiments, Student's ` t ' test was performed for comparing the mean values of each parameter between test and controls. Pearson's method was used for determining the correlation coefficients in the test group to assess the degree of association between serum levels of 25-OH-D_3 and other variables.

CHAPTER IV
RESULTS

CHAPTER IV

RESULTS

PART A - CASE CONTROL STUDY

Clinical Characteristics of the patients and controls are given in Table 1. Biochemical parameters are given in Table 2. Serum levels of 25-hydroxy vitamin D_3 were significantly higher in patients with angiographic evidence of coronary artery disease and acute myocardial infarction, when compared with controls.

Serum 25-hydroxyvitamin D_3 levels correlated with calcium and magnesium levels in serum, but not with serum levels of cholesterol, trigycerides and low-density lipoproteins (LDL). Vitamin D_3 levels in serum had an inverse relationship with serum levels of high-density lipoprotein. Patients with angiographic evidence of coronary artery disease had higher levels of low density lipoprotein, triglyceride and lower levels of high-density lipoprotein when compared to the controls. Cases had higher number of smokers and hypertensives. There was no significant difference in the number of persons with

Table I

Comparison of clinical characterisics of the study population

	CAD N=88	AMI N=55	CONTROL N=70
Mean age in years	54.7 ± 9.1	51.0 ± 10.3	50.1 ± 14.1
Hypertension %	47.6 *	30.8	31.9
Diabetes %	24.4	21.2	18.8
Family history Coronary artery Disease %	22	11.5	15.9
Smoking %	69.5 *	51.9*	33.3

CAD- Coronary artery disease , AMI – Acute myocardial Infarction. Data are mean ± S.D * P< 0.05

Table II

Comparison of biochemical parameters in the study population

	CAD	AMI	CONTROL
Cholesterol mg/dl	242.7 ± * 58.3 (86)	230.03 ± * 78.4 (55)	220.3 ± 46.0 (69)
Triglyceride mg/dl	228.8 ± * 82.4 (80)	216 ± * 70.6 (55)	185 .0 ± 52.9 (67)
High density lipoprotein mg/dl	33.3 ± * 9.6 (86)	32.5 ± * 8.2 (55)	49.3 ± 9.9 (69)
Calcium mg/dl	9.1 ± * 3.1 (81)	8.99 ± * 2.4 (53)	8.0 ± 2.1 (66)
Magnesium mg/dl	1.9 ± 0.7 (81)	1.84 ± 0.6 (54)	1.9 ± 0.5 (68)
Inorganic Phosphate mmol/l	1.97 ± * 0.6 (81)	1.7 ± * 0.6 (55)	1.62 ± 0.3 (64)
25 hydroxyvitamin D_3 ng/ml	115.9 ± * 42.8 (83)	92.2 ± * 44.2 (55)	73.4 ± 28.9 (68)

Data are mean values ± S.D. * $P < 0.05$
CAD – Coronary artery disease , AMI – Acute Myocardial Infarction.
Number of subjects are given in brackets.

TABLE III

Multivariate analysis of the study groups (N =213)

Variable	Odds Ratio	Significance
Hypertension	1.2802	0.5820
Diabetes mellitus	1.9044	0.1981
High density Lipoprotein (<35 mg %)	14.158	0.0001
Low density Lipoprotein (>160mg %)	1.618	0.2540
Smoking	2.4532	0.0280
25-hydroxyvitamin D_3 (>86ng/ml)	4.1607	0.0005

diabetes and those with a family history of coronary artery disease between cases and controls. Serum 25 hydroxy vitamin D_3 levels did not have any association with diabetes, but was positively associated with history of smoking.

In sub group analysis there was no difference in serum 25-OH-D_3 levels between patients who had coronary calcification and those who did not. Results of multivariate analysis are given in Table 3. Odds ratio for serum 25 hydroxy vitamin D_3 was 4.16 ($P < 0.0005$).

PART B - EXPERIMENTAL STUDY IN RABBITS

Morphological observations

Gross findings

Four of the 14 animals, which received vitamin D_3 injections, had extensive, gritty whitish lesions with adherent thrombi in both abdominal and thoracic aortas (Figure 17 c) . In another four animals aortas were dilated and intimal ulcers were also seen (Figure 17 b). Aortas were normal in appearance in the other six rabbits and all of the control animals (Figure 17 a). Aneurysmal changes were also observed in animals that had calcification (Figure 17 d).

Figure 17

Photomicrographs of normal aorta and aortas of rabbits given vitamin D_3 injections.

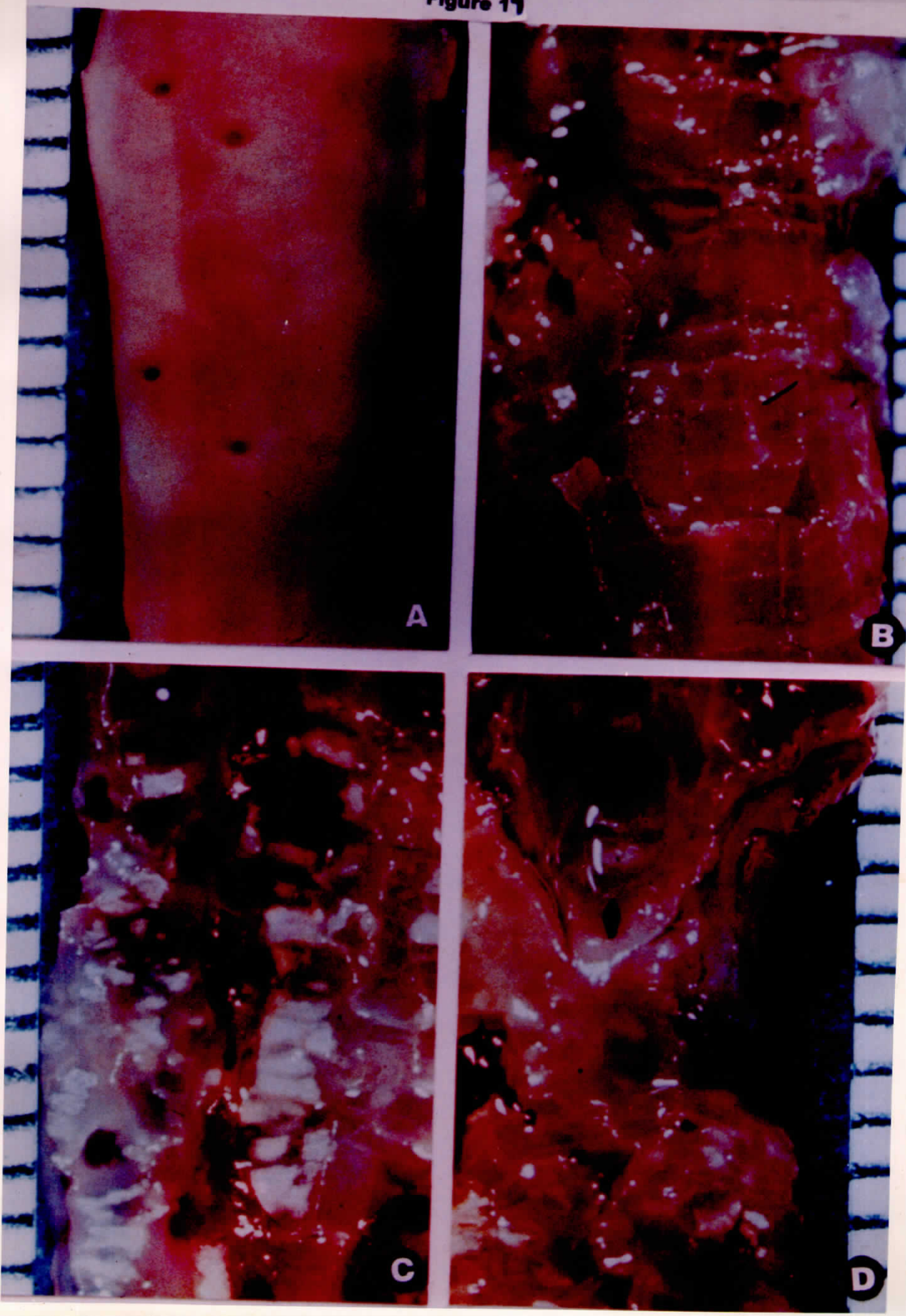
a. Normal aorta with smooth glistening intima.

b. Dilated aorta with spotty whitish calcific areas in intima.

c. Extensive whitish calcified plaques in the dilated aorta.

d. Aneurysmal lesion in the aorta.

Figure 17



Microscopic findings

Histological abnormalities were seen in the aortas of 8 test animals that had gross abnormalities. Fragmentation of internal elastic lamina, focal loss of elastic fibers and fragmentation and degeneration of elastic fibers in the media were seen in all the eight animals. In four of the test animals there was extensive calcification in the media (von Kossa positive) (Figure 19 c and 19 d). Calcified areas were also positive for the presence of iron (Figure 19 b). None of the animals had lesions resembling atherosclerosis. Inflammatory infiltrates were also absent. Thinning of the aorta along with calcific plaques were observed (Figure 18 d). Focal loss of elastic tissue (elastolysis) (Figure 18 c), thinning of media (Figure 19 a) and fragmentation of internal elastic lamina were observed in the aortas which appeared dilated on macroscopic examination (Figure 18 b). No histological abnormalities were observed in control animals (Figure 18 a).

Ultrastructural findings

Ultrastructural lesions in animals, which were given vitamin D were fragmentation of elastic fibers and degeneration of smooth muscle cells (SMC) in the media (Figure 21 d). Mitochondriae were vacuolated (Figure 21 a). Elastic fibers and SMCs were calcified (Figures 20 c and 21 c). Cellular debrii, degenerated cells

Figure 18

Photomicrographs of aortas of rabbits after periodic intramuscular injection of vitamin D_3

a. Normal aorta of control rabbits . parallel rows of elastic fibers are seen. (Vangieson's x 100)

b. Fragmentation and degeneration of elastic fibers (Vangieson's x100)

c. Focal loss of elastic fibers (elastolysis) (Vangieson' x100).

d. Thinning of aorta and intimal calcific plaques. (H & E x 10).

Figure 18

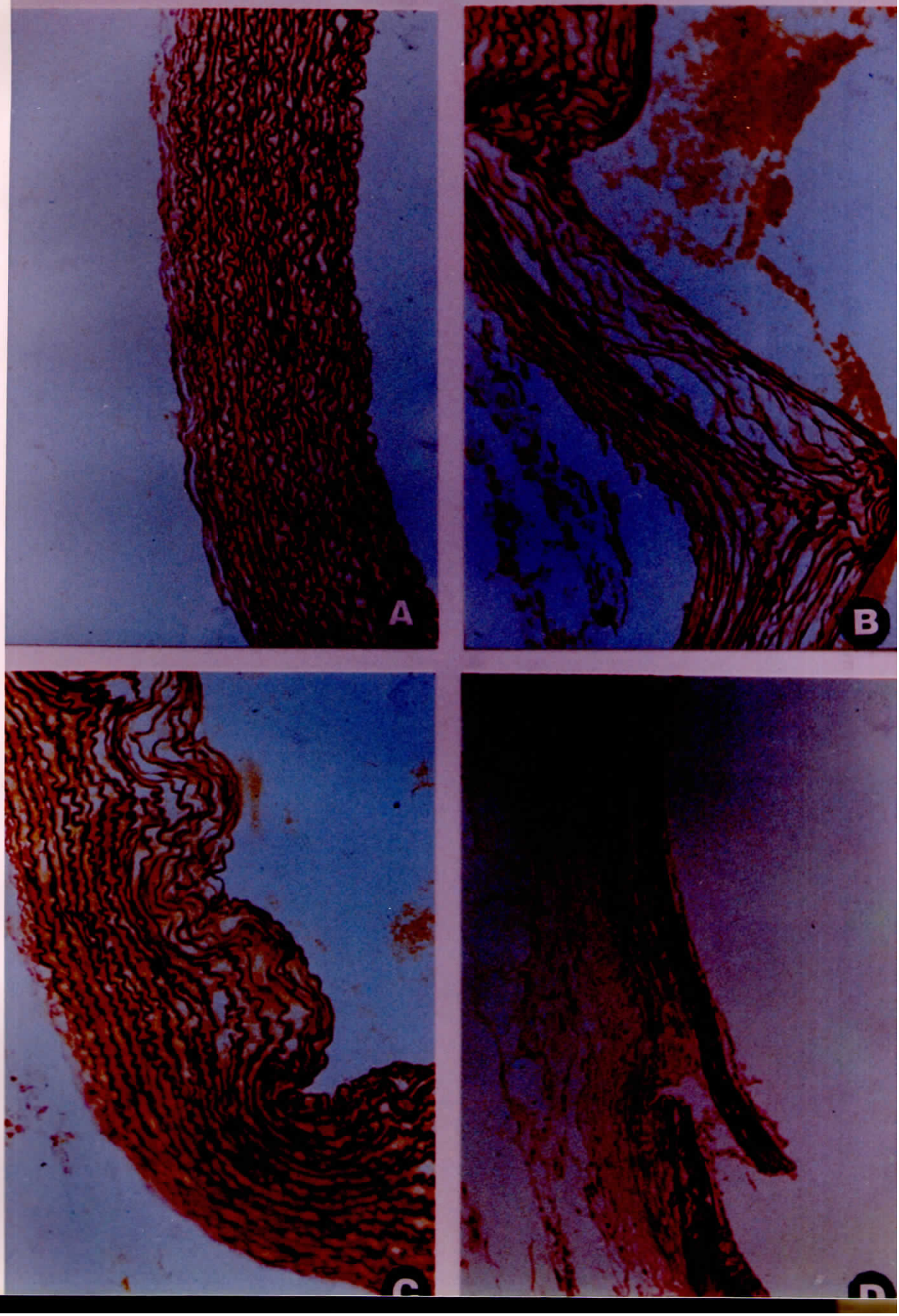


Figure 19

Photomicrographs of aortas of rabbits after periodic intramuscular injection of vitamin D_3.

a. Thinning of media (H & E x 10)

b. Bluish areas of Perl's staining positivity in the calcified intimal plaques (Perl's stain x 100)

c. Spotty calcium deposit in the media (von Kossa x 40).

d. Extensive medial calcification (von Kossa x 40).

Figure 19

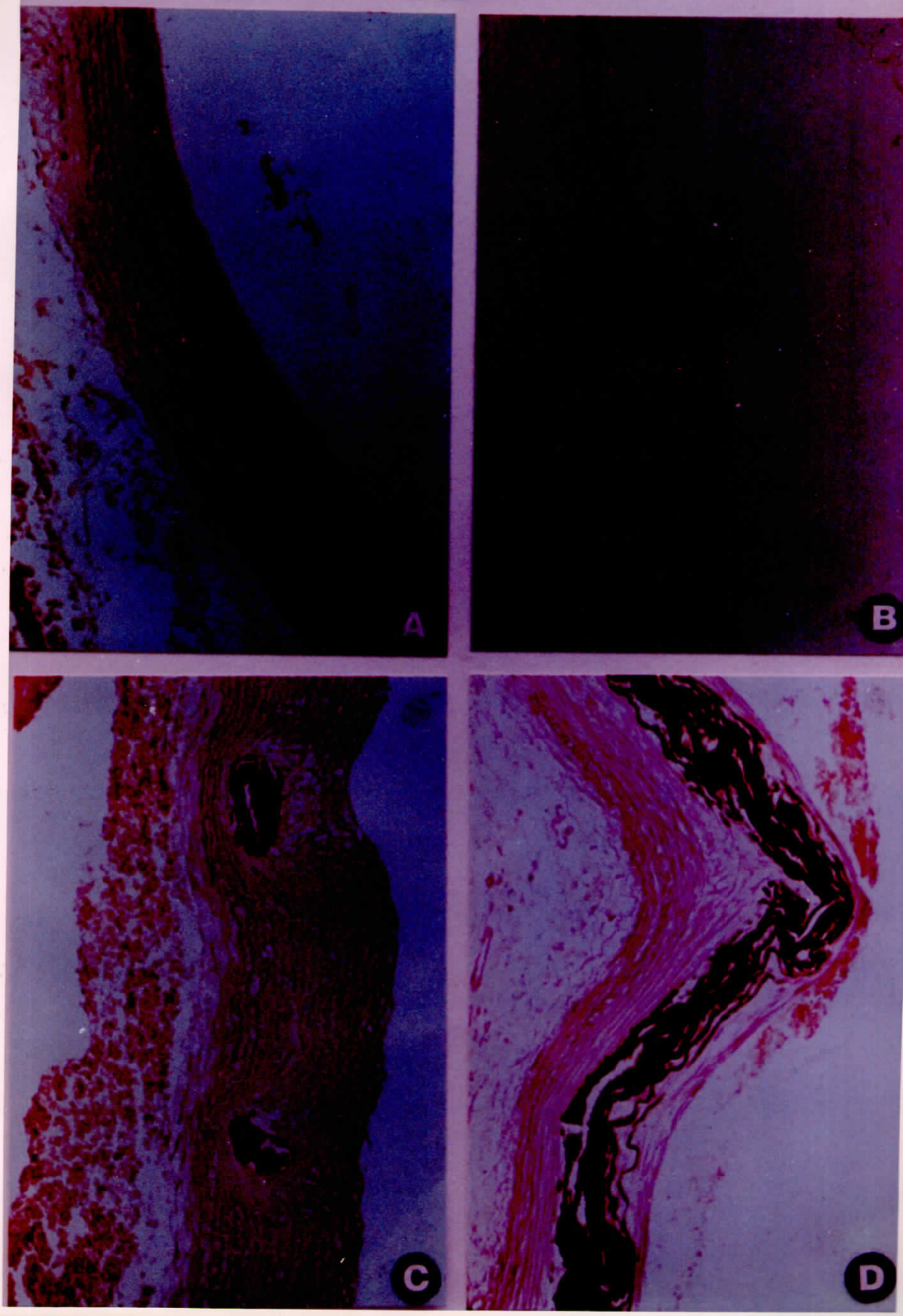


Figure 20

Electron micrographs of aortas of rabbits after periodic intramuscular injection of vitamin D_3 (Uranyl acetate-lead citrate).

a. Intact smooth muscle cells with intercellular junctions x 10,000.

b. Elastic fibers and smooth muscle cells in normal pattern x 17,000.

c. Calcified smooth muscle cells x 10,000

d. Bundles of collagen fibers x 20,000.

Figure 19

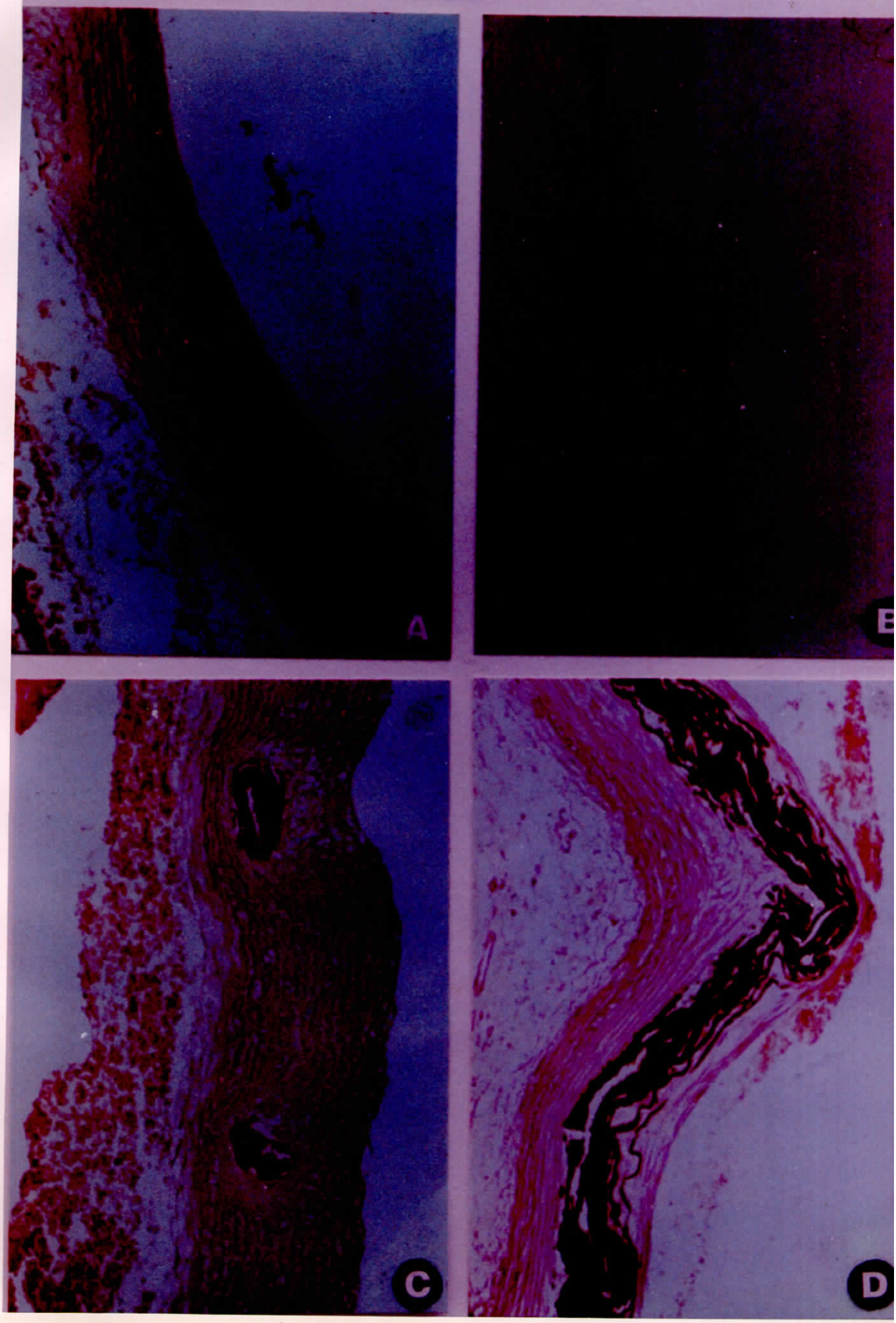
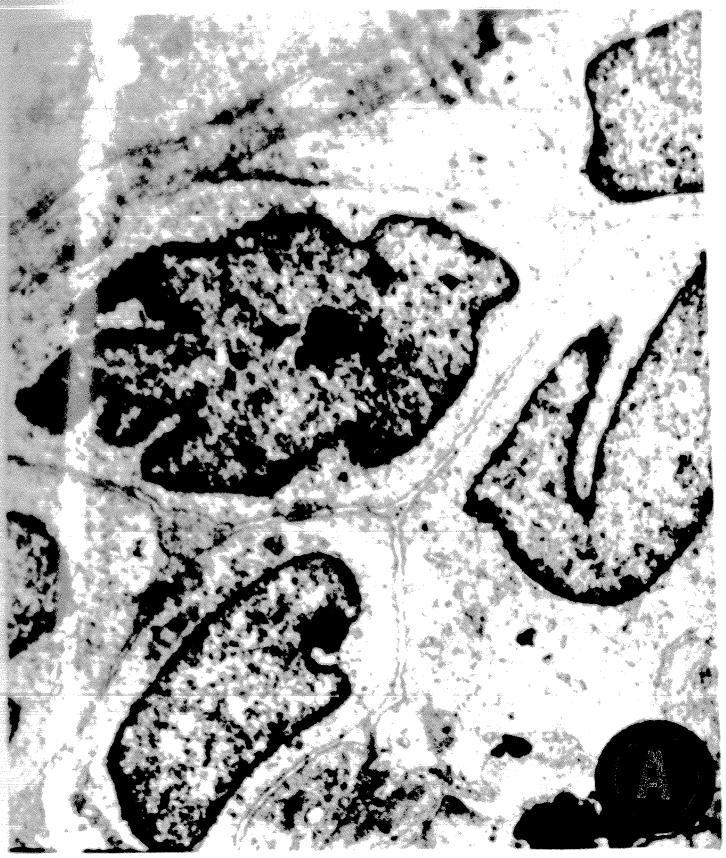
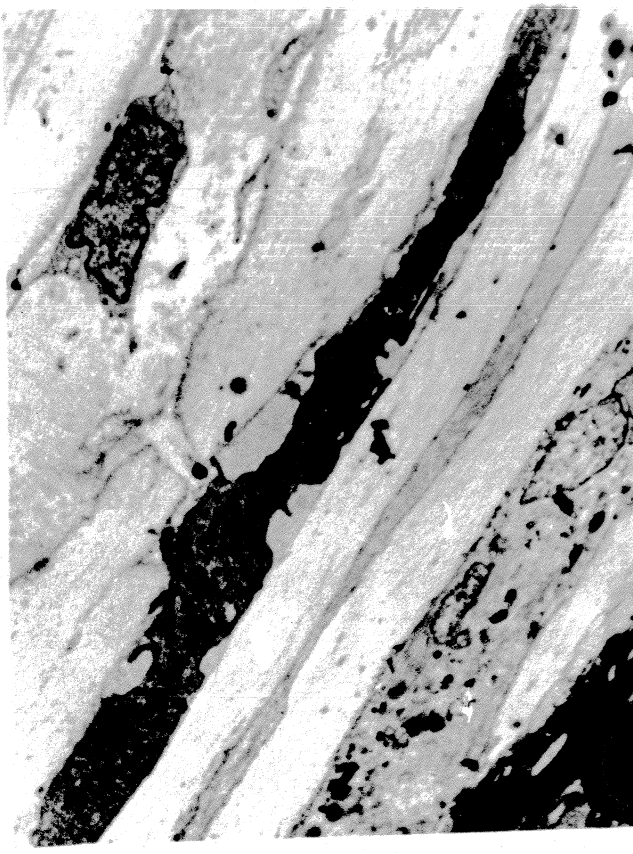


Figure 20





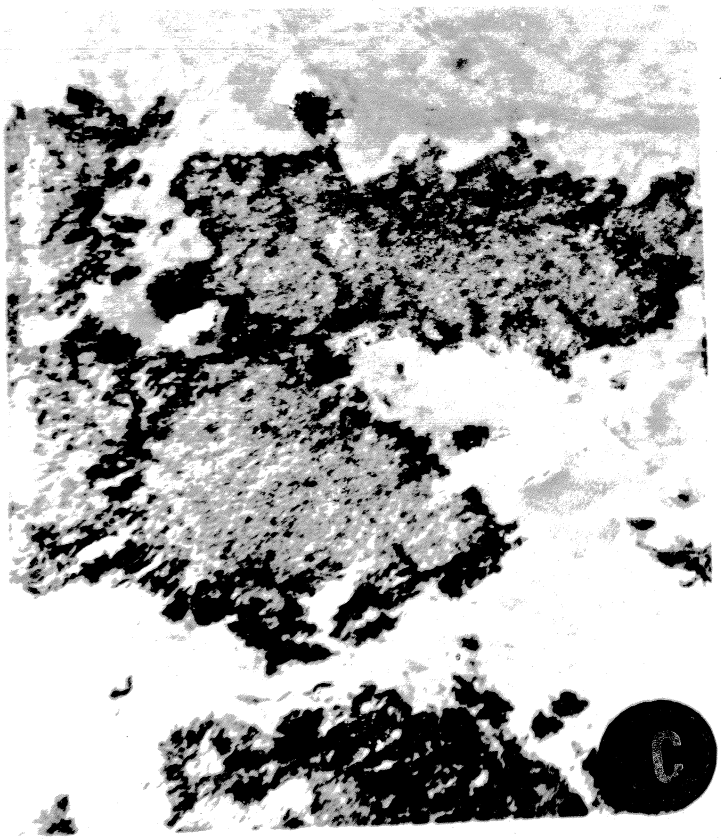


Figure 21

Electron micrographs of aortas of rabbits after periodic intramuscular injection of vitamin D_3 (Uranyl acetate-lead citrate).

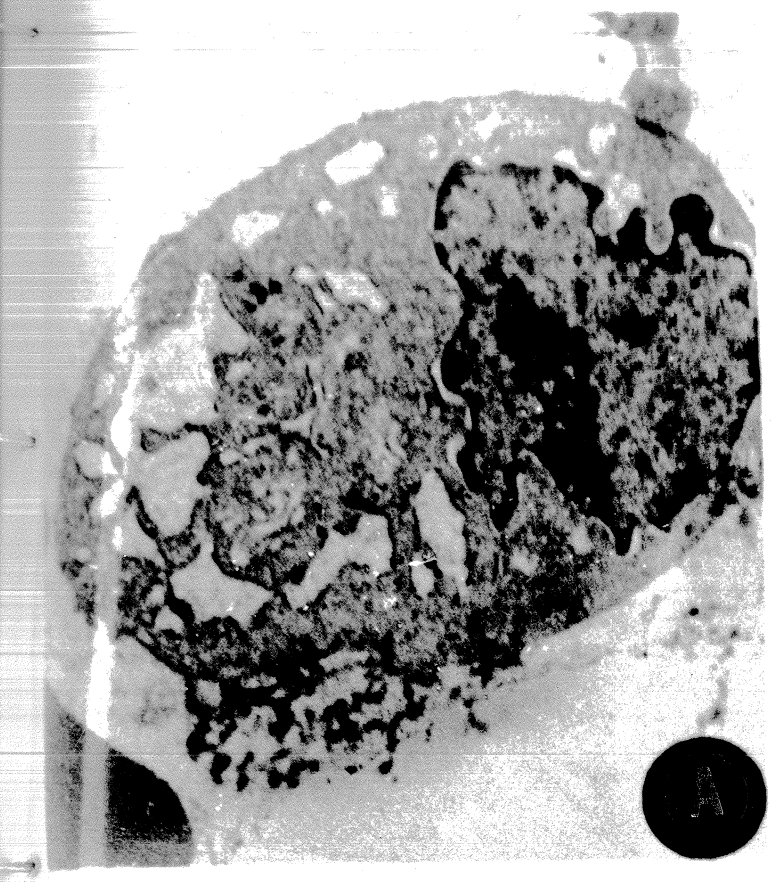
a. Degenerated smooth muscle cell with vacuolated mitochondria x 25,000.

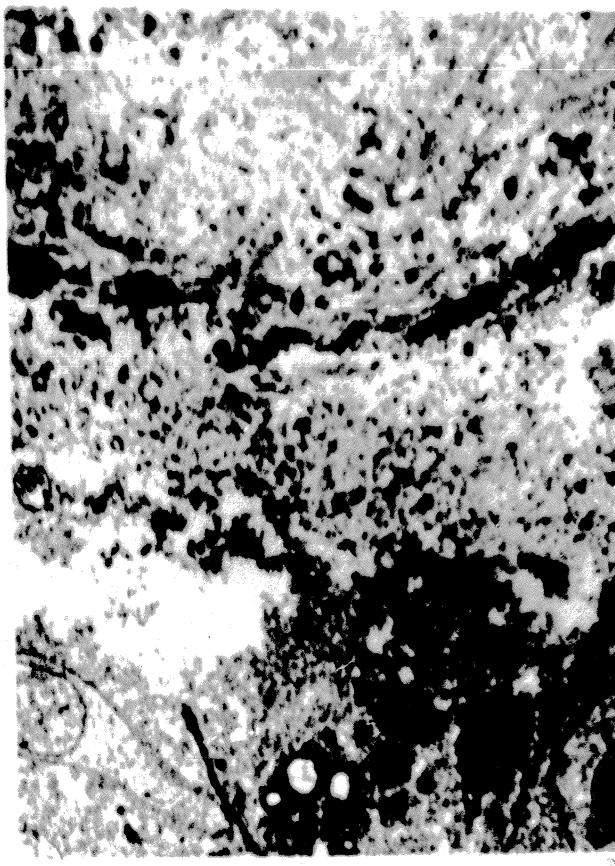
b. Cellular debrii in the media x 12,000.

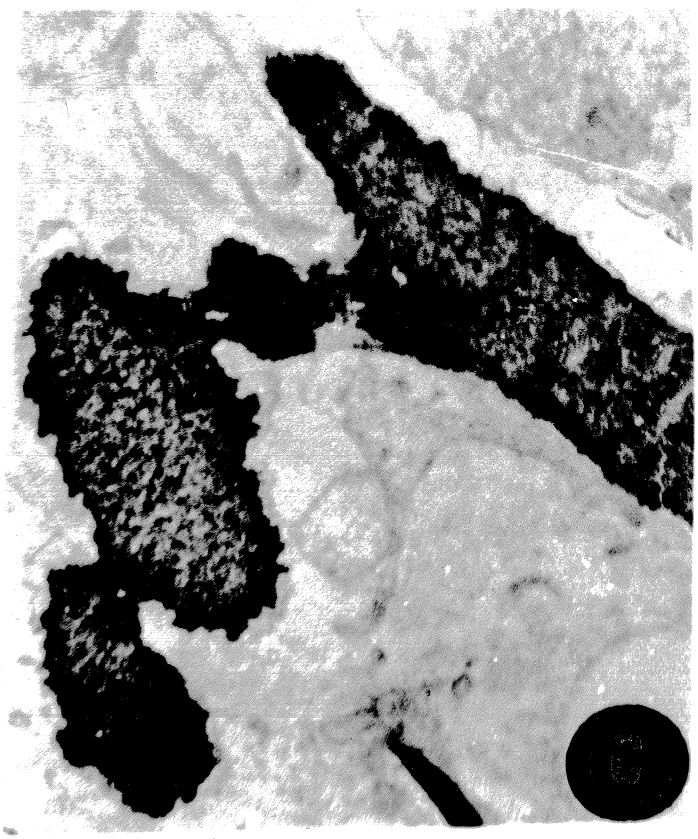
c. Calcified elastic fibers x 15,000.

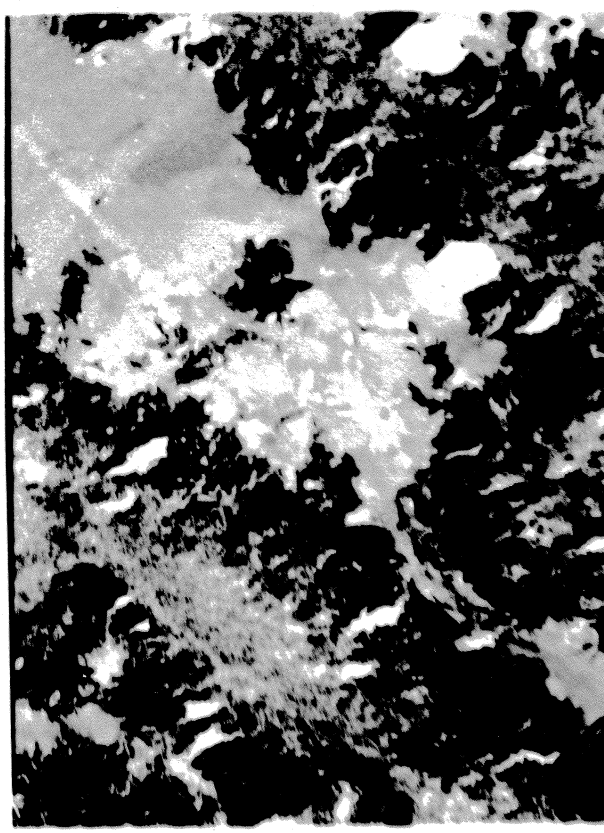
d. Calcified plaque x 30,000.

Figure 21









(Figure 21 b) and bundles of collagen were also seen (Figure 20 d). No ultrastructural lesions were observed in smooth muscle cells or elastic fibers of the aortas of control animals (Figures 20 a and b).

Biochemical parameters

The results of biochemical analyses of serum and aortas are given in table 4, table 5 and table 6. Serum levels of 25-OH-D_3 were seen increasing from week 1 to week 4 in animals, which received vitamin D_3. When compared with control animals (Group II) the animals in Group I had elevated serum levels of 25-OH-D_3 and calcium at the end of fourth week. Total cholesterol and magnesium levels were not significantly different. Serum levels of 25-hydroxyvitamin D_3 did not correlate with serum levels of cholesterol, triglyceride and high density lipoprotein. Serum 25-hydroxyvitamin D_3 levels were higher in rabbits with aortic calcification when compared with those rabbits without calcification in the aorta.

There was a significant increase in calcium levels in the aortas of test animals compared to that in control animals (Figure 22). Magnesium and lipid levels in the aortas were not significantly different (Figures 23 and 24). There was significant elevation of 1,25-dihydroxyvitamin D receptor (Figure 25) and an increase in calcium uptake in aortic smooth muscle cells of test animals when

Figure 22

Levels of calcium in the aortas of rabbits after periodic intramuscular injections of vitamin D_3

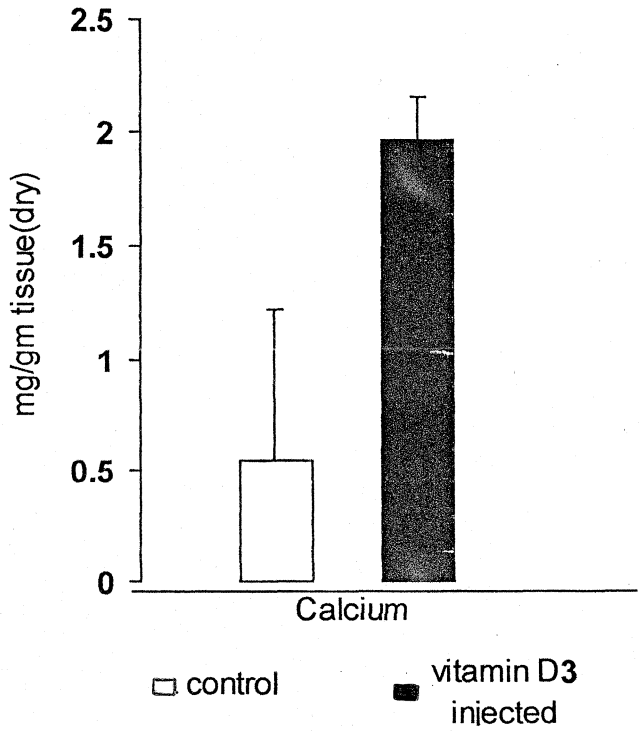


Figure 23

Levels of magnesium in the aortas of rabbits after periodic intramuscular injections of vitamin D_3

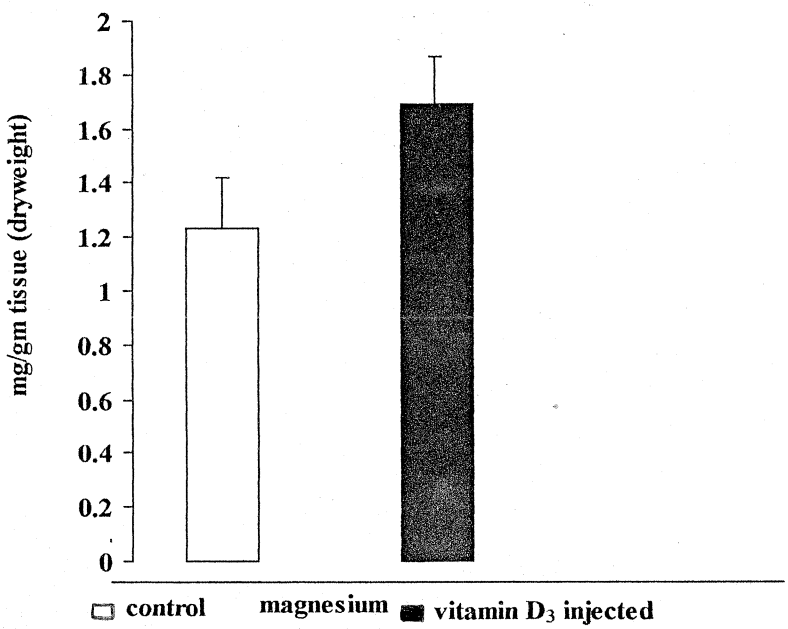


Figure 24
Total lipids in the aortas of rabbits after periodic intramuscular injections of vitamin D_3

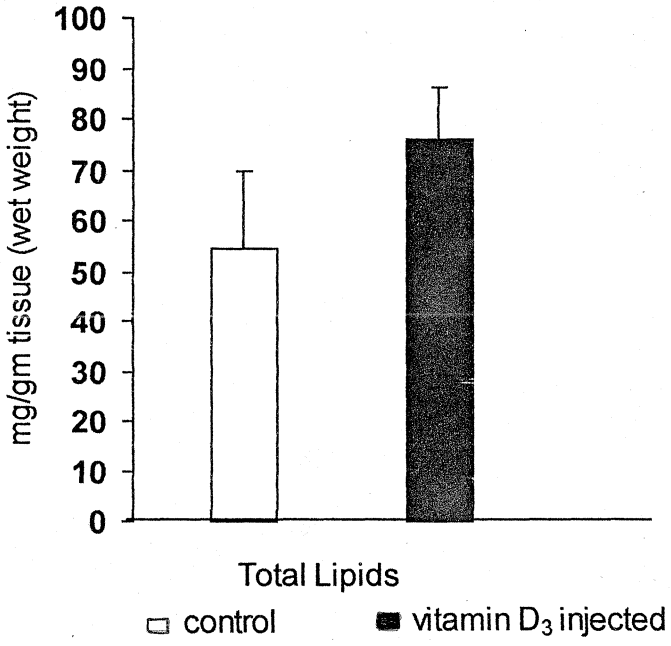


Table IV

Comparison of serum biochemical parameters in control rabbits (N=6) and rabbits given vitamin D_3 (N=14)

Parameters	Base line Level	wk1		wk2		wk3		wk4	
		C	T	C	T	C	T	C	T
25-OH- D3 ng/ml	67.8 ± 4.5	67.7 ± 1.7	87.7 ± 15.4	67.7 ± 8.9	105.3 ± 20.7	67.2 ± 7.1	108.7 ± 33.4	70.7 ± 3.1	180 ± 25.3
Calcium mg/dl	8.6 ± 1.9	7.5 ± 0.9	8.8 ± 1.9	8.6 ± 1.0	10.3 ± 2.7	7.9 ± 1.1	9.4 ± 2.2	9.4 ± 0.9	9.9 ± 2.7
Magnesium mg/dl	2.5 ± 0.4	1.9 ± 0.1	1.7 ± 0.5	2.3 ± 0.3	1.98 ± 0.5	1.98 ± 0.4	1.7 ± 0.2	2.8 ± 0.3	2.5 ± 0.5
Cholesterol mg/dl	105.4 ± 36.4	109.8 ± 25.9	110.1 ± 38.2	114.8 ± 35.1	143.8 ± 58.2	94.3 ± 10.7	116.9 ± 51.8	99.7 ± 24.8	112.0 ± 46.2
Triglyceride mg/dl	131.3 ± 26.8	107 ± 21.7	117 ± 27.3	89.3 ± 20.6	137.6 ± 48.4	98.5 ± 18.4	124.9 ± 46.7	91.7 ± 22.4	53.1 ± 30.7
High density Lipoprotein mg/dl	19.1 ± 4.4	13.5 ± 2.4	28.4 ± 12.8	26.2 ± 10.1	37.5 ± 14.3	17.3 ± 3.2	29.1 ± 15.2	17.17 ± 4.1	20.4 ± 10.8

Figure 25

Levels of $1,25(OH)_2\ D_3$ receptor in the aortas of rabbits after periodic intramuscular inject ions of vitamin D_3

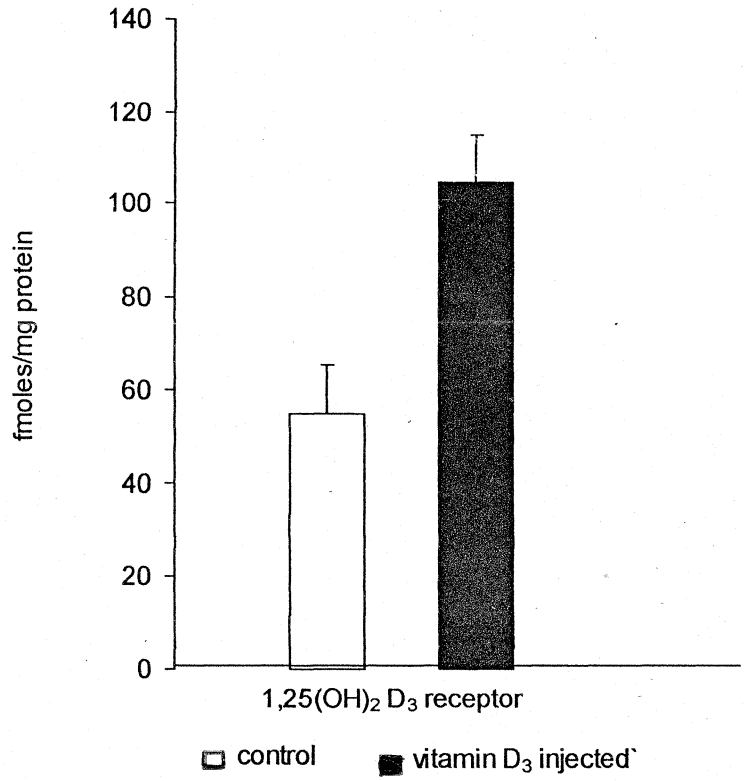


Figure 26

Kinetics of ^{45}Ca uptake by aortic smooth muscle cells of rabbits after periodic intramuscular administration of vitamin D_3

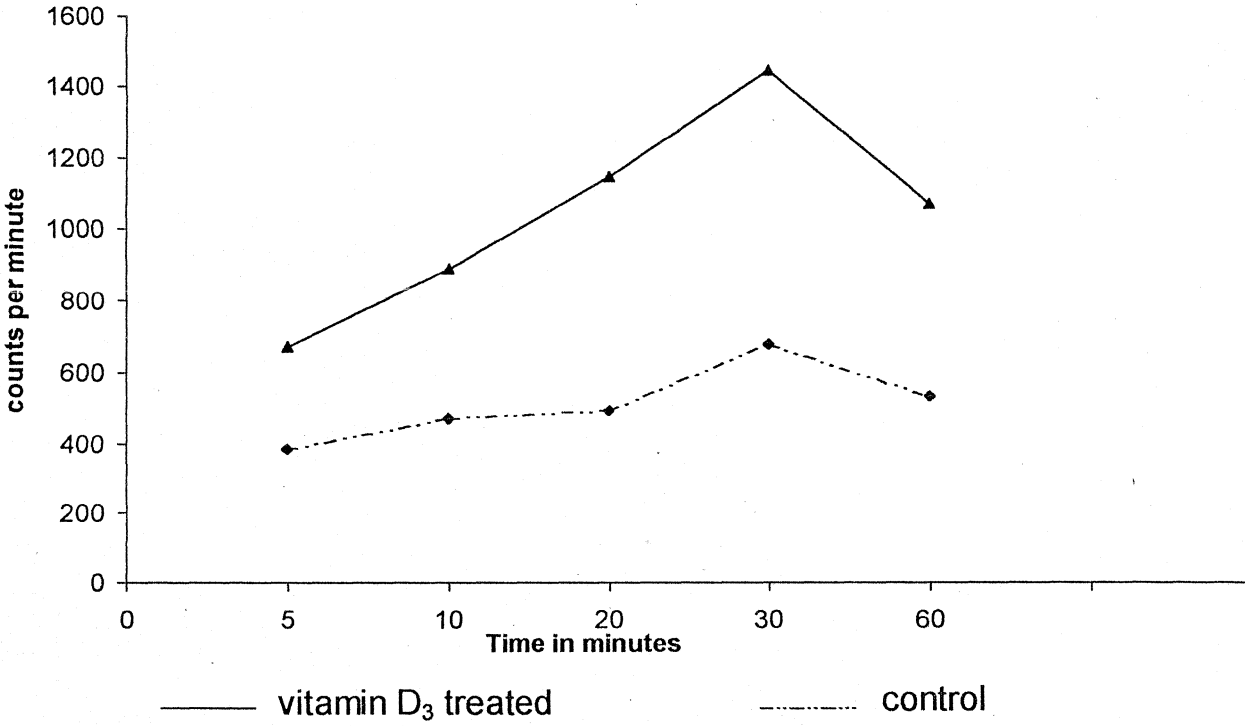


Table VI.

Comparison of biochemical parameters in aortas with calcification and aortas without calcification in rabbits given vitamin D_3

Variables	Group IA N= 4	Group IB N=10
Total lipids (mg/gm)	67.25 ± 22.7	79.6 ± 67.4
Calcium (mg/gm)	2.78 ± 0.87 *	1.53 ± 0.29
Magnesium (mg/gm)	2.05 ± 0.51	1.55 ± 0.71
1,25-dihydroxyvitamin D_3 receptor (fmoles/gm protein)	145.25 ± 13.02 *	96.64 ± 21.7
^{45} Ca uptake (cpm)	2534.6 ± 484.6 *	1016 ±738.4

Data are mean values ± S.D. *P < 0.05
Group IA - aorta with calcification Group IB - aorta without calcification

compared with control animals. Kinetics of ^{45} Ca uptake by the medial cells of aorta is shown in (Figure 26).

Calcium levels in aortas of test animals, correlated with 1,25- dihydroxyvitamin D_3 receptor as well as ^{45} Ca uptake.

In animals with calcification of the aorta, there was significant elevation of tissue calcium, 1,25-dihydroxyvitamin D_3 receptor and calcium uptake in the aortic medial cells than in animals without calcification. (Table 6)

CHAPTER V
DISCUSSION

CHAPTER V
DISCUSSION

Two models of arterial calcification were chosen in the present study: (1)atherosclerotic coronary artery disease (CAD) and coronary artery calcification in humans and (2) aortic calcification in animals induced by intramuscular injection of vitamin D. Inspite of several evidences from experimental studies that excess of vitamin D is arteriotoxic and can induce arterial calcification, the relationship between vitamin D status and arterial calcification in humans had not been explored when the present investigation was initiated. Very recently, two reports on correlation of serum levels of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) with the extent of coronary artery calcification were published. Coronary artery calcification in our patients with coronary artery disease did not correlate with serum levels of 25-hydroxyvitamin D_3 (25-OH-D_3) . Watson and colleagues (1997) reported an inverse correlation of $1,25(OH)_2D_3$ with the extent of vascular calcification in patients with CAD. They employed electron beam computed tomography for quantification of coronary calcification. Their results seem to suggest a protective role for vitamin D in vascular calcification. However, majority of the values reported by them are within the normal range.Serum 25-hydroxyvitamin D_3 , a serum marker for nutritional status is not documented.

Arad and coworkers (1998) on the other hand observed that serum concentrations of calcium, $1,25(OH)_2D_3$ and parathyroid hormone (PTH) did not correlate either with coronary calcification or the ratio of coronary calcification to the extent of coronary stenosis. They also measured the degree of coronary calcification using electron beam computed tomography.

High doses of vitamin D_3 when orally given to experimental animals do not always increase serum levels of $1,25(OH)_2D_3$ but levels of 25-OH-D_3 increases because $1,25(OH)_2D_3$ levels are closely controlled, its production being determined by parathyroid hormone (PTH), calcium and phosphate levels in blood. For evaluating vitamin D status, 25-OH-D_3 is considered to be the best index by most workers (Holick MF 1990). Therefore it is important to measure serum levels of 25-OH-D_3 to assess the status of individuals or animals. If serum levels of 25-OH-D_3 are increased beyond the normal range, vascular calcification may develop even within normal levels of $1,25(OH)_2D_3$ (Shepard RM and DeLuca H 1980). Hence we did not estimate serum levels of $1,25(OH)_2D_3$ and instead measured serum levels of 25-OH-D_3.

Fluoroscopic determination of calcification is not the most sensitive technique for detection of arterial calcification. Several recent studies reported

that use of digital subtraction cinefluoroscopy and electron beam computed tomography are superior to fluoroscopy in determining arterial calcification (Detrano RC et al., 1994; Wexler L et al., 1996 and Doherty TM et al., 1997). We used fluoroscopy for detecting the extent of calcification because of lack of access to superior methods.

An experimental study was conducted in rabbits to delineate the pathogenic mechanisms of arterial calcification. Out of 14 animals that received vitamin D_3 injections, 4 had extensive, gritty whitish calcified lesions with adherent thrombi in both abdominal and thoracic aorta. In another four animals aorta was dilated and intimal ulcers were also seen. Aneurysmal changes were also observed in animals, which had calcification. Microscopically histological abnormalities such as focal loss of elastic fibers and fragmentation and degeneration of elastic fibers and calcification in the media were seen.

Eisenstein and Groff (1957) had observed in rabbits challenged with 100,000 IU of vitamin D_3 twice in a week for 4 weeks, dense masses of medial calcification. Deposition of calcium appeared to be within elastic fibers. In another study, rabbits fed vitamin D_3 in conjunction with a cholesterol containing diet developed more severe atherosclerotic lesions than those rabbits fed a similar diet with omission of either vitamin D or cholesterol (Hass GM, Trueheart RE and Hemmens A 1960). Importantly, lesions similar to

human atherosclerosis were seen in monkeys receiving a combination of both cholesterol and vitamin D_3 (Pen SK et al., 1979) or cholesterol, vitamin D_3 and nicotine (Liu L B et al., 1979). Monkeys receiving D_3 alone had slightly increased thickening of the intima. Toda, Leszczynski and Kummerow (1983) noticed in rabbits fed vitamin D_3, macroscopic aortic lesions and extensive linear calcium deposition in the inner media of the aorta. Medial layer of the aorta contained calcium deposition around elastic fibers and in the cytoplasm of smooth muscle cells (SMCs). Toda and coworkers (1985) studied the effect of different dietary levels of vitamin D_3 on coronary arteries of Yorkshire swine. Severe degree of intimal thickening was observed in coronary arteries of animals fed the highest amount of vitamin D_3. Electron microscopic examination revealed a greater frequency of degenerated cells in the coronary arteries which did not have stainable lipid. Authors suggested a possible link between excessive daily intake of vitamin D_3 and the development of coronary atherosclerosis in humans. Fleckenstein and co workers (1994) produced severe degree of arterial calcium overload similar to Monckeberg's arteriosclerosis by administering overdoses of vitamin D_3 to rats and rabbits. In rats which were given vitamin D_3 and nicotine, severe calcium overload and pathological lesions similar to atherosclerosis were seen in the arterial wall. The lesions in our animals were not similar to atherosclerosis.

Possibly, this difference is because we did not use either hypercholesterolemic diet or give nicotine to the animals.

A fascinating finding in the aorta of rabbits given excessive vitamin D_3 in the present study is aneurysmal changes in the aorta. Norman, Wysocki and Lamawansa (1995) have hypothesized that vitamin D may be an aetiological factor in the pathogenesis of aortic aneurysms. They draw attention to the evidence from *in vitro* studies that vitamin D_3 inhibits production of elastin by SMCs. A consistent biochemical abnormality in aortic aneurysms is a significant reduction in the amount of elastin in arteries. Weber (1997) opines that vitamin D and or PTH mediated enhancement of metalloproteinase activity could contribute to elastic fiber degradation and together with the alkaline pH of tissue could predispose to calcium deposition.

We observed that calcified areas in the aortas of animals were also positive for the presence of iron. Moon, Brandy and Davison (1992) reported that in chicks orally given iron and fed a low calcium diet, vitamin D administration elevated the amount of iron in blood, liver and bone indicating an increase in iron absorption under the influence of vitamin D. He opined that vitamin D increased body iron stores and that iron overload can initiate free radical damage and contribute to atherosclerosis.

A consistent ultrastructural lesion, in the aortas of our rabbits fed excessive vitamin D_3 , was degeneration of SMCs in the media. Mitochondria in SMCs were vacuolated. Elastic fibers and SMCs were calcified. These observations are similar to those of Eisenstein and Zeruolis (1964) in vitamin D induced aortic calcification in rabbits. In vitamin D_3 treated animals, Pen et al. (1979) observed degenerated cells, cellular debris and proliferation of SMCs. Lipid deposition was not seen. With higher doses of vitamin D_3 intimal thickening was prominent and composed of SMCs, collagen fibers and lipid material. Extensive cellular damage, fragmentation and duplication of internal elastic membrane with spotty calcium deposits were also seen. Most striking changes were seen in those animals which received cholesterol and vitamin D_3 and the lesions closely, resembled atherosclerotic lesions seen in humans. Ito, Cho and Kummerow (1990) reported, in piglets fed a diet deficient in magnesium and supplemented with vitamin D_3, degeneration of SMCs and marked increase in the number of foam cells and macrophages. Toda, Leszczynski and Kummerow (1983) noticed in rabbits thickened intima which contained many degenerated SMCs and frequently dead SMCs characterized by mitochondrial swelling, dilation of endoplasmic reticulum and cytoplasmic vacuoles.

A significant elevation of $1,25(OH)_2 D_3$ receptor was found in aortic medial smooth muscle cells of the experimental animals in the present study

There was also significant elevation in ^{45} Ca uptake by the smooth muscle cells. Calcium level in the aorta correlated with 1,25(OH) $_2$ D $_3$ receptor as well as ^{45} Ca uptake.

Beckman et al. (1990) demonstrated in rats that up regulation of 1,25 $(OH)_2D_3$ receptor (VDR) occurs with vitamin D_3 excess. In their study, when rats were given high amounts of vitamin D_3, there was increase in plasma levels of 25-OH-D_3 and significant up regulation of intestinal VDR concentration. Koh et al. (1988) documented not only the presence of $1,25(OH)_2D_3$ receptor in rat coronary artery smooth muscle cells, but also change in shape and proliferation of SMCs *in vitro* under the influence of physiological levels of $1,25(OH)_2D_3$. There was also dose dependent enhancement of these effects at higher concentrations.

Hiruyuki (1988) observed that 1,25(OH)$_2$ D $_3$ stimulates Ca ATPase activity in a dose dependent manner at physiological concentrations. The stimulatory effect is exerted via genomic activation after binding to the receptor. Tsutomu and Hiroyuki (1988) showed that $1,25(OH)_2D_3$ stimulates ^{45} Ca uptake by vascular SMCs. The effect is dose dependent at physiological concentrations and specific for the sterol. An increase in calcium uptake in aortic myocytes of spontaneously hypertensive rats (SHR) but not in Wistar Kyoto normotensive rats was reported by Bukoski and Mc Carron (1987). In contrast to the above

in vitro investigations we have investigated the calcium uptake in the aortas removed from animals given excess vitamin D_3.

Most investigators have attributed increased calcium influx into tissue to the hypercalcemia that usually result from enhanced intestinal calcium absorption and bone resorption secondary to hypervitaminosis D (Norman AW 1979; Shepard RM, DeLuca HF 1980; Ganote CE et al.,1975). Hypercalcemia, however does not always accompany hypervitaminosis D (Holmes RP 1982; Gertner JM and Domenech M 1977). Hence some other mechanism must exist. The changes most directly correlated with hypervitaminosis D are increased levels of vitamin D metabolites in plasma, particularly 25-OH-D_3 and an increased deposition of 25-OH-D_3 and possibly other vitamin D metabolites in tissues. Once taken up by tissue cells, 25-OH-D_3 may promote increased membrane permeability to calcium by affecting cellular metabolic pathways. Alternatively, 25-OH-D_3 can directly incorporate into cellular membranes and alter their permeability to calcium(Holmes RP and Kummerow FA 1983). An increased calcium influx into cells would increase calcium sequestration by mitochondria and the endoplasmic reticulum, resulting in the formation of calcium deposits and organelle damage(Trump BJ, Berezesky IK, Osornio-Vargas AR 1981). An elevation in cytoplasmic calcium levels may occur perturbing many biochemical reactions regulated by calcium. The altered metabolism and organelle damage result in

cell necrosis and the formation of large calcium deposits, features, which are commonly observed with hypervitaminosis D.

Another suggestion is that 1,25-dihydroxyvitamin D_3 increases *in vitro* vascular calcification by modulating secretion of endogenous parathyroid hormone related peptide (PTHrP). Jono et al. (1998) investigated the effect of $1,25(OH)_2 D_3$ on calcification by bovine vascular smooth muscle cells *in vitro*. $1,25(OH)_2 D_3$ dose dependently increased bovine vascular SMC calcification and alkaline phosphatase activity in the cells. $1,25(OH)_2 D_3$ also decreased secretion of PTHrP by bovine vascular SMCs in a dose dependent manner and depressed PTHrP gene expression. Further, exogenous PTHrP antagonized the stimulatory effect of $1,25(OH)_2 D_3$ on bovine vascular SMCs. Finally, $1,25(OH)_2D_3$ in a dose dependent manner increased the expression of the osteopontin gene, one of the bone matrix proteins in bovine vascular SMCs, contributing to the stimulatory action on bovine vascular SMC by $1,25(OH)_2 D_3$.

Serum levels of 25-OH-D_3 were seen increasing in our animals given excessive vitamin D_3. There was significant difference in serum levels of calcium between rabbits given vitamin D and controls after four weeks. There was no statistically significant difference in serum cholesterol levels between both the groups.Our findings corroborate with those of Toda, Leszczynski and Kummerow(1983)in pigs and Ito, Cho and Kummerow (1990) in piglets. Pen

and coworkers (1979) however, observed that serum cholesterol significantly increases in those animals fed a combination of cholesterol along with vitamin D_3. Cholesterol levels in serum did not increase in the group of animals which were given only vitamin D_3.

In addition to the role of vitamin D in arterial calcification we also explored in patients the relationship of serum levels of 25-OH-D_3 with known risk factors for atherosclerotic heart diseases. In a case-control study, serum levels of 25-OH-D_3 was found significantly higher in patients with angiographic evidence of coronary artery disease (CAD) and acute myocardial infarction (AMI) when compared with controls.

Multivariate analysis after adjusting for confounding variables revealed that serum 25-OH-D_3 is an independent risk factor for coronary artery disease, with an odds ratio of 4.16.

Serum levels of 25-OH-D_3 correlated with calcium and magnesium levels in serum but not with serum levels of cholesterol, triglycerides and low-density lipoprotein. Serum vitamin D_3 levels however, inversely correlated with high-density lipoprotein. Serum 25-OH-D_3 levels did not have any association with diabetes but was positively associated with history of smoking.

Scragg et al., (1990) also found no correlation of serum levels of 25-OH-D_3 with serum cholesterol levels in a study of 179 patients with coronary artery diseases. Our finding that patients with AMI have high serum levels of

25-OH-D_3 is contradictory to that of Schmidt et al., (1977) who observed no difference in serum 25-OH-D_3 levels between patients with AMI and controls. In a community based case-control study an inverse association between plasma 25-OH-D_3 and myocardial infarction has been reported (Scragg R et al., 1990). There has not been any effort in the above mentioned two studies to correlate vitamin D levels with major risk factors for coronary artery disease or for sub group analysis to relate vitamin D levels with coronary artery calcification. They have also not done any multivariate analysis to adjust for confounding variables.

Our patients with angiographic evidence of coronary artery disease also had higher serum levels of low-density lipoprotein and triglycerides and lower serum levels of high-density lipoprotein when compared with controls. These findings are consistent with observations in patients with coronary heart disease by other workers (Gordon T et al., 1977; Austin MA et al., 1988; Assmann G and Schulte H 1992; Hokanson JS and Austin MA 1993; Jeppesen et al., 1998).

Our accidental discovery that serum levels of 25-OH-D_3 is an independent risk factor for CAD could possibly be significant in a tropical environment.

Coronary artery disease is a major cause of death all over the world. South Asians, however, have been found to have the highest mortality rates

due to CAD amongst all ethnic groups so far studied (Mc Keigu PM 1992). The magnitude of the problem of coronary artery disease in the Indian sub continent and whether there are any differences among various geographical regions are unknown. Studies done in India suggest that the prevalence of CAD in our population is about 10% (Chadha S et al., 1990) four times the prevalence of CAD in the United States of America (Destafano F et al.,1993). There is also a difference in the age group of the affected population. Coronary artery disease is more common in the younger South Asian population compared to other ethnic groups (Singh RB et al., 1998). Atherosclerotic narrowing in their coronary arteries is reported to be more severe and extensive (Enas EA and Mehta J 1995). When prevalences of smoking, blood pressure and diabetes in the Indian subcontinent are compared with the prevalences of these factors in other ethnic groups, no significant differences are found in the prevalence rates. Though a clear association between cholesterol levels and the rate of coronary artery disease has been demonstrated within and between populations, the serum cholesterol levels among South Asians have been found to be lower than the levels in other ethnic groups so far studied (Singh R B et al., 1998). Recently Uffe Ravinoskov (1998) reviewed a large number of investigations which included cohort, cross sectional, case control and ecological studies as well as randomised trials on the effect of fat reduction in patients with CAD.

He draws attention to important limitations in the data to support a role for dietary saturated fatty acids and polyunsaturated fatty acids in cardiovascular diseases. He questions the suggestion that dietary saturated fatty acids are harmful and that dietary polyunsaturated fatty acids are protective in atherosclerotic cardiovascular disease. Studies by Krishnaswamy and colleagues (1989; 1994) from Vellore in South India reveal that CAD is associated with low serum levels of lipids in Indian patients. Diffuse disease occurs at an earlier age than in the western populations. Coronary artery disease is seen even when lipid levels are not high. They observed a weak correlation between risk factors and severity of CAD. Krishnaswamy et al. (1989) opine that "presently known risk factors for CAD in no way explain the severity of the disease. Other factors, yet to be ascertained, could contribute additionally to atherosclerosis in our patients". While one unifying factor does not explain the differences between South Asians and the western population it appears that both genetic and environment might play their roles in the causation of CAD in South Asians. Several contributing factors have been proposed. Lipoprotein (a), a genetically determined lipoprotein, which not only promotes the early development of atherosclerosis but also of thrombosis, has been found to exist in higher proportions among the South Asians than among other ethnic groups and is considered to be a strong independent risk factor for coronary

artery disease (Enas EA and Mehta J 1995). Though South Asians as a group are not more obese than other ethnic groups as assessed by their body mass index (BMI), South Asians are more predisposed to develop abdominal obesity resulting in an increased waist to hip ratio (Mc Keigue PM, Shah B and Marmot MG 1991). Abdominal obesity is considered to be a strong independent risk factor for coronary artery disease among South Asians and may explain the high mortality due to coronary artery disease in this ethnic group.

Insulin resistance syndrome is characterized by impaired glucose tolerance or non insulin-dependent diabetes mellitus, increased waist to hip ratio, hypertriglyceridemia, low high density cholesterol levels, hypertension and an increased predilection for coronary artery disease. Dhawan (1996) observed that in South Asians living in the United Kingdom there is a correlation of insulin resistance syndrome with an increased risk of CAD, when compared to Caucasians. The predisposition to insulin resistance and its metabolic abnormality seems to be genetically determined.

There are evidences to suggest that chronic infection by *Helicobacter pylori, Cytomegalovirus or Chlamydia pneumonia,* may predispose to coronary heart disease (Danesh J, Collins R and Peto R 1997). The epidemiological evidence for *Chlamydia pneumonia* and vascular disease is strong, and the organism is able to infect human smooth muscle cells,

endothelial cells, and macrophages (Gupta S and Camm AJ 1997), although it is unclear if *C.pneumonia* causes coronary artery disease or is just present in the tissues. The possibility that chronic infections set in motion a process of inflammation is undeniable and the "response to injury" hypothesis argues that many of the changes that characterize the later stages of atherosclerosis and plaque formation are inflammatory in nature (Libby P 1997).

Results of the present study suggest that vitamin D may play a role as one of the causal risk factors for CAD in our patients. The causes of coronary artery disease are most likely to be multi factorial and it would be naïve to suggest that one factor alone is the causative agent. Since conventional risk factors have not been found to explain the high rates of CAD in South Indian population, the role of vitamin D needs to be considered. A link between serum 25-hydroxyvitamin D_3 levels and coronary artery disease in the tropical population can be established. Excessive UV exposure in the tropical environment can be a contributing factor in enhanced synthesis of vitamin D_3, elevated serum levels and progression of coronary artery disease. As vitamin D is not an essential dietary component for the vast majority of people, a relatively small excess may be toxic in the long term. An average sized adult probably needs a 100 or 200 units of vitamin D_3 a day from all sources including sunshine and a 10 kg baby requires about a hundred units of the vitamin. Holick (1992) has pointed out that brief casual exposure of the face, arms and hands to sunlight is equivalent to ingesting 200 IU (5 μg) of vitamin

D and repeated solar exposure (causing mild erythema) raises plasma 25-OH-D_3 concentrations as much as does the long term ingestion of 10,000 IU (250 μg) of vitamin D per day. Loomis (1967) calculated on this basis that mere exposure of the skin of the cheeks of fair, thin-skinned children provides about 400 units daily, a conclusion confirmed by Hollick , Mac Laughlin and Doppeet (1981).

Our study was prompted by earlier observations that serum 25-OH-D_3 levels are enhanced after short-term ultraviolet exposure either from natural sunlight (Sedrani SH, Elidrissy T, El Arabi KM 1983) or artificial ultravilolet irradiation (Matsuoka LY, Wortsman J, Hollis BW 1990). Increased plasma levels of 25-OH-D_3 has been reported in two individuals exposed to tropical sunlight during a vacation of one week (Shepard RH et al. ,1979). In another study, the average circulating levels of 25-OH-D_3 were found to be raised in life guards with a minimum of four weeks of prolonged exposure to sunlight (Haddad JG and Chyu KJ 1971). Skin pigment melanin is believed to act as a protective barrier against sunlight and UV exposure. Observations in Negroes and Asian subjects following repeated exposure to UV radiation suggest that pigmentation has no significant effect on cutaneous synthesis of vitamin D (Stamp TCB 1975). Clemens and coworkers (1982) also found that increased pigmentation is not an absolute barrier to vitamin D synthesis. The melanogenic response may be insufficient to inhibit cutaneous synthesis of

vitamin D in persons who have continuous and prolonged or repeated solar exposure as in tropical regions. The inborn variation in a man's ability to metabolize vitamin D and the possible variations in the range of individual reactivity to vitamin D may determine the susceptibility for coronary artery disease.

The above evidences are significant to the hypothesis that vitamin D is a risk factor for CAD in the tropics.

To summarize, this study demonstrates that (I) serum levels of 25-OH-D_3 are elevated in patients with coronary artery disease and (2) high levels of serum 25-OH-D_3 are arteriotoxic and increase 1,25-dihydroxyvitamin D_3 receptor expression and calcium uptake in aortic smooth muscle cells as well as induce calcification and aneurysms in the aorta of rabbits.

Given the earlier observations that increase in calcium in the vessel walls is an early event in atherosclerosis and that vitamin D together with cholesterol and nicotine can bring about typical atherosclerotic lesions in animals, the role of vitamin D in the causation of atherosclerotic vascular disease needs to be further explored.

CHAPTER VI

SUMMARY AND CONCLUSION

CHAPTER VI

SUMMARY AND CONCLUSION

The objectives of the present study were to explore the role of vitamin D in arterial calcification. The investigation was undertaken because of increasing interest in the mechanisms of arterial calcification and the suggestion that arterial calcification is not a passive phenomenon but possibly a regulated metabolic process similar to osteogenesis.

Serum levels of 25-OH-D_3 were measured in 143 male patients with either angiographic evidence of coronary artery disease or acute myocardial infarction diagnosed by WHO criteria and 70 male control subjects by competitive protein binding radio ligand assay. A case - control design and 't' test were employed to assess whether the vitamin D_3 levels were significantly different in patients with coronary artery disease (CAD). Whether there is difference in serum 25-OH-D_3 levels between patients with coronary artery calcification and those without calcification was probed. Twenty five hydroxyvitamin D_3 levels in serum were also correlated with serum lipid parameters, and other major risk factors for CAD as well as serum levels of calcium, magnesium and inorganic phosphate.

Pearson's method was used for determining correlation coefficients. Using Chi Square method association among different parameters was also checked.

In an experimental model of arterial calcification, produced by repeated intramuscular injections of excessive cholecalciferol (vitamin D_3) to rabbits, the relationship of serum levels of 25-OH-D_3 to serum lipid levels, lipid and calcium levels in aorta and morphological lesions in aorta were examined. Smooth muscle cells from the media of aorta of experimental animals were isolated and levels of 1,25 dihydroxyvitamin D_3 (1,25(OH)$_2$ D$_3$) receptor and calcium uptake were assayed to study the mechanisms of arterial calcification.

Significant results from the study are given below

(1) No difference was found in serum levels of 25-OH-D_3 between patients with coronary artery calcification detected by fluoroscopy and those without coronary artery calcification.

(2) Serum levels of 25-OH-D_3 was discovered to be an independent risk factor for coronary artery disease, with an odds ratio of 4.16.

(3) Serum levels of 25-OH-D_3 were inversely related to serum levels of high density lipoprotein (HDL) cholesterol and had a positive association with history of smoking.

(4) Intramuscular injections of excessive vitamin D_3 led to calcification and aneurysmal changes in aortas of rabbits. Serum levels of 25-OH-D_3 were elevated in these animals, but had no relationship to serum lipid levels. Histological lesions comprised of fragmentation and loss of elastic fibers in the media.

(5) 1,25-dihydroxyvitamin D_3 receptor levels were up regulated and calcium uptake enhanced in aortas of animals which were given excessive vitamin D_3

The evidences gathered suggest that excess of vitamin D is arteriotoxic and can induce arterial calcification through up regulation of $1,25(OH)_2D_3$ receptor and excessive calcium uptake in smooth muscle cells in arteries. Given the earlier observations that increase in calcium in vessel wall is an early event in atherosclerosis and that a combination of overdose of vitamin D, cholesterol and nicotine can produce atherosclerotic lesions in animals, the high levels of 25-OH-D_3 observed in our patients with CAD is possibly of pathogenic significance. Our findings support the hypothesis that vitamin D related arterial damage may be involved in the onset of atherosclerosis.

The study has some drawbacks. We have in our patients assayed only the levels of 25-OH-D_3 in the serum and not the levels of $1,25(OH)_2D_3$, the most active metabolite of vitamin D. We have also not determined the levels of parathyroid hormone (PTH), the regulatory hormone. Fluoroscopy was employed for diagnosing coronary artery calcification in patients. Fluoroscopy is not the most sensitive technique for detection of arterial calcification. Fluoroscopy was used because of lack of access to other superior methods such as digital subtraction cinefluoroscopy and electron beam computed tomography.

In the experiment in rabbits, we did not examine coronary arteries and hence could not relate coronary artery lesions with vitamin D status in animals.

There are a few salient clues from the present investigation, for further research. Whether the elevated serum levels of 25-OH-D_3 observed in patients with CAD are related to prolonged solar exposure that can occur in a tropical environment is to be probed. The mechanistic basis for vitamin D related susceptibility for coronary artery disease, which may involve genetic polymorphism in vitamin D receptor requires exploration. Hypervitaminosis D associated elastolysis and aneurysmal lesions in animals are possibly a good model to search for pathogenenic mechanisms in arterial aneurysms. It is likely that vitamin D may be an unrecognized causative factor in the pathogenesis of aortic aneurysms in humans and this possibility demands scrutiny.

BIBLIOGRAPHY

BIBLIOGRAPHY

Agatston As, Janowitz WR, Hildner FJ, Lusmer WR, Viamonta M, Detrano R. Quantification of coronary artery calcium using ultrafast computed tomography. Am Coll Cardiol 1990; 15:827-32.

Alberts B, Bray D, Lewis J, Raff M, Roberts K , Watson JD. Steroids and the cell. In : The molecular biology of the cell. New York, Garland , 1983.

Amati GD, Silver MD. Atherosclerosis of the aorta and its complications. In: Silver MD (ed). Cardiovascular Pathology. New York, Churchill Livingstone Inc., 1991; 267-306.

Anderson HC. Mechanism of mineral formation in bone. Lab invest 1989; 60: 320 - 30.

Anderson HC .Mechanisms of pathologic calcification. Rheum Dis Clin North Am 1988; 14: 303 - 13.

Anderson HC. Calcific disease. A concept. Arch Pathol Lab Med 1983; 107: 340- 48.

Arad Y, Spadaro LA, Roth M, Scordo J, Goodman K, Sherman S, Lerner G Newstein D. Serum cocentration of calcium, 1,25- vitamin D and parathyroid hormone are not correlated with coronary calcifications. An electron beam computed tomography study. Coron Artery Dis 1998; 9: 513- 18.

Association of Official Agricultural Chemists. Official Methods of Analysis. 5th ed. Washington DC, AOAC, 1960; 650-675.

Association of Official Analytical Chemists. Official methods of analysis 12th ed. Washinton DC, AOAC, 1975; 885.

Bajwa GS, Morrison LM, Ershoff BH. Induction of aortic and coronary athero-arteriosclerosis in rats fed hypervitaminosis D cholesterol- containing diet.Proc Soc Exp Biol Med 1971;138: 975-82.

Beckman MJ, Horst RH, Reinhardt TA, Beitz DC. Upregulation of the intestinal 1,25-dihydroxyvitamin D_3 receptor during hypervitaminosis D. A comparison between vitamin D_2 and vitamin D_3. Biochem Biophys Res Comm 1990;169: 910-15.

Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959; 37: 911- 17.

Boland RL. Plant as a source of vitamin D_3 metabolites. Nutr Rev 1986; 44:1-8

Bostrom K, Watson KE, Horn S, Wortham C, Horman M, Demer LL. Bone morphogenetic protein expression in human atherosclerotic lesion. J Clin Invest 1993; 91: 1800-09.

Bostrom K, Watson KE, Stanford WP, Demer L.. Atherosclerotic calcification: Relation to developmental osteogenesis. Am J Cardiol 1995; 75: 88B-91B.

Breen JF, Sheedy DF, Schwartz RS, Stanson AW, Kaufmann RB, Moll PP, Rumberger JA. Coronary artery calcification detected with ultrafast CT as an indication of coronary artery disease. Radiology 1992; 185: 435-39.

Brighton CT, Lorich DG, Kupcha R, Rully TM, Jones AR, Wood RA. The pericyte as a possible osteoblast progenitor cell. Clin Orthop 1992; 275: 287-99.

Bukoski RD, Mc Carron DA. Effect of 1,25(OH)2 vitamin D_3 and ionized Ca^{2+} on ^{45}Ca uptake by primary cultures of aortic myocytes of spontaneously hypertensive and Wistar kyoto normotensive rats. Biochem Biophys Res Commun 1987; 146 : 1330 - 35.

Chadha S, Radhakrishnan S, Ramachandran K, Kaul U, Gopinath N. Epidemiological study of coronary heart disease in urban population of Delhi. Indian J Med Res 1990; [B] 92:424-30.

Chen TC, Adrian KT, Holick MF. Methods for the determination of the circulating concentrations of 25-hydroxyvitamin D. J Nutr Biochem 1990; 1: 315-19.

Christakos S, Norman AW. Studies on the mode of action of calciferol. Evidence for a specific high affinity binding protein for 1,25-dihydroxyvitamin D_3 in chick kidney and pancreas. Biochem Biophys Res Commun 1979; 89: 56-63.

Clemens TL, Henderson SL, Adams JS, Holick MF. Increased skin pigment reduces the capacity of skin to synthesise vitamin D_3. Lancet 1982; 1: 74-76

Crowfoot D, Dunitz JD. Structure of calciferol. Nature (London). 1948;162: 608-610.

Danesh J, Collins R, Peto R. Chronic infections and coronary heart disease: is there a link ? Lancet 1997; 350: 430-36.

Davies H. Coronary Heart Disease. The significance of coronary pathology in infancy and the role of mitogens such as vitamin D. Medical Hypothesis 1989; 30: 179 - 85.

Davie M, Lawson DEM. Assessment of plasma 25-hydroxyvitamin D_3 response to ultraviolet irradiation over a controlled area in young and elderly subjects. Clin Sci 1980; 58: 235-42.

Destafano F, Merrit RK, Anda RF, Carper ML, Eaker ED. Trends in nonfatal coronary heart disease in the United States, 1980 through 1989. Arch Int Med 1993; 153: 2489-94.

Detrano R, Salcedo EE, Hobbs RE, Yiannikas J. Cardiac cinefluoroscopy as an inexpensive aid in the diagnosis of coronary artery disease. Am J Cardiol 1986; 56; 1041-46.

Detrano R, Markovic D, Simp Fendorfer C, Franco I, Hollman J, Gregera F, Stewart W. Digital subtraction fluoroscopy, a new method of detecting coronary calcification with improved sensitivity for the prediction of coronary disease. Circulation 1985; 71: 725-32.

Detrano R, Froelicher VF. A logical approach to screening for coronary artery disease•Arch Int Med 1993; 153: 2489-94.

Detrano RC, Wong ND, Tang W et al. Prognostic significance of cardiac cinefluoroscopy for coronary calcific deposits in asymptomatic high risk subjects. J Am Coll Cardiol 1994; 24: 354-58.

Dhawan J. Coronary heart disease risks in Asian Indians. Curr Opin Lipidol 1996; 7: 196-98.

Doherty TM, Detrano RC. Coronary arterial calcification as an active process, a new perspective on an old problem. Calcif Tissue Int, 1994; 54: 224-30.

Doherty TM, Tang W, Dascalos S, Watson KE, Demer LL, Shavelle RM, Detrano RC. Ethnic origin and serum levels of 1, 25-dihydroxyvitamin D_3 are independent predictors of coronary calcium mass measured by electron-beam computed tomography. Circulation. 1997; 96, 1477-81.

Donath W, De Langen C. Vitamin D sclerosis of the arteries and the danger of different forms of arteriosclerosis. Acta Med Scand 1956; 156:317-325.

Edelman GM, Jones PS. Outside and downstream of the home box. J Biol Chem 1993; 268: 20683-86.

Eisenstein R, Ellis J, Jacqueline R. In vitro studies of Vitamin D induced aortic calcification . Proc Soc Exp Biol Med 1969; 132: 58-62.

Eisenstein R and Groff W. Experimental hypervitaminosis D: Hypercalcemia, hypermucoproteinemia and metastatic calcification. Proc Soc Exp Biol Med. 1957; 94: 441-44.

Eisenstein R, Zeruolis BS . Vitamin D induced aortic calcification. An electron microscopic study. Arch Pathol 1964; 77: 27-35.

Eisman JA, Shepard RM, De Luca HF. Determination of 25 hydroxyvitamin D_2 and hydroxyvitamin D_3 in human plasma using high pressure liquid chromatography. Anal Biochem 1977; 80:298-305.

Emtage JS, Lawson DEM, Kodicek E. Vitamin D induced synthesis of mRNA for calcium binding protein. Nature 1973; 246: 100-101.

Enas EA, Mehta J. Malignant coronary artery disease in young Asian Indians: Thoughts on pathogenesis, prevention and therapy. Clin Cardiol 1995;18:131-35.

Fiske CH, Subbarow Y. The colorimetric determination of phosphorous. J Biol Chem 1925; 66: 375-400.

Fitzpatrick LA, Severson A, Edwards WD, Ingram RT. Diffuse calcification in human coronary arteries, association of osteopontin with atherosclerosis. J Clin Invest 1994; 94: 1597-604.

Fleckstein GG, Thimm F, Frey M, Czirfusz A. Role of calcium in arteriosclerosis-Experimental evaluation of antiarteriosclerosis potencies of Ca antagonists. Basic Research in Cardiology. (Suppl 1). 1994; 89: 145-59.

Franceschi RT, Simpson RV, Deluca HF, Binding protein for vitamin D metabolites. Serum carriers and intracellular receptors. Arch Biochem Biophys 1981; 210: 1-13.

Fraser DR, Kodicek E. Unique biosynthesis by kidney of a biologically active vitamin D metabolite. Nature 1970; 228: 764-66

Fraser DR. Regulation of the metabolism of vitamin D. Physiol Rev 1980; 60: 551-613.

Freedman FW, Roberts W. Vitamin D and the supravalvar aortic stenosis syndrome. Circulation 1966; 34: 77-86.

Fuster V. Human lesion studies. Ann NY Acad Sci 1997; 811: 207-25.

Giachelli CM, Bae N, Almeida M, Denhardt DT, Alpers CE, Schwartz SM. Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. J Clin Invest 1993; 92:1686-96.

Gimblet EG, Marney AF, Bonsnes RW. Determination of calcium and magnesium in serum, urine, diet and stool by atomic absorption spectrophotometry. Clin Chem 1967;13: 204-14.

Gotlieb AT, Havenith MG. Atherosclerosis: Lesions and pathogenesis. In: Silver MD (ed). Cardiovascular Pathology, New York, Churchill Livingstone Inc., 1991, 225-65.

Gupta S, Camm AJ. Chlamydia pneumoniae and coronary heart disease. Br Med J 1997; 314: 1778-79.

Haddad JG, Chyu KJ. Competitive protein binding radio assay for 25-hydroxy cholecalciferol. J Clin Endocrinol 1971; 33: 992-95.

Hass MG, Trueheart RE, Hemmens A. Experimental arteriosclerosis due to hypervitaminosis D. Am J Pathol 1960; 37: 521-47.

Hass M G, Trueheart RE, Taylor BC, Stump M. An experimental histological study of hypervitaminosis D. Am J Pathol 1958; 34: 395-431.

Hausler MR, Mc Cain T. Basic and clinical concepts related to vitamin D metabolism and action. N Eng J Med 1977; 297: 974-83 & 1041-50.

Henry HL, Norman AW. Vitamin D: metabolism and biological actions. Annu Rev Nutr 1984; 4: 493-520.

Hess AF, Weinstock M. (1924, 1925). Cited In: Norman AW (ed). Vitamin D. The calcium homeostatic steroid hormone. New York, Academic Press Inc., 1979; 26-36.

Hibberd KA , Norman AW (1969). Cited In: Norman AW (ed). Vitamin D. The calcium homeostatic steroid hormone. New York, Academic Press Inc., 1979; 26-36.

Hirota S, Imaketa M, Kohri K et al. Expression of osteopontin messenger RNA by macrophages in atherosclerotic plaque. A possible association with calcification. Am J Pathol 1993; 143: 1003-1008.

Hiroyuki K . 1,25 dihydroxyvitamin D_3 stimulates Ca ATPase in a vascular smooth muscle cell line. Biochem Biophys Res Commun 1988; 15: 1139-42.

Holick MF. The use and interpretation of assays of vitamin D and its metabolites. J Nutr 1990; 120: 1464-69.

Holick M F. Vitamin D - Solar rays, the milky way, or both? New Engl J Med 1992; 326: 1213-14.

Holick MF, Mac Laughlin JA, Doppeet SH. Regulation of cutaneous previtamin D_3 photosynthesis in man: Skin pigment is not an essential regulator. Science 1981; 211: 590-92.

Holick MF, MacLaughlin JA, Clark MB, Holick SA, Potts JR, Anderson RR, Blank IH, Parrish JA, Elias P. Photosynthesis of previtamin D_3 in human skin and the physiologic consequences. Science 1980; 210: 203-05.

Holick MF, Schoes HK, DeLuca HF, Sudha T, Cousins RJ. Bichemistry 1971;10: 2799.

Holmes R, Kummerow F A. The relationship of adequate and excessive intake of vitamin D to health and disease. J Am Coll Nutr 1983; 2: 173-99.

Hughes MR, Baylink DJ, Jones PG, Haussler MR. Radioligand receptor assay for 25 hydroxyvitamin D_2/ D_3 and 1,25 dihydroxyvitamin D_2/ D_3. Applications to hypervitaminosis D. J Clin Invest 1976; 58: 61-70.

Ikeda J, Shirasana T, Esaki Y, Yoshiki ST, Horokawa K. Osteopontin mRNA is expressed by SMC derived foam cells in human atherosclerotic lesions of the aorta. J Clin Invest 1993; 92: 2814-20.

Imai H, Lee SK, Pastori SJ, Thomas WA . Degeneration of arterial smooth muscle cells. Ultrastructural study of smooth muscle cell death in control and cholesterol fed animals. Virchows Arch Abt A Pathol Anat 1970; 350: 183-204.

Ingram RT, Fitzpatrick LA, Edwards WD, Frye RL, Fisher LW, Schwartz RS.Calcification in human coronary atherosclerosis is specifically associated with osteopontin, a bone matrix protein. J Am Coll Cardiol 1993; 21: 363 A.

Ito M, Cho B H S, Kummerow F A. Effects of dietary magnesium deficiency and excess vitamin D_3 on swine coronary arteries. J Am Coll Nutr. 1990; 9: 155-63.

Jacobus CH, Holick MF, Shao Q et al. Hypervitaminosis D associated with drinking milk. N Engl J Med 1992; 326: 1173-77.

Jeppesen J, Hein H O, Suadicani P, Gyntelberg F. Triglyceride concentration and ischemic heart disease: eight year follow up in the Copenhagen Male Study. Circulation 1998; 97:1029-36.

Jono S, Nishizawa Y, Shioi A, Morii H. 1,25-dihydroxyvitamin D_3 increases in vitro vascular calcification by modulating secretion of endogenous parathyroid hormone-related peptide. Circulation 1998; 98: 1302-06.

Karl E Waston, Demer L L. The atherosclerosis-calcification link ? Curr Opin Lipidol 1996; 7: 101-104.

Khouri RK, Koudsi B, Reed H. Tissue transformation into bone invivo. A potential practical application. JAMA 1991; 266: 1953-55.

Koh E, Morimoto S, Fukuo K et al. 1,25 dihydroxyvitamin D_3 binds specifically to rat vascular smooth muscle cells and stimulates their proliferation in vitro. Life Science 1988; 42: 215-23.

Krishnaswami S, Jose J, Joseph G. Lack of correlation between coronary risk factors and CAD severity. Int J Cardiol 1994; 47: 37-43.

Krishnaswami S, Prasad N K, Jose J. A study of lipid levels in Indian patients with coronary arterial disease. Int J cardiol 1989; 24: 337-45.

Kwatra MS, Hothi DS, Singh A, Chawla RS. Atherosclerosis with metastatic calcification in Corriedale sheep in Punjab. Atherosclerosis 1974; 19: 521-28.

Lewis W, Brundage B, Crouse J et al. Coronary artery calcification : Pathophysiology, epidemiology, imaging methods, and clinical implications. A statement for health professionals from the American heart association. Circulation 1996; 94: 1175-92.

Libby P, Egen D, Skarlatos S. Role of infectious agents in atherosclerosis and restenosis: An assessment of evidence and need for future research. Circulation 1997; 96: 4095-4103

Lightwood R, Stapleton T. National policies for the prevention of rickets. Ann Pediat 1957; 188: 1957- 64.

Lightwood R, Sheldon W, Harris, Stapleton T.- (Committee for study of hypercalcemia in infants and vitamin D). Hypercalcemia in infants and vitamin D. Br Med J 1956; 2: 149-56.

Linden V. Vitamin D and myocardial infarction. Br Med J 1974;3:647-50.

Liu LB, Taylor CB, Peng SK, Mikkelson B. Experimental arteriosclerosis in Rhesus monkeys induced by multiple risk factors: cholesterol, vitamin D and nicotine. Arterial Wall 1979; 5: 25-32.

Loomis W F. Skin pigment regulation of vitamin D biosynthesis in man. Science. 1967; 57: 501– 06.

Lowry OH, Rosebrough NJ, Farr Al, Randall RJ. Protein measurement with folin phenol reagent. J Biol Chem 1951; 193: 265-75.

Lyons KM, Petton RN, Hogan BLM. Organogenesis and pattern formation in the mouse. RNA distribution patterns suggest a role for bone morphogenetic protein - 2A. Development 1990; 109: 833-44.

Matsuoka LY, Wortsman J, Hollis BW. Sun tanning and cutaneous synthesis of vitamin D_3. J Lab Clin Med 1990; 116: 87-90.

Madhok TC, DeLuca HF. Characteristics of the rat liver microsomal enzyme system converting cholecalciferol into 25-hydroxycholecalciferol . Biochem J 1979; 184: 491-99.

MC Collumn EV, Simonds N, Shipley PG, Park EA (1922) Cited In: Norman Aw (ed). The calcium homeostatic steroid hormone. New York, Academic Press Inc., 1979; 70-92.

Mc Keigue PM, Sha B, Marmot MG. Relationship of central obesity and insulin resistance with high diabetes prevalence and cardiovascular risk in South Asians. Lancet 1991; 337: 382-86.

Mellanby E. An experimental investigation on rickets. Lancet 1919; 1:407-12.

Mellanby E. The present state of knowledge concerning accessory food factors.Med Res (GB), Spec Rep Ser 1919; SRS-8.

Merke J, Milde P, Lewicka S et al. Identification and regulation of 1,25-dihydroxyvitamin D_3 receptor activity and biosynthesis of 1,25-dihydroxyvitamin D_3. Studies in cultured bovine aortic endothelial cells and human dermal capillaries. J Clin Invest 1989; 83: 1903-15.

Moon J,Bandy B, Davison AJ: Hypothesis: Etiology of atherosclerosis and osteoporosis: are imbalances in the calciferol endocrine system implicated ? J Am Coll Nutr 1992; 11: 567-83.

Norman AW. Vitamin D : The calcium homeostatic Steroid Hormone. NewYork, Acad Press Inc., 1979; 134-196.

Norman AW, Roth J, Orch L. The vitamin D endocrine system. Steroid metabolism, Hormone receptors and biological response. Endocr Rev 1982; 3: 331-36.

Norman P E, Wysocki S J, Lamwansa M. The role of vitamin D_3 in the aetiology of abdominal aortic aneurysms. Medical Hypothesis 1995 ; 45: 17-20.

Olson EB, Jr., Knutson JC, Bhattacharyya MH, DeLuca HF. The effect of hepatectomy on the synthesis of 25-hydroxyvitamin D_3 J Clin Invest 1976; 57: 1213 –20.

Pen S k, Taylor B C. Probable role of excesses of vitamin D in genesis of arteriosclerosis. Arterial Wall 1980; 6: 63-68.

Pen S K, Bruce T, Philip T, Belma M . Role of mild excesses of vitamin D_3 in arteriosclerosis. A study in squirrel monkeys. Arterial Wall 1979; 4: 229-43.

Ponchon G ; DeLuca HF (1969).Cited In: Norman AW (ed). Vitamin D. Calcium homeostatic steroid hormone. New York., Academic Press Inc., 1979; 134-96.

Prema TP , Raghuramulu N. Vitamin D-like activity in *Cestrum diurnum* grown in Hyderabad, India. J Sci Food Agric 1993; 62: 21-27.

Ravinoskov U . The questionable role of saturated and polyunsaturated fatty acids in cardiovascular disease. J Clin Epidemiol 1998; 51; 443-60.

Rooney P, Kumar S. Inverse relationship between hyaluronin and collagen in development and angiogenesis. Differentiation 1993; 54: 1-9.

Ross AG. Hypertension and diseases of medium-sized arteries. In : Silver MD(ed). Cardiovascular pathology.Vol. 1. 2nded. New York, Churchill Livingstone, 1991; 385-443.

Ross R, Glomset JA. Pathogenesis of atherosclerosis. N Engl J Med 1976; 295: 369-77, 420-25.

Ross R. The pathogenesis of atherosclerosis – an update. N Engl J Med 1986; 314: 488-500.

Ross R. Atherosclerosis: A defense mechanism gone awry. Am J Pathol 1993; 143: 987-1002.

Schmid K, MC Sharry WO, Pameizer CH, Binettee JP. Chemical and physico chemical studies in the mineral deposits of the human atherosclerotic aorta. Atherosclerosis 1980; 37: 199 -210.

Schmidt G H, Goossen J, Lindle F, Seidel D. Serum 25 hydroxy cholecalciferol in myocardial infarction. Atherosclerosis 1977; 26: 55-58.

Scragg R, Jackson R, Holdaway I M, Lim T, Beaglehole R. Myocardial infarction is inversely associated with plasma 25 hydroxyvitamin D_3 levels. A community based study. Int J Epidemiol 1990; 19: 559-63.

Sedrani SH, Elidrissy T, El Arabi KM. Sulight and vitamin D status in normal Saudi subjects. Am J Clin Nutr 1983; 38: 129 – 32.

Seelig. MS, Vitamin D and cardiovascular, renal and brain damage in infancy and childhood. Ann NY Acad Sci 1969; 147: 539-82.

Shanahan CM, Carry WR, Metcalfe K, Weissberg PL. High expression of genes for calcification regulating proteins in human atherosclerotic plaques. J Clin Invest 1994; 93: 2393-402.

Shepard R, Horst RL, Hamstra, De Luca. Determination of vitamin D and its metabolites in plasma from normal and anephric man. Biochem J 1979; 182: 55-69.

Shepard RM, DeLuca HF. Plasma concentration of vitamin D_3 and its metabolites in the rat as influenced by vitamin D_3 or 25 hydroxyvitamin D_3 intakes. Arch Biochem Biophys 1980; 202: 43-53.

Siebert P, Hunziker W, Norman AW. Cell free translation analysis of the Vitamin D dependent calcium binding protein mRNA activity present in total RNA and polysomal extracts from chick intestine. Arch Biochem Biophys 1982; 219: 286-96.

Singh RB, Rastogi V, Niaz MA, Ghosh S, Rody GS, Janus ED. Serum cholesterol and coronary artery disease in populations with low cholesterol levels: The Indian paradox. Int J Cardiol 1998; 65: 81-90.

Sklan D, Budowski P, Katz M.(1973) Cited In: Norman AW (ed). Vitamin D. The Calcium homeostatic steroid hormone. New York, Academic Press Inc., 1979; 70-92.

Stamp TCB, John GH, Twigg CA. Comparison of oral 25-Hydroxy-cholecalciferol vitamin D and ultraviolet light as determinants of circulating 25 hydroxy vitamin D. Lancet 1977; 1: 1341-43.

Stamp TCB. Factors in human vitamin D nutrition and in the production and cure of classical rickets. Proc Nutr Soc 1975; 34: 119-30.

Stary H E. The sequence of cell and matrix changes in atherosclerotic lesions of coronary arteries in the first forty years of life. Eur Heart J 1990; II (suppl E): 3-19.

Stumpf WE, Sar M, Deluca HF. Sites of action of 1,25 $(OH)_2$ D_3 identified by thaw-mount autoradiography. In: DV. Cohn, RV Talmage J.L Mathews (Eds). Hormonal control of calcium metabolism. Amsterdam. Excerpta Medica 1981; 222-29.

Sunita RD, Raghuramulu N. Provitamins D and vitamin D in plankton. (Abstract) 9th Workshop on vitamin D. 1994; 226.

Tanenbaun SR, Kondor Gt, Vaselik KE. Detection of calcific deposits in coronary arteries by ultrafast computed tomography and correlation with angiography. Am J Cardiol 1989; 63: 870-72.

Tanimura A, Mc Gregon DH, Anderson HC. Calcification in atherosclerosis. 1. Human studies. J Exp Pathol 1986 (a); 2: 261-72.

Tanimura A, Mc Gregon DH, Anderson HC. Calcification in atherosclerosis II. Animal studies. J Exp Pathol 1986 (b) ; 2: 275-97.

Tanimura A, MC Gregor DH, Anderson HC. Matrix vesicles in atherosclerotic calcification. Proc Soc Exp Biol Med 1983; 172: 173-77.

Taussig HB. Possible injury to the cardiovascular system from vitamin D. Ann Int Med 1966; 65:1195-99.

Toda T, Ito M, Toda Y, Smith T, Kummerow F. Angiotoxicity in swine of a moderate excess of dietary vitamin D_3. Food Chem Toxicol 1985; 23: 585-92.

Toda T, Leszczynski DE , Kummerow F A. The role of 25 hydroxy vitamin D_3 in the induction of atherosclerosis in swine and rabbit by hypervitaminosis D. Acta Pathol Jpn 1983; 33: 37-44.

Trump BJ, Berezesky IK, Osornio-vargas AR: Cell death and the disease process. The role of calcium. In : Bowen ID, Lockshin RA (eds). Cell death in Biology and Pathology. New York, Chapmen and Hall, 1981; 209-42.

Tsutomu I, Hiroyuki K . 1,25 dihydroxyvitamin D_3 stimulates $^{45}Ca^{2+}$ uptake by cultured vascular smooth muscle cells derived from rat aorta. Biochem Biophys Res Commun 1988; 152: 1388-94.

Virchow R. (1863) .Unabridged reprinting. Cellular Pathology as based up on physiological and pathological histology. Newyork, Dover, 1971; 404 -408.

Walters MR, Hunziker W, Norman AW, Unoccupied 1,25 dihydroxyvitamin D_3 receptors. Nuclear cytosol ratio depends on ionic strength. J Biol Chem 1980; 255: 6799-805.

Walters MR, Ilenchuk TT, Claycomb WC. 1,25-dihydroxyvitamin D_3 stimulates^{45} Ca 2+ uptake by cultured adult rat ventricular cardiac muscle cells. J Biol Chem 1987; 262: 2536- 41.

Walters M R, Wicker D C, Riggle P C. 1,25-dihydroxyvitamin D_3 receptors identified in the rat heart. J Mol Cell Cardiol 1986; 18: 67-72.

Walters MR, Cunea DL, Jamison AP. Possible significance of new target tissues for 1,25-dihydroxyvitamin D_3. Steroid Biochem 1983; 19: 913-20.

Wasserman RH, Corradino RA, Krook LP. *Cestrum diurnum*: A domestic plant with 1,25 dihydroxycholecalciferol like activity. Biochem Biophys Res Commun 1975; 62: 85-91.

Wasserman RH, Nobel TA. Vitamin D related compounds as a toxic principle in calcinogenic plants. In : Norman AW (ed). Vitamin D – Molecular Biology and Clinical Nutrition. Vol 1. New York, Marcel Decker Inc., 1980; 457-87.

Wasserman RH, Taylor AW. Vitamin D_3 induced calcium-binding protein in chick intestinal mucosa. Science 1960; 152: 791-93.

Watson E Karl, Demer L Linda. The atherosclerosis - Calcification link ? Curr Opin Lipidol 1996; 7: 101-104.

Watson K E, Abrolat M L, Malone L L, Hoeg J M, Doherty T, Detrano R, Demer L. Active serum vitamin D levels are inversely correlated with coronary calcification. Circulation 1997; 96: 1755-60.

Weber K T. Soft tissue, hard times a mordant affair. Cardiovascular Res 1997; 36; 291-92.

Wexler L, Brundage B, Crouse J et al. Coronary artery calcification: Pathology, epidemiology, imaging methods, and clinical implications. A statement for health professionals from the American heart association. Circulation 1996; 94: 1175-92.

Windaus A, Linsert O, Luttringhaus A , Weidlick G (1932). Cited In: Norman AW (ed). The calcium homeostatic steroid hormone. New York: Academic Press Inc. 1979; 37-69.

Zetter A, Seligson D. Estimation of calcium and magnesium using atomic absorption spectrophotometer. Clin Chem 1964; 10: 869-71.

APPENDICES

ABBREVIATIONS

AMI	- Acute myocardial infarction
CABP	- Calcium binding protein
CAD	- Coronary artery disease
Cpm	- Count per minute
DNA	- Deoxyribonucleic acid
DPX	- Dibutyl phthalate xylene
$1,25(OH)_2 D_3$	- 1,25-dihydroxyvitamin D_3
DSC	- Digital subtraction cinefluoroscopy
EDTA	- Ethylene diamine tetraacetic acid
$25\text{-}OH\text{-}D_3$	- 25-hydroxyvitamin D_3
HDL	- High density lipoprotein

IHD	- Ischemic heart disease
µCi	- Microcurie
µg	- Microgram
mCi	- Millicurie
mmol	- Millimole
mRNA	- Messenger ribonucleic acid
NADH	- nicotinamide adenic dinucleotide
ng	- nanogram
nmol	- nanomol
PEG	- Polyethylene glycol
POPOP	- 1,4 – Bis (5 phenyl oxazol- 2yl) benzene
PPO	- 2,5 diphenyl oxazole
RNA	- Riboucleic acid
S.D	-Standard deviation
VLDL	- Very low denisity lipoprotein