UCSF
UC San Francisco Electronic Theses and Dissertations

Title
Fluoride concentration in mineralizing tissues in rats with renal insufficiency

Permalink
https://escholarship.org/uc/item/11n9t7ff

Author
Amin, Urmi,

Publication Date
1998

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

# Fluoride Concentration in Mineralizing Tissues in Rats with Renal Insufficiency

by

Urmi Amin

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Oral Biology

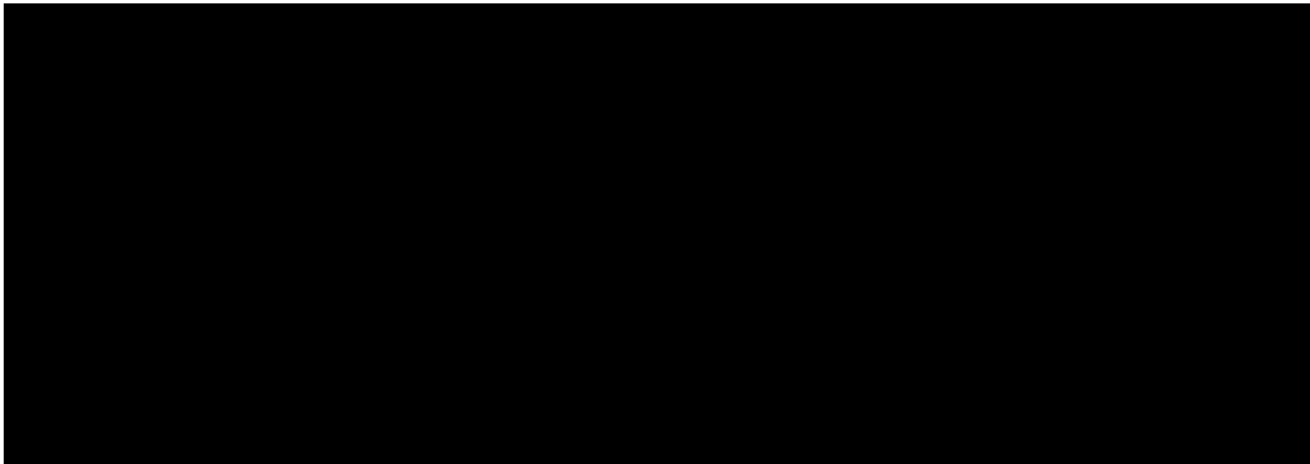
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



Date University Librarian

Degree Conferred: . . . . . . . . . . . . . . . . . . . . . . . . . . .

**TO MY DAD**

## ACKNOWLEDGMENTS

This work would not have been possible but for collaboration of Dr. Robert Mathias, Dr. Aki Jikko, and for the dedication and perseverance of my mentor, Dr Pamela Den Besten, to whom I am deeply grateful. If not for their generosity of time and patience, this thesis would not have been possible.

I give special thanks to Tineka Mathews for her technical assistance in helping me "think through a problem" and Dr. Anita Baron for introducing me to lab life. Additional thanks to Cen Gao for assistance in fluoride analysis. I would also like to thank Dr.Stuart Gansky and Dr. Hilen Amin for their invaluable guidance on the statistical analysis.

To my committee members, Dr. John Featherstone and Dr. Sunil Kapila, I also express my gratitude for their assistance in helping to bring this project to its final form.

Lastly, I would like to thank my husband Manish and my family, for without their support none of this would have been possible.

This work was supported in part by a research grant from the California Society of Pediatric Dentists.

UCSF LIBRARY

<u>**Fluoride Concentration in Mineralizing Tissues in Rats with Renal Insufficiency**</u>

**Urmi Amin, B.D.S.**

## ABSTRACT

In patients with progressive renal insufficiency, 1,25-dihydroxy-vitamin D deficiency and hyperparathyroidism develop and can cause disorders of mineral ion homeostasis. Such disturbances can result in renal osteodystrophy and growth failure. Renal insufficiency has also been associated with elevated plasma fluoride (F) levels which may interfere with normal bone mineralization. In addition, previous studies have found that total F content is increased in bone in experimental renal insufficiency. However, there is no information regarding the distribution of F content in both the growth plate and the cortical bone of rats with experimental chronic renal insufficiency. We hypothesized that there would be an increased F content in both the growth plate and the cortical bone of rats with experimental chronic renal insufficiency. The aim of this study was to determine whether F content was increased in the growth plate and the cortical bone of rats with experimental chronic renal insufficiency when compared to normal rats. Fifteen rats weighing 250-300g underwent either a single stage 5/6 nephrectomy (Nx) or sham surgery. The rats were fed normal rat chow (22 ppm F) and *ad libitum* tap water (0.16 ± 0.02 ppm F). At 4 weeks and at 8 weeks after surgery, 18 animals (9 Nx, 9 sham) and 12 animals (6 Nx, 6 sham), respectively, were sacrificed. At the time of sacrifice, blood was collected for analysis of creatinine (Cr) and F, and the right femur was removed for analysis of F content at the growth plate and mid-femur region.

The serum Cr levels were significantly higher in both Nx groups when compared to the levels in the sham group ($p < 0.05$). The serum F levels were higher in the Nx group when compared to the levels in the sham group, but were only significantly

increased at 4 weeks ($p < 0.05$). In the Nx group, accumulation of F was significantly increased in the growth plate at 4 and 8 weeks ($p < 0.01$), and in cortical bone at 8 weeks ($p < 0.05$).

Since F bone content was increased in the growth plate in the Nx rats, we hypothesized that increased extracellular F concentration would inhibit chondrocyte cell proliferation and proteoglycan synthesis. We therefore determined in cultured pig chondrocytes the effects of extracellular F concentration (0.1 - 50 μM F) on chondrocyte cell proliferation (thymidine ($^{3}H$) incorporation) and proteoglycan synthesis (incorporation of ($^{35}S$) sulfate into glycoaminoglycans). We found that increased extracellular F concentration had no significant effect on either ($^{3}H$) thymidine uptake or ($^{35}S$) sulfate incorporation in GAG in cultured chondrocytes.

In summary, in rats with experimental renal insufficiency, there is a rapid accumulation of F in the growth plate at 4 weeks with a slower increase of F levels in cortical bone at 8 weeks. This increased F content in bone may play a role in the pathogenesis of disordered mineral metabolism in chronic renal insufficiency.

## TABLE OF CONTENTS


| | |
|---|---|
| **TITLE** | **i** |
| **DEDICATION** | **iii** |
| **ACKNOWLEDGMENTS** | **iv** |
| **ABSTRACT** | **v** |
| **TABLE OF CONTENTS** | **vii** |
| **LIST OF FIGURES** | **ix** |
| **LIST OF TABLES** | **xi** |

**I. BACKGROUND, SIGNIFICANCE AND SPECIFIC AIMS**

| | |
|---|---|
| INTRODUCTION | 1 |
| A. HYPOTHESIS AND SPECIFIC AIMS | 1 |
| B. FLUORIDE | 2 |
| 1) Background and significance | 2 |
| 2) Fluoride metabolism | 3 |
| 3) Mechanism and site of absorption | 4 |
| 4) Fluoride in blood plasma | 4 |
| 5) Pharmacokinetics of fluoride | 5 |
| C. THE KIDNEY | 6 |
| 1) Renal clearance of fluoride | 6 |
| 2) Renal metabolites and regulation | 8 |
| 3) Chronic renal failure | 11 |
| 4) Fluoride and chronic renal failure | 11 |
| 5) Fluoride, renal osteodystrophy and renal insufficiency | 12 |
| 6) Renal osteodystrophy | 12 |
| a) Low-turnover bone disease | 13 |
| b) High-turnover bone disease | 14 |
| D. FLUORIDE AND BONE | 14 |
| 1) Skeletal fluorosis | 14 |
| 2) The effect of fluoride on bone mineralization | 15 |
| 3) Fluoride effect on bone crystals | 16 |
| 4) Cell biology of bone growth | 18 |
| 5) Skeletal morphogenesis and growth | 18 |

**II. MATERIALS AND METHODS**

| | |
|---|---|
| A. PRELIMINARY STUDIES | 20 |
| 1) Rationale for using a rat model for renal deficiency | 20 |
| 2) Measurement of ionic fluoride in bone | 21 |
| B. STATISTICAL METHODS | 23 |
| 1) Error of Method | 23 |
| C. EXPERIMENTAL DESIGN | 24 |
| 1) Surgical induction of uremia | 24 |
| 2) Renal function tests | 25 |
| 3) Blood biochemistry | 25 |




...USE LIBRARY

4) Fluoride analysis of mineralizing tissues 26
5) Fluoride analysis of urine, serum and drinking water 28
6) Biochemical determination 29
D. FLUORIDE EFFECTS ON CHONDROCYTES IN VITRO 30
1) Fluoride effect on the rate of cell proliferation 31
2) Fluoride effect on glycosaminoglycan synthesis 31
E. STATISTICAL ANALYSIS 32

III. RESULTS
A. FLUORIDE CONCENTRATION IN RAT FOOD AND DRINKING WATER 33
B. RENAL BIOCHEMICAL MEASUREMENT IN THE 4 WK GROUP 34
C. FLUORIDE BIOCHEMICAL MEASUREMENT IN THE 4 WK GROUP 35
D. RENAL BIOCHEMICAL MEASUREMENT IN THE 8 WK GROUP 37
E. FLUORIDE BIOCHEMICAL MEASUREMENT IN THE 8 WK GROUP 38
F. FLUORIDE BONE MEASUREMENT BETWEEN 4 & 8 WK GROUP 40
G. CHONDROCYTE CELL CULTURE 40

IV. DISCUSSION 43

V. REFERENCES 48

UCSF LIBRARY

## LIST OF FIGURES


| Figure | | Page |
|---|---|---|
| Fig 1. | The proposed mechanism for the re-absorption of F, as hydrofluoric acid (HF), from the renal tubule (Whitford, 1990) | 8 |
| Fig 2. | F analysis using a micro-diffusion method for concentrating minute amounts of fluoride using a ion-specific electrode (Orion, Model 94-09). | 21 |
| Fig. 3 | F analysis at the growth plate, bone marrow and mid-diaphysis region of the femur. | 26 |
| Fig 4. | F content in the cortical bone and the growth plate between Sham and 5/6 Nephrectomy (Nx) animals in the 4 week group (mean $\pm$ SD). F levels in the growth plate were significantly increased (p-value $<0.01$). There was an increase in F concentration in the cortical bone of the Nx animals but it was not found to be significant. | 36 |
| Fig. 5. | F content in cortical and the growth plate between Sham and Nx animals in the 8 wk group (mean $\pm$ SD). Eight week post surgery showed a significant increase in F content in the cortical bone and growth plate in the Nx group compared to the sham-operated group ($p<0.05$). | 39 |
| Fig. 6. | F content in the cortical bone and the growth plate between 4 wk Nx and 8 wk Nx animals (mean $\pm$ SD). There was a significant difference in final concentration of F in the cortical bone between the four and eight week post-surgery group ($p< 0.05$). Even though the concentration of F in the growth plate in the eight week was higher than that of the four week Nx group, it was not found to be significant. | 41 |

| **Figure** | **Page** |
|---|---|
| Fig. 7. F effects on the rate cell proliferation (mean ± SD). No significant difference was found in any of the F levels vs control. | 42 |
| Fig. 8. F effect on GAG synthesis (mean ± SD). No significant difference was found in any of the F levels vs control. | 42 |

## LIST OF TABLES


| Table | Page |
|---|---|
| Table 1. Composition of Prolab Rat/Mouse/ Hamster 2000 (a high energy diet). | 33 |
| Table 2. Biochemical determination for the 4 week post-surgery group. All values are the mean ± (SD). | 34 |
| Table 3. F content in mineralizing tissues, serum and urine in the 4 week post-surgery group. All values are the mean ± (SD). | 35 |
| Table 4. Biochemical determination for the 8 week post-surgery group. All values are the mean ± (SD). | 37 |
| Table 5. F content in mineralizing tissues, serum and urine in the 8 week post-surgery group. All values are the mean ± (SD). | 38 |
| Table 6. Comparison of F content in the cortical bone and the growth plate between the 4 week and 8 week Nx group. All values are the mean ± (SD). | 40 |


UCSF LIBRARY

# I. BACKGROUND, SIGNIFICANCE, AND SPECIFIC AIMS

## INTRODUCTION

Patients with progressive renal failure are prone to develop renal osteodystrophy as a result of disturbances of mineral ion homeostasis such as 1,25 dihydroxy-vitamin D deficiency and secondary hyperparathyroidism. Similar histological changes in bone to those obversed in renal osteodystrophy have been noted in patients treated with high doses of fluoride (F) for osteoporosis and in patients suffering from skeletal fluorosis. Secondary hyperparathyroidism has also been noted in some of these patients, indicating that F in high levels can cause a disruption in mineral ion homeostasis, the cause of which is unknown.

In patients with renal insufficiency, urinary F excretion decreases as a result of a reduction in the glomerular filtration rate of the kidney. This decrease of urinary excretion results in an increase in serum F levels and subsequent increase in the bone F concentration. The relationship between the accumulation of F in bone and the development of renal osteodystrophy and growth failure in children has not been established and must be more clearly defined.

## A. HYPOTHESIS AND SPECIFIC AIMS

We hypothesized that there would be an increased F content in both the growth plate and the cortical bone of rats with experimental chronic renal insufficiency. The aim of this study was to determine whether F content was increased in the growth plate and the cortical bone of rats with induced renal insufficiency when compared to normal rats. The animals were not supplemented with additional F, only that which is found in San Francisco drinking water (assumed to be fluoridated at $< 1.0$ ppm F) and in their diet.

In rats drinking water containing 100 ppm F histological changes in the hypertrophic zone were shown (Kameyama, 1974), however, the effect of F on chondrocytes in culture have not been studied. We hypothesized that increased extracellular F concentration would inhibit chondrocyte cell proliferation and proteoglycan synthesis. We therefore determined in cultured pig chondrocytes the effects of extracellular F concentration (0.1 - 50.0 μM F) on chondrocyte cell proliferation (thymidine ($^{3}$H) incorporation) and proteoglycan synthesis (incorporation of ($^{35}$S) sulfate into glycoaminoglycans).

As a way of providing background the following topics will be reviewed: F pharmacokinetics and its relation to renal function and bone deposition, kidney function and the effects of chronic renal insufficiency on mineral ion homeostasis (with the associated forms of renal osteodystrophy), and the chronic effects of high levels of F on bone formation.

## B. FLUORIDE

### 1. Background and significance

Fluorine is classified as a trace element found in very small amounts and concentrations in biological materials, generally in the parts per million (ppm) range or less. It is a highly active element, and is not found naturally in the free form. Fluorides are found in sea water, in all sea animals, in the earth's crust, notably as fluorspar (calcium fluoride), and also in drinking water (Anand et al., 1990).

When consumed in excessive quantities F interacts with body tissues to produce chronic disease states, primarily in mineralizing tissues. The difference between a desirable F dose and a toxic dose is narrow. The recommended range for systemic F ingestion for



UCSF LIBRARY

caries prevention in humans is generally considered to be 0.02 to 0.04 mg/kg/day (Turner et al., 1996). However, consumption of F at levels above this dose range frequently occurs, especially in young children (Pendrys and Stamm, 1990). In recent years, several problems resulting from excessive F intake (hyperfluoridation) have arisen, including dental and skeletal fluorosis. Hyperfluoridation usually results from increased F uptake from multiple fluoridated sources including fluoridated drinking water, salt, sugar, toothpaste and mouthwash, and F tablets (Pendrys and Stamm, 1990; Whitford, 1992). There are high concentrations of F in dental products (ranging from 229-22,900 ppm), and depending on frequency of use and the type of vehicle and delivery system used, there is a danger of excess ingestion of F (Ekstrand et al., 1990). Whereas the plasma F levels in humans in non-fluoridated drinking water areas range from 0.2 - 0.3 μmol/l, regular fluoridation (water fluoridation at 0.7-1.0 ppm) will produce levels of 1.0 μmol/l, and hyperfluoridation such that it will be more than 3.0 μmol/l, and sometimes exceeding values of 10 μmol/l (Willinger et al., 1994). The frequency and degree of dental fluorosis in human populations is associated with excessive F intake during childhood when the crowns of the teeth are mineralizing (Singer and Ophaug;Angmar-Mansson and Whitford, 1990)

## 2. Fluoride Metabolism

Fluoride metabolism can be divided into absorption, distribution, and elimination. The major route of absorption is ingestion via the gastrointestinal tract. Fluoride may also enter the body fluids via the lungs from airborne F. Absorption depends on the physical and chemical properties of the compound, its solubility, the pH of the stomach, and co-ingestion of foods (Fejerskov et al, 1996).



LIBRARY

3. Mechanism and site of absorption

Fluoride is absorbed by passive diffusion from both the stomach and the intestine. The mechanism and the rate of gastric absorption is inversely related to the pH so that factors which promote the secretion of gastric acid increase the rate of F absorption, which leads to earlier and higher peak plasma F levels, and vice versa. Fluoride is absorbed in the form of the undissociated weak acid hydrogen fluoride (HF, pKa = 3.45), which is readily absorbed through cell membrane lining of the stomach and into the plasma. Most of the F that is not absorbed from the stomach will be rapidly absorbed from the small intestine - with the remaining small amount of unabsorbed F excreted in the feces (Fejerskov et al., 1996.; Whitford, 1990).

4. Fluoride in blood plasma

It is through plasma by which F is distributed to all tissues and organs, including the kidney which is the primary site for elimination of F. Plasma is often referred to as the central compartment. Fluoride in plasma exists in two forms: a) ionic (also known as inorganic F or free F) and b) non-ionic or bound F, which together form the "total" plasma F levels. The ionic form of F can be detected using an ion-specific electrode (Fejerskov et al, 1996). Studies have indicated that ionic F is not bound by plasma proteins or by any other constituent of plasma. Therefore, it can be assumed that interstitial fluid and plasma F concentrations are virtually identical (Whitford, 1990). The concentration of F in plasma is a variable. It is dependent on the level of intake and several physiological factors such as the solubility of the F compound, pH of the stomach and renal clearance rate of the F ion, which in turn will influence the rate of bone accretion and dissolution (Whitford, 1990).

Plasma F levels increase in proportion to the chronic level of F intake, such that body fluid F levels are not homeostatically controlled, and, therefore, plasma F levels can be used as an index of immediate exposure to the ion (Whitford, 1990; Ekstrand, 1978).

## 5. Pharmacokinetics of fluoride

The pharmacokinetics of F can be described as a two compartment open model. In the two-compartment open model, F enters the system via the central compartment namely, plasma. It is then distributed to the peripheral compartment which consist of organs and tissues and is then eliminated from the central compartment by the kidneys (Fejerskov et al, 1996).

The rates of delivery of F to organs and tissues are generally determined by the blood flow rate to the organ or tissues. The various soft tissues are distinguished by their tissue-to-plasma (T/P) F concentration ratios. The T/P ratios for kidney and whole femur are very high (4.16 and 7.52, respectively), compared to other well-perfused organs, such as liver and heart (T/P ratios at 0.98 and 0.46, respectively). These tissues belong to the central compartment. The high F levels in kidney and bone have been explained by the fact that F in the tubular fluid of the distal nephron of the rat is normally about 100 times more concentrated than that of plasma, and that F is an avid bone-seeker (Whitford, 1990).

Fluoride is more slowly distributed to poorly perfused tissues, such as, muscle and adipose tissue, which belong to the peripheral compartment. The concentration of F increases in the peripheral compartment until a steady-state is reached. There is a net flux from the peripheral compartment to the central compartment, as F is continuously eliminated from the central compartment by the kidneys (Fejerskov et al., 1996).

## B. THE KIDNEY

The major route of removal of F from the body is through the kidneys. The active units of the kidney are the nephrons, within the cortex and medulla, which filter the blood and then reabsorb water and selected substances back into the blood. The urine thus formed is responsible for the excretion of waste products of protein metabolism, excess hydrogen ions and several other toxic substances including many drugs from the blood. The kidneys are also endocrine organs, and are responsible for the synthesis and release of renin, renal erythropoietic factor and 1,25 dihydroxy vitamin D. Since the kidney is responsible for producing 1,25 dihydroxy vitamin D, it plays an improtant role in regulating calcium and phosphorus balance, both of which are vital for maintaining the structural integrity of bones (Guyton, 1991).

### 1. Renal clearance of fluoride

F is freely filtered from the plasma in the glomerular capillaries into the urinary space of Bowman's capsule, after which it undergoes a variable degree of tubular re-absorption in the distal nephron (Whitford and Pashley, 1991). Since the binding of F to plasma proteins or macromolecules is negligible, the concentration in the glomerular filtrate is similar to that in serum. Tubular secretion of F does not occur under normal conditions (Whitford, 1990; Schiffl and Binswanger, 1980).

The renal clearance of F in the adult typically ranges from 30-50 ml/minute. The percentage of the filtered F reabsorbed from the renal tubules can range from about 10% to 90%. The final renal clearance of F is determined by both the urinary pH and urinary flow (Fejerskov et al., 1996; Whitford, 1990).

The proposed mechanism for F reabsorption by the renal tubules is by passive diffusion of hydrofluoric acid (HF), since the epithelium is essentially impermeable to ionic

F (Fig. 1). Thus, as the tubular fluid becomes more acidic, the ionic F is converted to HF. This increases the electro-chemical gradient for HF and promotes its diffusion from the tubular space into the interstitial fluid. In the interstitial fluid, where the pH is near neutrality, HF is promoted to dissociation to the F ion. The F ion diffuses into the relatively " leaky" capillaries and is returned to the systemic circulation. In contrast, as the tubular fluid becomes more alkaline, the F exists in the ionic form and the electro-chemical gradient for HF diffusion decreases. Thus, urinary alkalization promotes F ion excretion (Whitford, 1990).

F excretion depends on several important factors: total daily consumption of the element, degree of renal function, age, physiological state, and interaction of F with other factors (i.e., binders). A typical person excretes between 0.1 to 0.5 mg of F in a 24 hour period (Whitford, 1990; Fejerskov et al., 1996). However in humans consuming high enough levels of F to result in dental or skeletal fluorosis, urinary F varies between 1.2 and 10.0 mg per 24 hours. The elevated F in the urine would continue for a long time after the source of exposure has been eliminated as F equilibration throughout the body compartment continues (Krishnamachari, 1987).

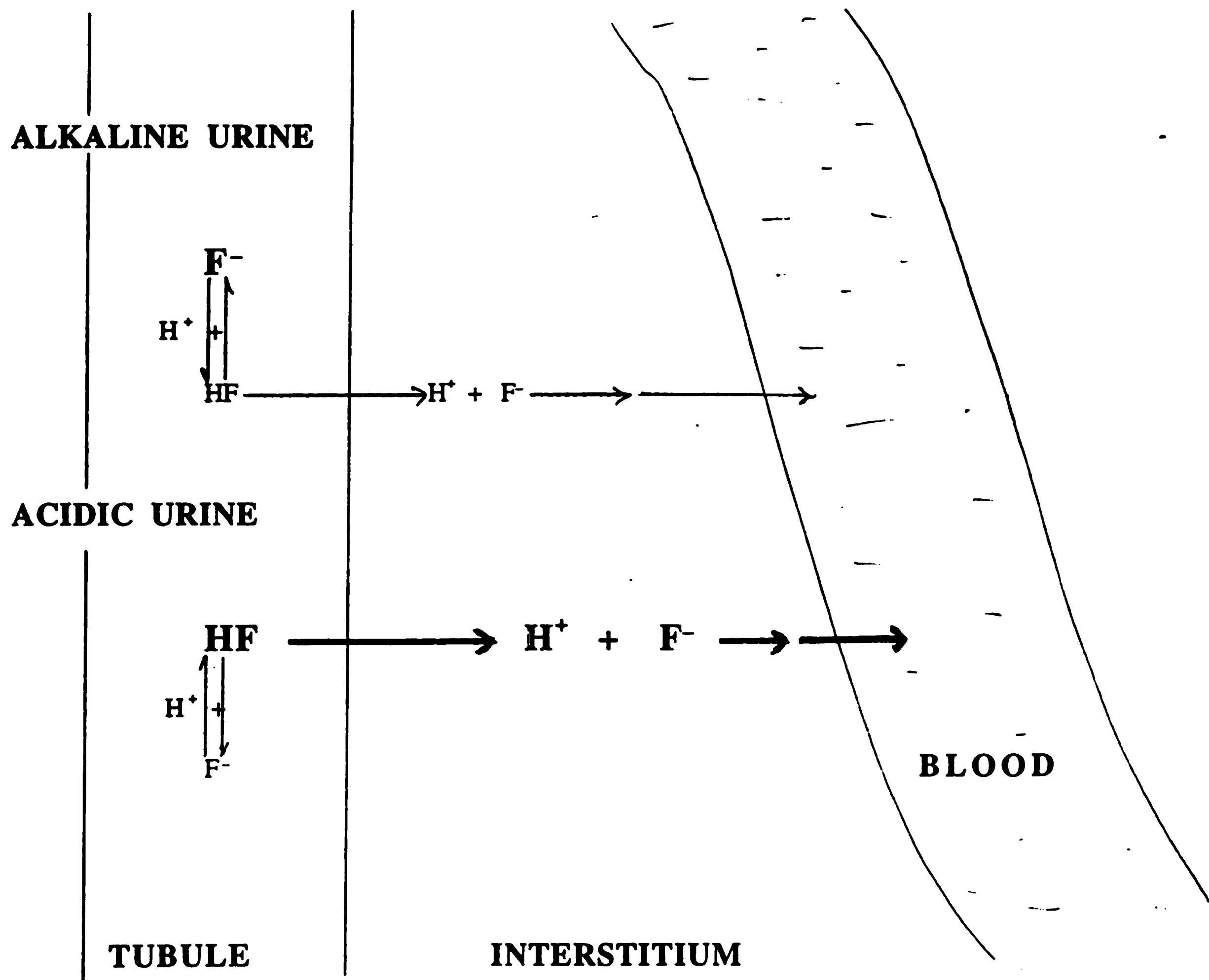



**Fig. 1- The proposed mechanism for the re-absorption of F, as hydrofluoric acid (HF), from the renal tubule (Whitford, 1990).**

2. Renal metabolites and regulation

*Calcium*

Disturbances in $Ca^{2+}$ metabolism are common in patients with renal insufficiency. Normal calcium homeostasis is maintained by the interaction of the GI tract, the bones and the kidneys, under the influence of parathyroid hormone (PTH), vitamin D, and other hormones (Guyton, 1991). While 99% of the total body $Ca^{2+}$ is found in the bones, the small portion remaining is dissolved in the blood plasma and interstitial fluid and is of great

importance (Suki and Rouse, 1991). The normal serum total $Ca^{2+}$ is approximately 9.0 - 10.2 mg/dl (2.25-2.55 mmol/l) (Sutton and Dirks, 1991). Forty percent of total serum calcium is bound to protein, mostly to albumin, while 50% is free or ionized. It is the ionized form that is physiologically active, it is necessary for blood coagulation, normal cardiac and skeletal muscle contraction, and nerve function (Guyton, 1991).

$Ca^{2+}$ is absorbed from the gastrointestinal (GI) tract, stored in the bone and excreted by the kidneys. In the intestine, $Ca^{2+}$ is actively transported across the epithelium by a 1,25-dihydroxy vitamin D-dependent calcium- ATPase system in the brush border. In addition, a small percentage of $Ca^{2+}$ absorption occurs by passive diffusion (Guyton, 1991)

The $Ca^{2+}$ in bone is of two types: a readily exchangeable reservoir and a larger pool of stable $Ca^{2+}$ that is only slowly exchangeable. The plasma $Ca^{2+}$ is in equilibrium with the readily exchangeable bone $Ca^{2+}$ (Guyton, 1991). A large amount of $Ca^{2+}$ is filtered in the kidneys, but 98-99% of the filtered $Ca^{2+}$ is reabsorbed. Tubular reabsorption is under hormonal regulation namely by parathyroid hormone, and 1,25 dihydroxy-vitamin D. Serum ionized $Ca^{2+}$ concentration is maintained within a narrow range of 4.0 - 4.9 mg/dl (1.0 - 1.25 mmol/l) (Sutton and Dirks, 1991).

*Parathyroid hormone*

Parathyroid hormone (PTH) secreted by the parathyroid glands maintains serum ionized $Ca^{2+}$ levels within a narrow range. When the serum ionized $Ca^{2+}$ level decreases, this results in the secretion of PTH into the blood (Brunier, 1994). PTH then directly stimulates bone resorption and increases renal $Ca^{2+}$ reabsorption in the distal tubules, both of which results in normalization of serum $Ca^{2+}$ concentration. PTH can also mobilize



LIBRARY

$Ca^{2+}$ in the intestine indirectly by stimulating the production of 1,25-vitamin $D_3$ in the kidney (Brunier, 1994).

Secondary hyperparathyriodism is commonly found in patients with chronic renal insufficiency. The mechanism is thought to be partly due to the reduced synthesis of 1,25-dihydroxy vitamin $D_3$ in the kidney, but also from a reduced sensitivity of the parathyroid gland to the suppressive effect of chronic increased serum $Ca^{2+}$ ions on PTH secretion (Llach, 1995).

*Vitamin D*

Vitamin D is synthesized in the skin following exposure to sunlight and is also absorbed from food in the proximal small bowel. Vitamin $D_3$ is converted into 25-hydroxy vitamin $D_3$ by liver enzyme, which in turn is converted into 1,25-dihydroxy vitamin $D_3$ by $1\alpha$-hydroxylase in the kidney. The $1\alpha$-hydroxylase is regulated by $Ca^{2+}$, Pi, and PTH (Guyton, 1991).

The half-life of 1,25-dihydroxy vitamin $D_3$ in plasma is 5-20 hours and the turnover rate is 1 - 2 µg per day. 1,25-Dihydroxy vitamin $D_3$ acts directly on the intestine and bone to increase plasma calcium and phosphate (Guyton, 1991).

*Hormonal regulation of calcium and phosphate*

Serum ionized calcium is tightly regulated in the body. PTH increases plasma calcium concentration, primarily by promoting bone resorption and by increasing its reabsorption in the renal tubules. 1,25-dihydroxy vitamin $D_3$ increases plasma calcium ion by increasing intestinal absorption of calcium and mobilizing calcium ion from bone (Ganong, 1989; Coburn and Slatopolsky, 1991).

3. Chronic renal failure

Chronic renal failure is a slow deterioration of renal function caused by a variety of renal and urological diseases resulting in abnormalities of body metabolism, hormonal regulation and organ system functioning. In the majority of patients under the age of five the cause is due to an anatomical defect. In patients over the age of five, chronic renal failure is usually due to an acquired glomerular disease or hereditary disorder.

A patient with chronic, progressive renal failure may be considered to pass through four stages. In the first stage approximately 50% of normal function may be lost before the concentration of urea nitrogen or creatinine in the plasma rises above the normal range. The second stage is that of mild renal insufficiency. Manifestations include mild azotemia (mild increase in concentration of urea nitrogen and creatinine in plasma), impaired ability to concentrate the urine, nocturia, and mild anemia. The third stage is the development of frank renal failure with advancing anemia, acidosis, and other clinical and biochemical manifestations (creatinine clearance decreases below 25 ml/min) . The fourth and final stage is also referred to as the development of end-stage renal disease and the symptoms are referable to multiple body systems. Once this stage of the disease has been reached, treatment by maintenance dialysis and/or kidney transplantation is necessary in order to prolong the life of the individual (First, 1982).

4. Fluoride and chronic renal failure

Parsons et al., 1975, reported the inability of the kidney to excrete F in chronic renal failure, resulting in elevated serum F levels with a tendency for accumulation in bone. When subjects where studied with either normal renal function or variable renal insufficiency, it was found that patients with advancing chronic renal disease tend to continue to excrete normal dietary loads of F until creatinine clearance decreased below 25

ml/min (third stage of renal failure). This accompanied with a rise in serum F concentrations. The mean urinary F excretion per 24 hours decreased significantly in patients with impaired renal function (Schiffl and Binswanger, 1980).

5. Fluoride, renal osteodystrophy and renal insufficiency

The relationship between increased plasma F levels and increased bone F levels have been shown to be nonlinear in both humans and animals with renal insufficiency (Kekki et al., 1982; Turner et al., 1996). Turner et al. (1996), showed that in animals with renal insufficiency, there was an increased volume of unmineralized osteoid, which was dose-dependent with F intake. Osteoid volume was increased over 20-fold in animals with renal insufficiency that received 15-50 ppm F, suggesting osteomalacia. The cause of the F-induced increases in osteoid volume appeared to impair mineralization. It is not exactly clear how F inhibits bone mineralization. Femoral bone strength in these animals with renal insufficiency were found to be reduced when compared to the control.

6. Renal osteodystrophy

In patients with renal insufficiency, 1,25-dihydroxy vitamin $D_3$ deficiency and secondary hyperparathyroidism are observed. These disturbances result in disorders of mineral ion homeostasis and are clinically expressed as renal osteodystrophy and growth failure.

With the increase in life expectancy, the morbidity associated with renal bone disease will assume a greater significance. It is characterized by hyperphosphatemia, hypocalcemia, 1,25-dihydroxy vitamin $D_3$ deficiency and secondary hyperparathyroidism (First, 1982; Coburn and Slatopolsky, 1991).

Renal osteodystrophy can be divided into two broad categories: high-turnover bone disease and low-turnover bone disease (Coburn and Slatopolsky, 1991).

6a. Low-turnover bone disease.

Osteomalacia

Osteomalacia is characterized by decreased numbers of osteoblasts and osteoclasts, resulting in a decrease in bone turnover along with an increase in osteoid, unmineralized bone tissue, although it is now relatively rare. It was previously associated with high bone aluminum content (Llach and Coburn, 1989; Sutton and Cameron, 1992). Aluminum toxicity, caused by excessive use of aluminum-based phosphorus binders and, in the past, by aluminum in water used for dialysis, is thought to be the causal factor with this type of lesion (Hruska, 1997)). The bone aluminum content was found to correlate closely with degree of osteomalacia in these patients (Coburn and Slatopolsky, 1991).

In osteomalacia, serum calcium levels are typically normal or high in the presence of low PTH levels. Phosphate levels vary depending on the use of phosphate binders used but they may be high. Serum alkaline phosphatase, osteocalcin, and PTH levels tend to be low, and serum aluminum levels are usually elevated (Sutton and Cameron, 1992). Common symptoms, associated with osteomalacia include muscle weakness, severe bone pain, and fractures involving the ribs and femoral neck. The bone fractures, in general, often do not heal well (Sherrard and Andress, 1989).

Aplastic or Adynamic bone disease

Aplastic or adynamic bone disease is associated with relative hypoparathyroidism and low rates of bone formation with flat osteoblasts, few osteoid stems and rare osteoclasts and resorption cavities. The pathophysiology of aplastic disease is not

completely understood, factors such as high-$Ca^{2+}$ dialysate, excessive use of $Ca^{2+}$-based phosphorus binders, and aggressive vitamin D therapy have been implicated (Hruska, 1997).

6b. High-Turnover Bone Disease. Osteitis fibrosa

Osteitis fibrosa is the most common type of renal bone disease. It is characterized by an increased bone turnover and elevated PTH levels. The mild form of this disease is shown by: localized areas of increased osteoblastic and osteoclastic activity, a slight increase in osteoid tissue and resorption cavities, but no evidence of peritrabecular fibrosis. The moderate form results in a more general increase in osteoblastic and osteoclastic activity, with increased osteoid tissue, and resorption cavities with clearly defined but limited peritrabecular fibrosis. The severe form results in extensive osteoblastic and osteoclastic activity with increased osteoid tissue, usually of normal thickness, resorption cavities which are increased in both extend and depth, and areas of woven bone and extensive peritrabecular fibrosis (Fletcher et al., 1997).

Hypocalceamia, hyperphosphateamia, and low levels of 1,25-dihydroxy vitamin $D_3$ are thought to lead to the high levels of PTH implicated in "high turnover" bone disease (Sherrard and Andress, 1989; Sutton and Camerion, 1992; Fletcher et al., 1994).

FLUORIDE AND BONE

1. Skeletal fluorosis

Bone changes observed in chronic skeletal fluorosis in man include osteosclerosis, osteomalacia, secondary hyperparathyroidism, and osteoporosis. The extent of which histological type predominates depends on several factors of which the dose and duration

of exposure to F and calcium nutrition status are important determinants. Secondary hyperparathyroidism can occur in individuals who have been chronically exposed to environmental F, chiefly due to a failure of bone resorption to maintain a satisfactory concentration of ionized calcium at the parathyroid gland level (Krishnamachari, 1987; Srivastava et al., 1989).

In 1937, Roholm reported osteosclerosis in miners of cryolite, a mineral composed of sodium aluminum and F[ $Na_3AlF_6$]. He showed that small doses of F given over a long period would cause osteosclerosis, while larger doses given acutely would lead to osteomalacia and osteoporosis. As a result of this observation sodium fluoride (NaF) was indicated for the treatment of osteoporotic patients (Rich and Ensick, 1961). It has been suggested that high F doses used to treat osteoporosis can cause secondary hyperparathyroidism and subsequent calcium deficiency that may lead to mineralization defects (Dure et al., 1996).

2. <u>The effect of fluoride on bone mineralization</u>

Fluoride is deposited in mineralizing tissues such as enamel, dentin, cartilage, and bone. Following an injection of radioactive F in laboratory animals, the skeleton was clearly labeled within a few minutes, and approximately 99% of all F was found in mineralized tissues (Ericsson and Hammarstrom, 1962 ).

Neuman and Neuman (1958) proposed that the uptake of F from the extracellular fluid by bone occurs by a three-stage process: by ionic exchange in the hydration shell of the crystallite, by exchange with an ion or group on the surface of the crystallite, and by the migration of surface ions into vacant spaces deeper within the crystallite. Fluoride associated with bone is not irreversibly bound, but rather may be mobilized by iso-ionic or hetero-ionic exchange, between interstitial fluid and the hydration shell or the crystallite surface, through the continuous process of bone remodeling. The clearance rate of F from

plasma is essentially equal to the sum of the renal and skeletal clearance rates (Ekstrand et al., 1980).

3. Fluoride effects on bone crystals

Bone is made up of unit cell of hydroxyapatite represented by the formula $Ca_{10}(PO_4)_6(OH)_2$. In this structure the F ion substitutes for the hydroxyl ion (OH) giving rise to fluoroapatite with the formula: $Ca_{10}(PO_4)_6F_2$. This substitution of the F ions for the OH ions has consequences on the structural stability of the apatite crystal, on the shape of the crystal, as well as on their kinetics of precipitation and dissolution. The structure of fluoroapatite is simpler, making it more stable and a less soluble crystal. In bone mineral, F has been shown to affect bone crystal structure by increasing crystallinity and reducing specific surface area. Bone treated with F has been shown to be more resistant to acid dissolution than normal bone. The distribution of F in bone is not uniform, but its net effect is to increase bone mineral density probably by an increased packing of bone crystals. Fluoride is shown to accumulate around Haversian systems and in mottled osteons (Grynpas, 1990)

The incorporation of F in bone mineral is dependent on age (Zipkin and McClure, 1952; Miller and Philips, 1956; Suttie and Philips, 1959), the duration of F exposure and bone type. This observation has been confirmed by x-ray microprobe analysis (Grynpas, 1990). The most plausible explanation for the age-dependency of F retention is that the crystallites of younger bone are smaller, more numerous, and loosely organized. They are heavily hydrated, and therefore, they offer a much larger surface area for the uptake of F than does more mature bone (Whitford, 1990). It is, therefore, a concern that the rate of F accumulation in bone in children with chronic renal insufficiency may be higher than the normal population and may contribute to growth failure.



LIBRARY

In the elderly, it is likely that a net loss of F from bone occurs due to the increased rate of bone resorption relative to that of bone accretion (Whitford, 1990). This is consistent with the observation that plasma F levels tend to rise during the last two decades of life (Grynpas, 1990).

The results of a 20 month longitudinal study of the pharmacokinetics of F in the dog indicated that at the time of weaning, approximately 90% of an administered F dose was taken up by the skeleton. During the first year of life, when the rate of growth steadily declines, the fractional uptake of the F dose by bone fell progressively until it reached a value of approximately 50% at one year of age, with little change thereafter. The renal clearance rate was independent of age and remained within the 1-3 ml/min/kg range throughout the study. Thus, in the weaning pup, the renal elimination of F accounted for only 10% of the dose while, in the mature animal, this fraction increased to approximately 50% (Whitford, 1990).

In 1937, Roholm reported that F accumulates in the skeleton and induces, at high doses, an increase radiopacity of the bones. Rich and Ensinck (1961) first reported on the use of sodium fluoride to treat patients with metabolic bone diseases. It has been used in combination with calcium and vitamin D, to reduce the incidence of vertebral crush fractures in osteoporotic individuals (Gruber and Baylink, 1991; Pak et al., 1994). Therapeutic F doses range from about 15 to 40 mg/day. At these doses, serum F levels are sufficient to stimulate new bone formation (Pak et al., 1994). The amount of new bone formed correlated with serum F levels, which was inversely correlated with glomerular filtration rate. It is still controversial whether the increase in trabecular bone mass is accompanied by a commensurate increase in bone strength, since, at high levels of F in addition to its positive effect on the bone cellular forming process, it has a negative affect on the mineralization process (Caverzasio and Bonjour, 1996).



LIBRARY

4. Cell Biology of bone growth

Bone is composed of two basic architectural structures, cortical and trabecular bone. Cortical bone accounts for 80% of skeletal mass, and is largely found in the shafts of the long bones and forms the outer wall of all bones. The remaining 20% of skeletal mass is trabecular bone. Bone is continually remodeled. Trabecular bone has a more active remodeling system when compared to cortical bone. The turnover of trabecular bone is 26 % versus 3 % for cortical bone, thus trabecular bone tends to be more sensitive to metabolic influences (Price et al., 1994).

5. Skeletal morphogenesis and growth

Growth of the long bones is by a mechanism of endochondral ossification, whereby the bones are first modeled in cartilage and then transformed into bone by ossification that begins in the shaft (diaphyses) and in the ends of the bone (epiphyses). Osteoblasts form a network of collagen fibers at the site where the cartilage cells and matrix have begun to disintegrate. Trabecular bone is then deposited on cartilaginous remnants. The embryonic bone increases in width by appositional growth, and the central cancellous bone core gradually becomes resorbed to form a marrow cavity (Price et al., 1994; Ganong, 1989).

The epiphyseal ossification centers is responsible for increasing the length of bone. The width of the epiphyseal plate (growth plate) is proportionate to the rate of growth. In the growing child this is a site of many complex cellular events; namely the proliferative, maturation, resorption and bone formation. Disturbances of any one of these processes may be reflected in growth retardation (Price et al., 1994).

Ream in 1981 showed that in rats given 120 ppm F in their drinking water for 4 weeks, rat femurs showed a histological increase in width in the osteoid seams on the

periosteal surface of the femoral diaphysis. This was in part due to an increase in the number of osteoid-producing cells and delayed mineralization of this tissue. It was also noted that a delay in mineralization in the distal femoral epiphyseal plate resulted in an increase in the number of hypertrophied cells. Concurrent with these changes in bone, the serum levels of calcium, phosphorus, and alkaline phosphatase remained within normal ranges (Ream, 1981).

In 1974, Kameyama showed that in rats chronically exposed to 100 ppm F in drinking water caused an increase in thickness of the cartilage layer in the rat mandibular condyle. This increase in thickness has also been observed in the growth plates of long bones by other investigators (Ream and Pendergrass, 1982; Mizohata and Kameyama, 1988). The increased thickness was due to an increase in the lower hypertrophic cell zone of the cartilage (Harbrow et al, 1992).

The effect of F on the chondrocyte function is unclear. It is hypothesized that it may stimulate chondrocyte growth, or inhibit calcification of the cartilage matrix. Stimulation of growth in chondrocytes may result in increased metabolic activity and maturation status of these cells.

## MATERIALS AND METHODS

### A. PRELIMINARY STUDIES

1. Rationale for using a rat model for renal deficiency -

The rat was chosen for these studies since renal insufficiency (Ittel et al., 1992) and the F effects on bone metabolism (Narita et al., 1990; Cheng et al., 1990; Turner et al., 1996; Grynpas and Rey, 1992) have been well characterized in previous studies in this animal model. A standardized procedure was used that allowed several levels of renal failure to be established in the rat and definite parameters to be monitored for the degree of renal failure as defined by Ormrod and Miller (1980). Three levels of stable renal failure were experimentally attained by glomerular filtration rate (GFR) of creatinine. Mild - 5.9 ml/min/kg or 46-54% of normal clearance; moderate - 3.0 ml/min/kg or 24% of normal clearance, and severe - 1.48 ml/min/kg or 11.6 % of normal clearance. The 5/6 nephrectomy animal model would fall into the moderate renal insufficient category.

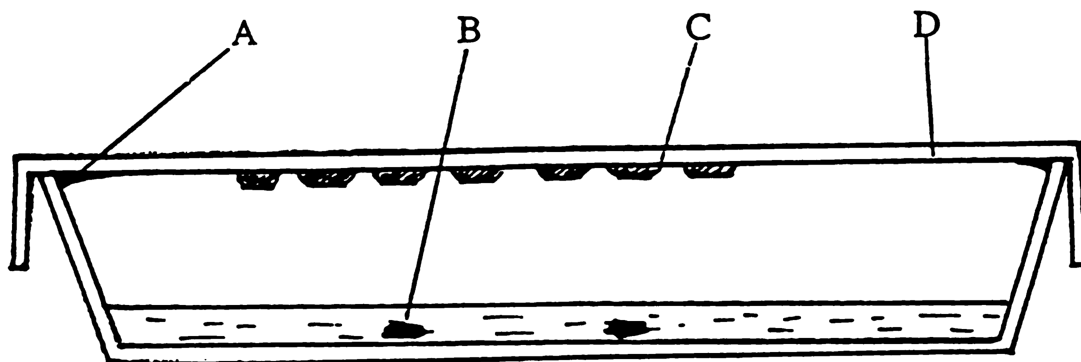
In addition, numerous F pharmacokinetic studies have been performed on laboratory animals, including the rat, dog, rabbit, hamster, guinea pig, and mouse (Whitford et al., 1991). In a comparison study of F metabolism in young adult dogs, cats, rabbits, rats, and hamsters, major quantitative differences in the metabolic handling of F among the five species was observed. In contrast, the results of F metabolism in rats resemble those found in humans. The value of plasma clearance of F attributed by kidney removal and uptake by calcified tissues was similar to that observed in humans (Whitford et al, 1991). Therefore, it was felt that the rat animal model would be appropriate to study the effects of renal insufficiency on F metabolism.



LIBRARY

2. Measurement of ionic F in bone

Preliminary studies were conducted to determine the time required for complete demineralization of the bone specimens, to ensure optimal F ion release for measurement of ionic F levels. A micro-diffusion method for concentrating minute amounts of F, using an ion-specific electrode (Orion, model 94-09), following acid-hexametyldisiloxane diffusion was used (Whitford and Reynolds, 1979; Dunipace et al, 1995) (Fig. 2).




**A - Vaseline seal  B - Sample in acidic media**

**C - 70 µl of 0.05 M Na(OH) on the lid in 7 equal drops  D - Lid**

**Fig. 2 F analysis using a micro-diffusion method for concentrating minute amounts of F using a ion-specific electrode (Orion, Model 94-09).**

Micro-diffusion method for concentrating minute amounts of fluoride following acid-hexametyldisiloxane diffusion.

Samples of cortical bone, bone marrow and growth plate from the right femur were weighed using a Cahn C-31 micro balance. The samples were placed in the Falcon plastic petri dish containing 2 ml of deionized water. Petroleum jelly was placed around the edge of the lid (using a syringe). Next 70 µl of 0.05 M NaOH were placed on the bottom of the lid in 7 equal drops. The lid was then everted and placed on the petri dish. Two ml of saturated 3 N $H_2SO_4$ (HMDS) was placed through the hole previously created in the lid with a soldering iron. The hole was sealed over with petroleum jelly and paraffin wax and placed on a rocker. The F ion is released from the specimen in the acidic environment and diffused to the NaOH "trap" on the lid of the petri dish. Following diffusion, the "trap" was buffered with 28 µl of 0.2 N acetic acid. The drop volume was maintained at 98 µl with the addition of deionized water, to offset any evaporation. The total volume allows good conductivity to be maintained between the F electrode and the reference electrode. Standards containing 5, 10 and 50 nM NaF were made up in triplicate. The procedure was repeated as described above.

To measure the time required for complete demineralization of the samples, F was measured in the NaOH "trap" at 5, 24, 30, and 48 hours after start of the experiment. At 5 hours, the F on the "trap" was measured as described above. A new lid containing fresh 70 µl of 0.05 M NaOH was immediately placed over the petri dish and returned to the rocker, and F measurement was repeated at 24, 30 and 48 hours after the initial start of experiment. There was negligible F release after 30 hours from the bone samples. It was therefore decided that a minimum of 30 hours was required for complete demineralization of the samples.

## B. STATISTICAL METHODS

### 1. Error of Method

To reduce error arising from the electrode, the electrode was allowed to "warm up" prior to use. The electrode was placed in a solution of TISAB buffer for approximately 30 minutes. Standards were run in triplicate each time, and were used to bracket the range of concentrations of the samples.

In the reading of the samples, the 98 μl drop, containing the diffused F ion in the NaOH/ HAc buffed solution, was "bridged" by the F electrode and the reference electrode so that good conductivity was maintained. The millivolt readings were recorded after one minute when a stable reading was achieved. Extreme care was taken to prevent F contamination from sample to sample. The electrode was rinsed after each reading with deionized water and gently dried using a Kim wipe.

The measurements for F in cortical bone and growth plate were repeated four times. The measurements for F in bone marrow were repeated three times. The measurements of F in serum and urine were done only once because of limited sample volume.

## C. EXPERIMENTAL DESIGN

### 1. Surgical induction of Uremia

Thirty-nine week old male Sprague-Dawley rats with a weight of 242.5 ± 6 g, were randomly divided into two groups. One group underwent a single stage 5/6 nephrectomy (Nx) (Reyes et al., 1992; Ingram et al., 1995), while the other group had a sham surgery performed as described below.

#### 5/6 Nx

Renal insufficiency was created using a modified surgical procedure described by Ormrod and Miller, 1980. Prior to all surgical procedures, the animals were anaesthetized with a cocktail of ketamine/xylazine (40 mg/kg/ 3 mg/kg IP). An incision was made in the skin and body wall along the midline. The left kidney was exposed, removed from the capsule and the upper and lower renal arteries were tied off with suture material (6.0 silk). The right kidney was then exposed, decapsulated, and the renal pedicle was ligated and the kidney removed. The wound and skin were closed with sutures (6.0 silk) or clips. All surgical procedures were done under aseptic conditions.

#### Sham Nephrectomy

Prior to all surgical procedures, the animals were anaesthetized with a cocktail of ketamine/xylazine (40 mg/kg/ 3 mg/kg IP). An incision was made in the skin and body wall along the midline. The left kidney was exposed and removed from the capsule. The procedure was then repeated with the right kidney. The wound and skin was closed with sutures (6.0 silk) or clips.

2. Renal function test

The rats were fed normal rat chow (Prolab RMH 2000) throughout the study and were allowed to drink *ad libitum* tap water. The animals were placed in metabolic cages, whereby urine was collected over a 24 hours period, two days prior to euthanasia. The final 24-hr urine samples were collected for measurement of creatinine (Cr), total $Ca^{2+}$ (section C. 6) and F ion (section C. 5), and were stored at -70 °C. Cr clearance (mg/min/Kg), a measure of glomerular filtration rate (GFR), was calculated as:

Urine flow (ml/min) x urine Cr (mg/dl)/plasma Cr (mg/dl)/ weight (Kg).

At 4 weeks and at 8 weeks after surgery, 18 animals (9 Nx, 9 Sham) and 12 animals (6 Nx, 6 sham) respectively, were sacrificed. The 9 Nx animals with the highest serum creatinine levels were sacrificed at 4 weeks with the remaining 6 Nx animals killed after an additional 4 weeks. An equal number of control animals were included with each group. Following anesthesia with a fluorinated anesthetic agent, the animals were given pentobarbital sodium 300 mg/kg IP and killed by bilateral thoractomy. Blood was drawn by cardiac puncture, and death was assured by bilateral pneumothorax. The procedures were approved by the Committee on Animal Research of the University of California, San Francisco.

3. Blood biochemistry

Blood samples were centrifuged immediately upon collection at 4000 r.p.m. at 4°C for 15 mins., and the serum was separated out and stored at -70°C. Serum samples from

each animal were analyzed for creatinine (Cr), total $Ca^{2+}$ ion ($TCa^{2+}$), phosphorus (P), PTH, 1,25-$(OH)_2$D3 and F (F) (see section 6 below).

4. Fluoride analysis of mineralizing tissues

The right femur was removed for F analysis. An x-ray image of the distal portion was taken to locate the growth plate. Bone F analyses were measured at two specific points along the femur namely, at the growth plate and midway along the shaft of the bone (Fig 3).

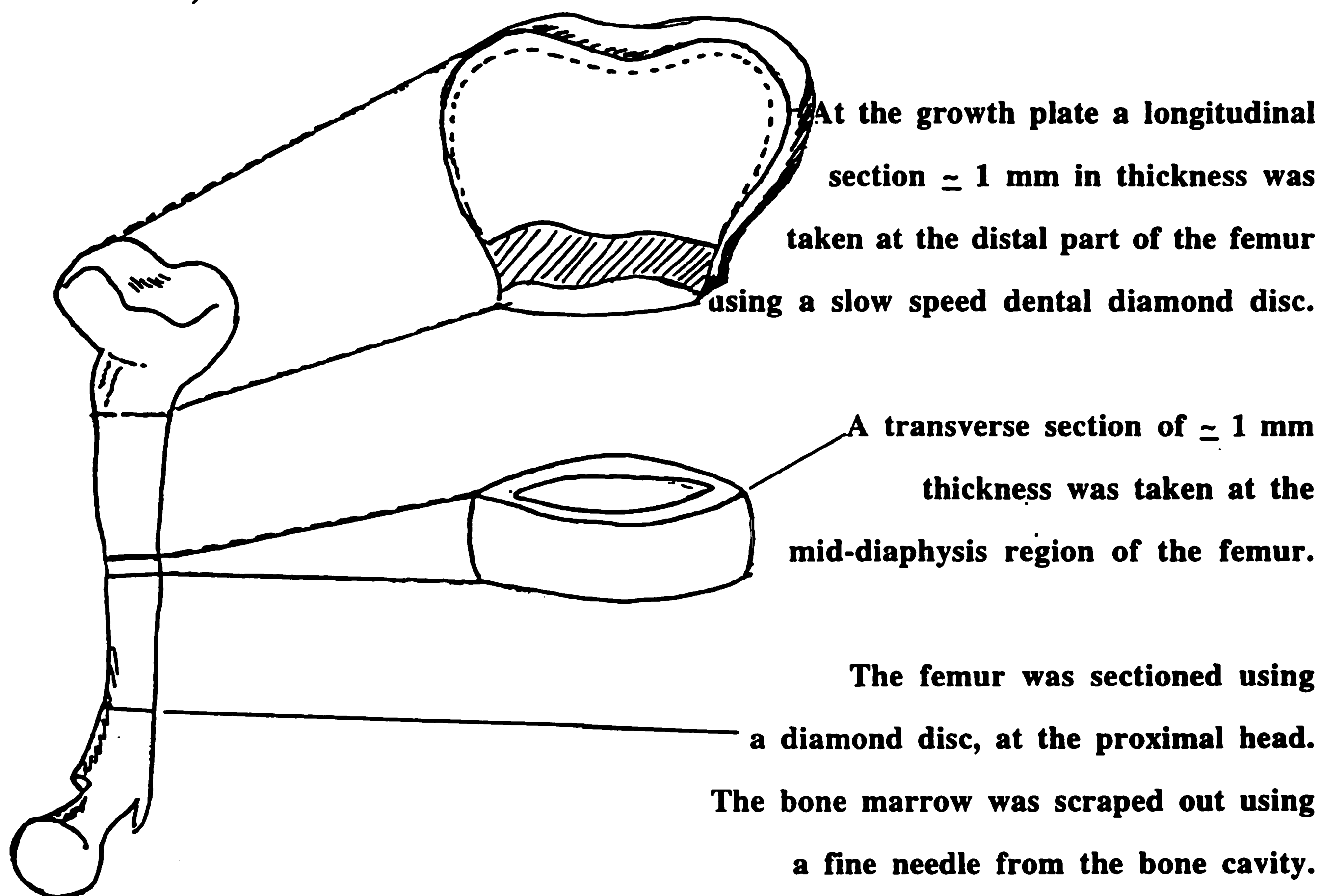



**Fig. 3 F analysis at the growth plate, bone marrow and mid-diaphysis region of the femur**

Bone Specimen Preparation

The right femur of each animal (15 Nx and 15 S, total of 30 femurs) was freshly excised, cleaned of muscle and soft tissue using a scalpel blade. The femur was dried by lyophilization for a minimum of 24 hours and stored at - 20 °C.

Bone fluoride was analyzed using a modification of the hexamethyldisiloxane micro-diffusion method of Taves. (Whitford and Reynolds, 1979; Dunipace et al., 1995) (as described on p.22).

Bone Marrow

Each femur was sectioned using a slow speed dental diamond disc (Diamond 911H, Brasseler) at the proximal head. The bone marrow was scraped out using a fine needle from the bone cavity. The sample was divided into three specimens for F analysis and the results averaged.

Cortical bone

A transverse section of approximately 1 mm in thickness was taken at the mid-diaphysis region of the femur. The cortical bone was cut using a slow speed dental diamond disc (Diamond 911H, Brasseler). The section was divided into four pieces, that were individually analyzed and the results were averaged.

Growth Plate

At the growth plate a longitudinal section approximately 1 mm in thickness was taken at the distal part of the femur using a slow speed dental diamond disc (Diamond 911H, Brasseler). The section was divided into four pieces, that were individually analyzed and the results were averaged.

5. Fluoride analysis of urine, serum and drinking water

F urine and serum were measured using a modification of the hexamethyldisiloxane microdiffusion method of Taves, 1968 (description on p. 22).

Urine

F analysis of urine followed the same procedure as described above except 0.5 ml of urine was measured and the volume made up to 2.0 ml with deionized water. Standards of 5, 10 and 50 nM of NaF were placed on the rocker with the specimens. Time allocated for acid induced diffusion F was 24 hours.

Serum

Serum F levels were analyzed in the same way using 0.25 - 0.5 ml of serum depending on amount of specimen available for analysis. The volume was made up to 2.0 ml with deionized water. Standards of 5, 10 and 50 nM of NaF were placed on the rocker with the specimens. Time allocated for acid induced diffusion of F was 24 hours.

Drinking Water

The F content in drinking water was analyzed directly. Five ml of drinking water was added to 5 ml of TISAB buffer, the solution was shaken well and F was then measured using the F electrode. One minute was allowed for the electrode to equilibrate. Standard solutions of 1.0, 0.1, and 0.01 mM of NaF were used for calibration of the drinking water.

6. Biochemical determination

Plasma $TCa^{2+}$, P, and Cr and urine $Ca^{2+}$ and Cr concentrations were determined by use of an autoanalyzer (Hitachi 747, Boehringer Mannheim, Indianapolis, IN).

Serum immunoreactive N-terminal PTH (iPTH) was determined in duplicate with a rat PTH (IRMA) Kit (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). It is a two-site immunoradiometric assay, which consists of two different goat antibodies to the N-terminal region (1-34) portion of the rat PTH. The goat antibodies are purified by affinity chromatography. One of the antibodies is immobilized onto plastic beads to capture the PTH molecules and the other is radiolabeled for detection. The sample containing the rat PTH is incubated simultaneously with an antibody coated bead and the $^{125}I$ labeled antibody. Both intact PTH (1-84) and N-terminal (1-34) contained in the sample are immunologically bound by both the immobilized antibody and the radiolabeled antibody to form a "sandwich" complex.

The beads are washed after the incubation period to remove any unbound labeled antibody and other components. The radioactivity of the antibody is then measured in a gamma counter. The radioactivity of the antibody complex bound to the bead is directly proportional to the amount of rat PTH in the sample. A standard curve is generated by plotting the CPM versus the respective rat PTH concentration for each standard on logarithmic scales. The concentration of rat PTH in the samples is determined directly from this curve.

Serum levels of 1,25-$(OH)_2D_3$ were determined in duplicate by radioreceptor assay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). One ml of serum sample was kept frozen (-20°C or below) until analysis was performed. A modified column $C^{18}OH$ extract of 1 ml samples are incubated with a sensitive 1,25-$(OH)_2D_3$ binding protein for one hour and then titrated 1,25-$(OH)_2D_3$ is added. After one hour, dextran coated charcoal suspension is introduced to accomplish the bound/free separation. The tube is

incubated for 30 minutes at 2-8 °C, following which they are centrifuged at 1500 x g for 20 minutes at 2-8 °C. The supernatants are decanted or transferred to scintillation vials. The radioactivity of the titrated protein-bound vitamin $D_3$ is then measured in a liquid scintillation counter. The concentration of 1,25-$(OH)_2D_3$ in the sample serum is determined by comparison with counts from the standard curve vials. Values are expressed in picograms per milliliter of serum after corrections for the sample concentration factor and the sample recovery from the column chromatography step.

D. F EFFECTS ON CHONDROCYTES IN VITRO

Plastic culture dishes were coated with Vitrogen 100 ( type 1 collagen at 30μg/ml) at 37°C for at least 16 hours. The dishes were washed with sterile water.

The chondrocytes were isolated from the growth plates of rib of a one week old pig (wt. 3 kg), as identified by a X-ray. The chondrocytes cells were harvested by incubation of the growth plate cartilage in PBS (calcium and magnesium free) containing 0.125% trypsin and 0.1% EDTA for one hour and then stopped by the addition of 10% fetal calf serum (FCS). The extracellular matrix was separated from the cells by washing the isolated cells with PBS three times. The chondrocyte cells were seeded at high density (1 x $10^5$ cells/ well in a 96 well tissue culture plate ). The cells were maintained in 0.2 ml of alpha MEM supplemented with 10% fetal calf serum, 18 units/ml penicillin G and 18 mcg/ml of streptomycin, until day of confluence (two days later). The fetal calf serum was analyzed for F and was found to be negligible.

1. Fluoride effect on the rate of cell proliferation

[$^3$H] thymidine incorporation into DNA was used as a marker for cell proliferation (Takebayashi et al., 1995). On the day of confluence the medium was changed to 0.3% FCS in a MEM with 50μg/ml of ascorbic acid (to arrest growth). Twenty-four hours after confluence varying concentrations of F (0.1, 0.5, 1.0, 5.0, 10.0, and 50 μM) were added to the cell culture. Twenty-one hours after the addition of F, $^3$H was incorporated at 1 μCi/well. The reaction was stopped three hours after $^3$H incorporation. The medium was aspirated and the cell layer washed with PBS three times. The cell layer was washed with 10% TCA (Tricholoroacetic acid) and then with EtOH:Ether 3:1 ratio three times. After the EtOH:Ether was aspirated the last time, the cell layer was allowed to air dry for 2-3 mins. and then dissolved with 200μl of 1N NaOH. This mixture was collected in vials containing 50μl of 6N HCl to create a neutral pH. Four milliliter of Sciniti Verse was added and the photon energy of $^3$H was measured in a scintillation counter.

2. Fluoride effects on glycosaminoglycan synthesis

[$^{35}$S] sulfate incorporation into GAG (glycosaminoglycan) was used as a marker for proteoglycan synthesis (Takebayashi et al., 1995). On day of confluence the medium was changed to 0.3% FCS in a MEM with 50 μg/ml ascorbic acid. Twenty-four hours after achieving confluence, varying concentrations of F (0.1, 0.5, 1.0, 5.0, 10.0, and 50 μM) were added to the cell culture. Three hours after addition of F, $^{35}$S was incorporated at 2 μCi/well. The reaction was stopped 24 hours after incorporation of $^{35}$S. The medium was collected and stored at -4° C to prevent degradation of GAG. Proteinase E solution (100μ/well of actinase/0.1 M tris pH 7.5/2.5 mm $CaCl_2$) was added to the cell layer and incubated at 37°C overnight. The following day, the cell layer in the proteinase solution plus the medium was added to 930 μl of mixture A (4.5 ml of 1% cetyl peridinium chloride

(CPC), 4.5 ml of 2 mM $MgSO_4$, 4.5 ml of 100 mM Tris/$CaCl_2$ 2.5 mM at pH 7.4, 0.45 ml chondrotin sulfate 0.1 mg/ml). The mixture was then incubated at 37° C for 30 mins. The mixture was passed through a No. 32 glass millipore filter. The filter was placed in a tube containing 4 ml of Sciniti Verse and the photon energy read for $^{35}$S in a scintillation counter.

## E. STATISTICAL ANALYSIS

All data are expressed as means ± SD Statistical analysis was performed by unpaired t-test of unknown variance using Microsoft Excel. A p-value of $< 0.05$ was taken to indicate a statistically significant difference. Analysis of variance (ANOVA) was used to analyze the accumulative affect of F between the 4 and 8 week groups. The ANOVA model contained two factors: 4 week class (sham and 5/6 nephrectomized animals) and 8 week class (sham and 5/6 nephrectomized). Analysis of variance (ANOVA) was used to the analysis the varying concentrations of F on $^3$H incorporation in cell proliferation and $^{35}$S incorporation in GAG production.

# III. RESULTS

## A. FLUORIDE CONCENTRATION IN RAT DIET AND DRINKING WATER.

Total F in the rat chow as reported by the manufacturer was 22 ppm. Analysis of F in the drinking water was 0.16 ± 0.02 ppm (mean ± SD).

Table 1. Composition of Prolab Rat/Mouse/ Hamster 2000 (a high energy diet).

**Nutrients**

| | |
|---|---|
| Minerals, ash | 6.7% |
| Calcium | 0.93% |
| Phosphorous | 0.81% |
| Phosphorous (non-Phytate) | 0.59% |
| F[1] ppm | 22 ppm |
| Protein | 19.0 % |
| Fat | 9.5% |
| Nitrogen-Free extract | 51.3% |
| Fiber (Crude) | 3.6% |

[1] Fluoride content as reported by the manufacturer.

B. RENAL BIOCHEMICAL MEASUREMENTS IN THE 4 WK GROUP

Four weeks after surgery, the body weights of the Nx animals were significantly lower than those of sham-operated animals ($p<0.05$). Results from Table 2 show that there was a significant increase in serum PTH and serum $TCa^{2+}$ concentration in the Nx group compared to the S group (p-value <0.05). There was a significant reduction of vitamin D (p-value <0.05), but no significant change in the value of Pi between the two groups.

Table 2. Biochemical determination for the 4 week post-surgery group. All values are the mean ± (SD).

| | Sham | Nx | p-value |
|---|---|---|---|
| **Numbers** | 9 | 9 | |
| **Serum** | | | |
| **Total Ca (mg/dl) | 9.9(0.1) | 10.2(0.1) | <0.05 |
| P (mg/dl) | 9.3(0.2) | 9.7(0.2) | NS |
| ** iPTH (pm/ml) | 53.0(5) | 72(6) | <0.05 |
| **$1,25(OH)_2D_3$ (pg/ml) | 46.0(3) | 33(4) | <0.05 |
| **Urine*** | | | |
| **Cr Cl (ml/min/kg) | 10.2(0.6) | 1.9(0.4) | <0.05 |
| **24 h urine Ca (mg/kg) | 3.7(0.5) | 1.4(0.5) | <0.05 |

*** calculated using body weight at time of sacrifice.**

**** values found to be significantly different between S & Nx.**

## C. FLUORIDE BIOCHEMICAL VALUES IN THE 4 WK GROUP

F levels in the growth plate and serum were significantly increased (p-value <0.01), while F levels in urine were significantly reduced in the Nx group (p-value <0.01). There was an increase in F concentration in the cortical bone of the Nx animals but it was not found to be significant. There was no significant difference in F content in the bone marrow (Table 3 and Fig. 4).

Table 3. Fluoride content in mineralizing tissues, serum and urine in the 4 week post-surgery group. All values are the mean ± (SD).

| | Sham | Nx | p value |
|---|---|---|---|
| **Numbers** | 9 | 9 | |
| **Bone F** (ppm) | | | |
| cortical | 442(82) | 531(106) | NS |
| **growth plate | 353(63) | 550(167) | <0.01 |
| bone marrow | 54(29) | 27(28) | NS |
| **Urine** (ppm) | 4.50(1.65) | 1.07(0.36) | <0.01 |
| ** [2] **Serum** (ppm) | 0.62(0.29) | 1.19(0.44) | 0.01 |

**[2] Serum levels were affected by the fluoridated anesthetic agent given just prior to sacrifice.**

**** values found to be significantly different between S & Nx.**

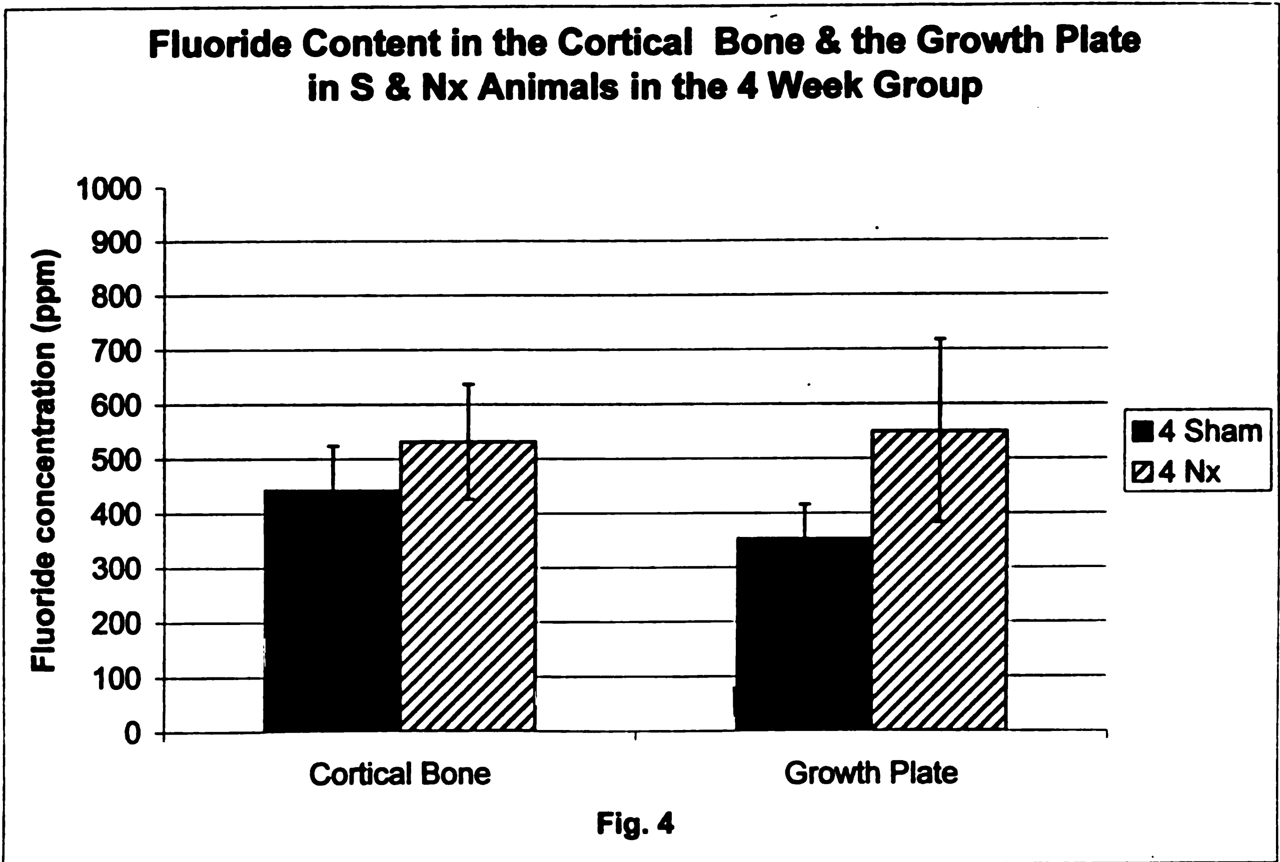



**Fig. 4. F content in the cortical bone and the growth plate between S and Nx animals in the 4 week group. (mean ± SD).**
**F levels in the growth plate were significantly increased (p-value <0.01). There was an increase in F concentration in the cortical bone of the Nx animals but it was not found to be significant.**

## D. RENAL FUNCTION IN THE 8 WK GROUP

Eight weeks after surgery, the body weight of the Nx animals were significantly lower than those of sham-operated animals ($p<0.05$). Results from Table 4 show that there was a significant increase in serum PTH and serum $TCa^{2+}$ concentration in the Nx group as compared to the S group (p-value $<0.05$). There was no significant change in the value of Pi between the two groups. 1,25-$(OH)_2D_3$ was not measured in the 8 week group.

Table 4. Biochemical determination for the 8 week post-surgery group. All values are the mean ± (SD).

| | **Sham** | **Nx** | **p value** |
|---|---|---|---|
| **Numbers** | 6 | 6 | |
| **Serum** | | | |
| **Total Ca mg/dl | 9.5(0.3) | 10.4(0.6) | <0.05 |
| Pi mg/dl | 7.4(0.6) | 8.1(1.6) | NS |
| **iPTH pm/ml | 28.8(20.1) | 72(13.3) | <0.05 |
| **Urine*** | | | |
| **Cr Cl ml/min/kg | 3.5 (0.44) | 1.4 (0.59) | <0.001 |
| **24 h urine Ca mg/kg | 0.66(0.1) | 0.47(0.1) | <0.010 |

*** calculated using body weight at time of sacrifice.**

**** values found to be significantly different between the S & Nx group.**

## E. FLUORIDE BIOCHEMICAL MEASUREMENTS IN THE 8 WK GROUP

Eight week post surgery showed a significant increase in F content in the growth plate and cortical bone in the Nx group compared to the sham operated group. Fluoride levels in urine were significantly elevated in the Nx group compared to the sham operated group. Even though there was an increase in F levels in the serum in the Nx group, it was not found to be significant (Table 5 and Fig.5).

Table 5. F content in mineralizing tissues, serum and urine in the 8 week post-surgery group. All values are the mean ± (SD).

| | Sham | Nx | p value |
|---|---|---|---|
| **Numbers** | 6 | 6 | |
| **Bone F** (ppm) | | | |
| **cortical | 479(80) | 725(175) | <0.05 |
| **growth plate | 396(97) | 654(135) | <0.01 |
| bone marrow | 16(7.2) | 16(9.2) | NS |
| ****Urine** (ppm) | 2.48(0.61) | 4.24(1.1) | <0.01 |
| **[2]**Serum** (ppm) | 0.89(0.40) | 1.09(0.2) | NS |

**[2]Serum levels were affected by the fluoridated anesthetic agent given just prior to sacrifice.**

**** values found to be significantly different between the S & Nx group.**

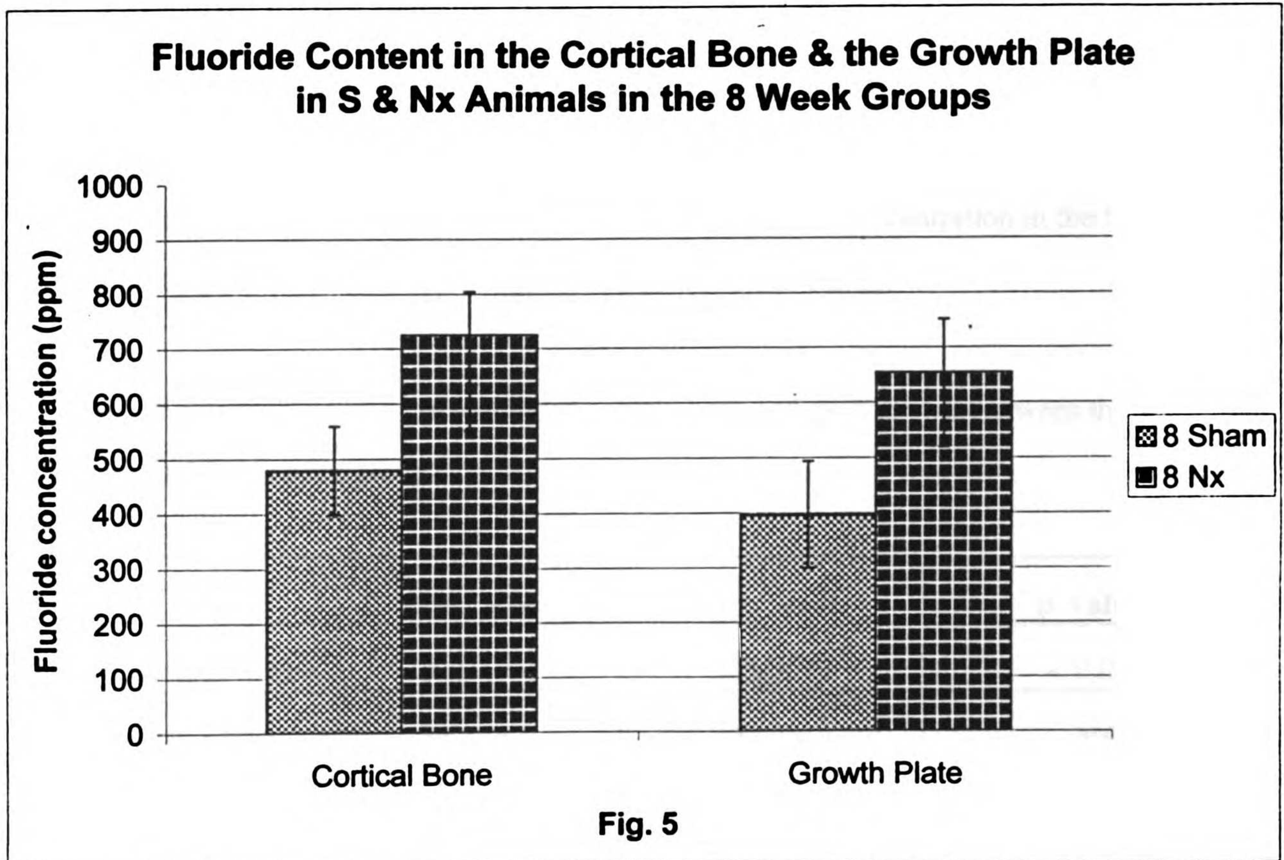



**Fig. 5. F content in the cortical bone and the growth plate between S and Nx animals in the 8 wk group.(mean ± SD)**
**Eight week post surgery showed a significant increase in F content in the cortical bone and growth plate in the Nx group compared to the sham operated group ($p<0.05$).**

## F. FLUORIDE BONE MEASUREMENTS BETWEEN 4 & 8 WK GROUP

The final concentration of F in the cortical bone between the four and eight week Nx group was significantly different ($p < 0.05$). Even though the final concentration of F in the growth plate in the eight week was greater than the F concentration in the four week Nx group, it was not found to be significant (Table 6 and Fig 6).

Table 6. Comparison of F content in the cortical bone and growth plate between the 4 week and 8 week Nx group.

| | **4Nx week** | **8Nx week** | **p value** |
|---|---|---|---|
| ****Cortical bone** | 531(106) | 725(175) | < 0.05 |
| **Growth plate** | 550(167) | 654(135) | NS |

**** value found to be significantly different between the 4Nx & 8Nx group.**

## G. CHONDROCYTE CELL CULTURE

There was no significant difference in ($^3H$) thymidine uptake or in $^{35}S$ incorporation in GAG at any of the F levels in the chondrocyte primary culture as compared to controls (Fig. 7 and 8 below).

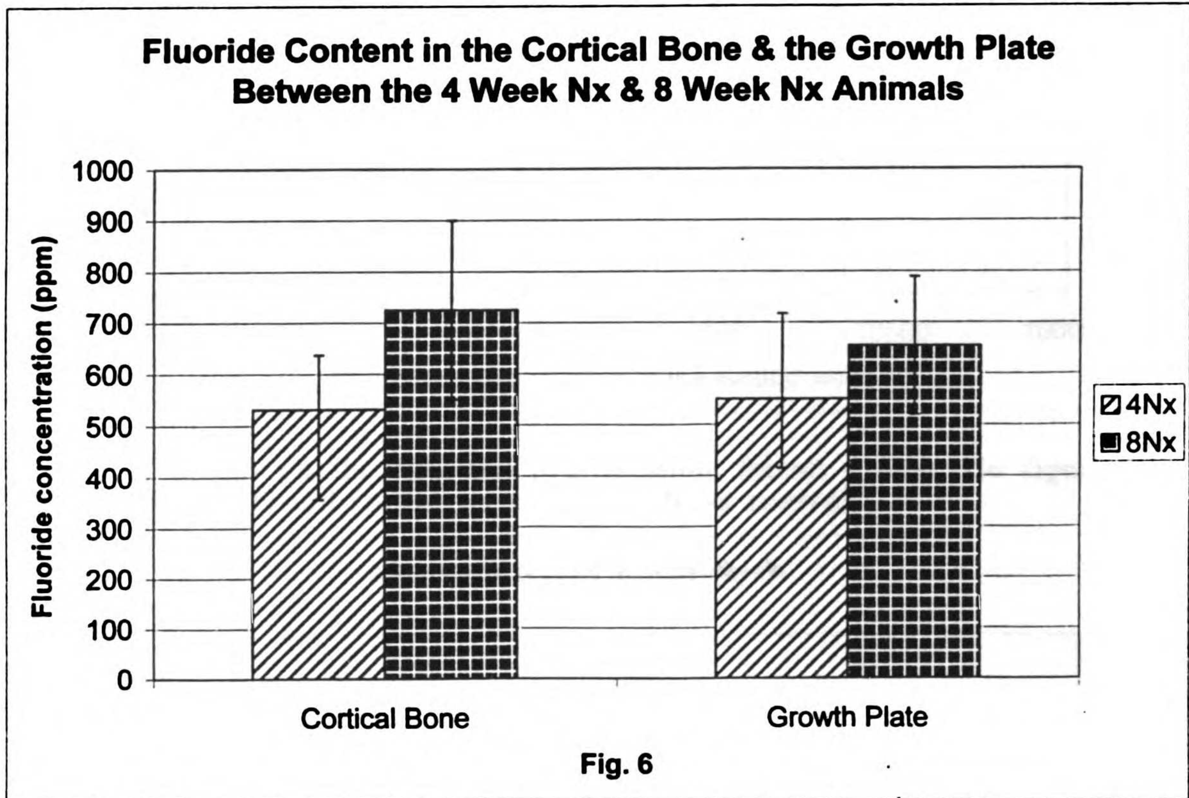



**Fig. 6. F content in the cortical bone and the growth plate between 4 wk Nx and 8 wk Nx animals. (mean ± SD)**
**There was a significant difference in the final concentration of F in the cortical bone between the four and eight week Nx group ($p < 0.05$). Even though the concentration of F in the growth plate in the eight week was higher than that of the four week Nx group, it was not found to be significantly different.**

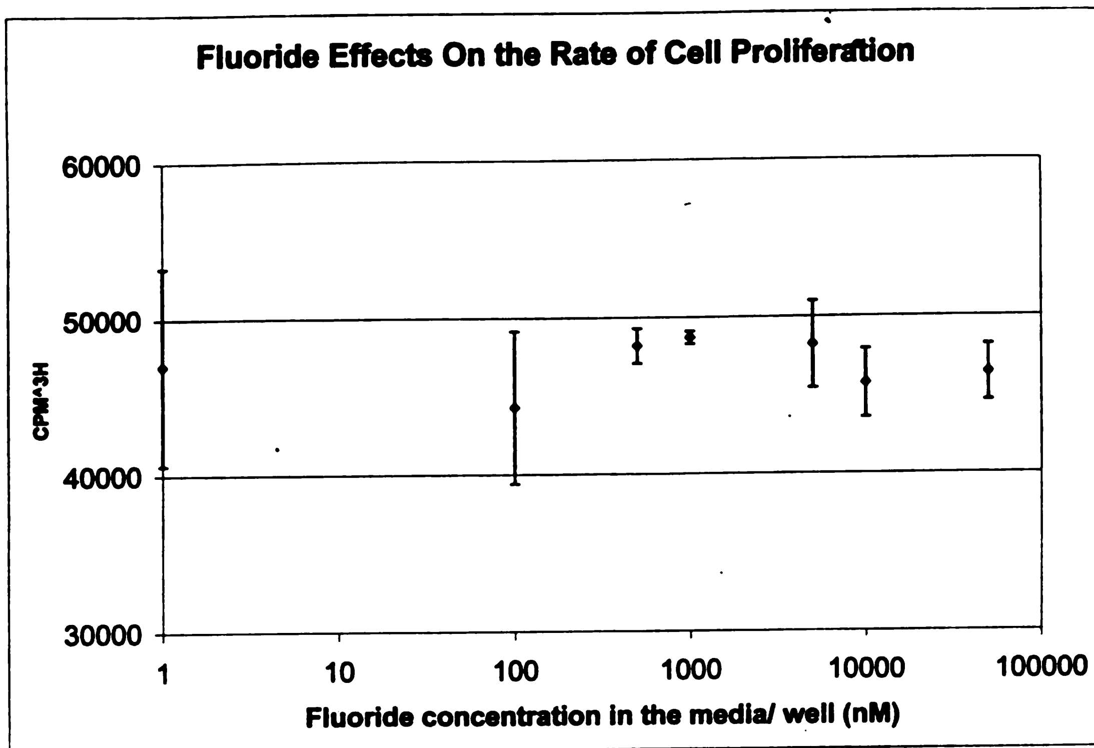



Fig. 7. F effects on the rate cell proliferation. (mean ± SD) No significant difference was found in any of the F levels vs control.

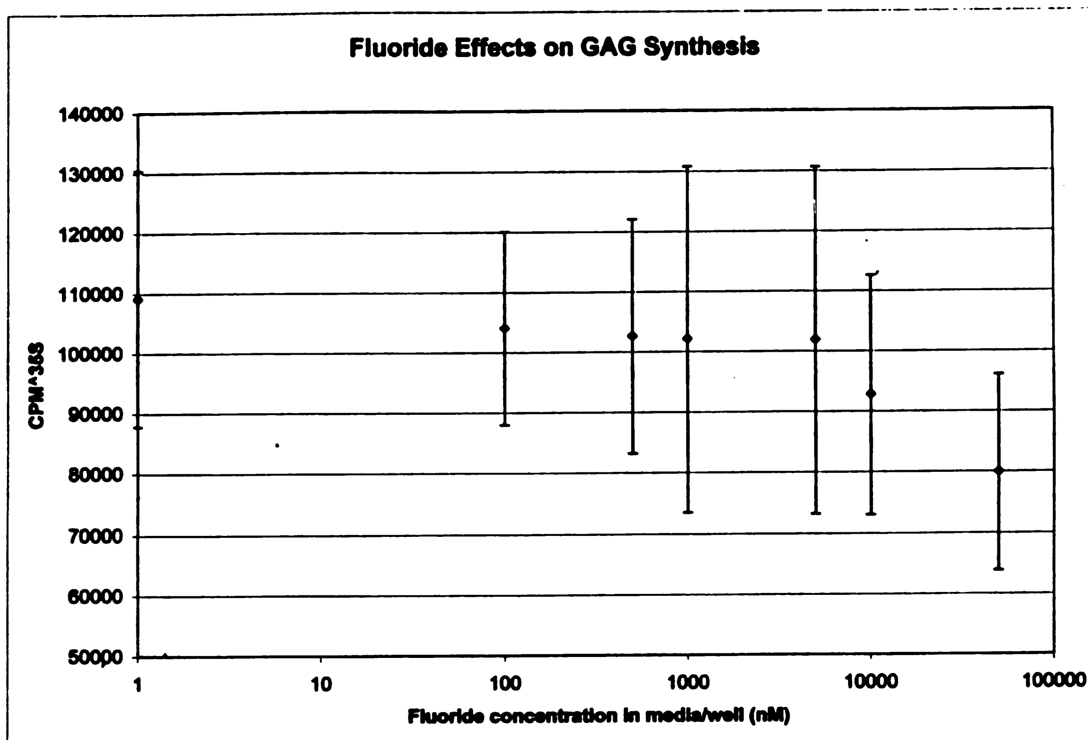



Fig. 8. F effect on GAG synthesis. (mean ± SD) No significant difference was found in any of the F levels vs control.

## IV. DISCUSSION

One of the many tests carried out that quantify residual renal function in the nephrectomized animals was that which measured the glomerular filtration rate (GFR) of creatinine and blood creatinine levels. At both 4 and 8 weeks after surgery, there was a significant decrease in the GFR and a significant increase in serum creatinine levels in the Nx animals compared to that of the of sham-operated animals. In the 4 week Nx group there was a significant reduction of urine creatinine resulting in 18.6 % of normal clearance which is consistent with a definition of moderate/severe uremic group (24 -11.6% of normal clearance) (Ormrod and Miller, 1980). In the eight week group, there was a significant decrease in creatinine clearance levels resulted in 40% of normal clearance which is consistent with a definition of mild/moderate uremic animal model (46-24% of normal clearance) (Ormrod and Miller, 1980). The degree of renal failure as measured by creatinine clearance in the eight week Nx group was not as severe as found in the four week group. This is consistent with the fact that the animals with the highest serum creatinine were assigned to the 4 week group, with a corresponding number of sham operated animals. In both the 4 and 8 week group the body weights of the Nx animals were significantly reduced compared to those of the sham-operated animals. This is commonly found in animals with experimental renal failure.

The levels of serum $Ca^{2+}$ and PTH were significantly elevated in both 4 and 8 week Nx animals as compared to the sham-operated animals. Secondary hyperparathyroidism and hypercalcemia is commonly found in patients with chronic renal insufficiency (First, 1982; Coburn and Slatopolsky, 1991). PTH is secreted to maintain serum $Ca^{2+}$ levels. In patients with chronic renal insufficiency, there is a disruption of this mechanism, which is partly due to the reduced secretion of 1,25-vitamin $D_3$ in the kidney, but also from a reduced sensitivity of the parathyroid gland to the suppressive effect of chronic increased serum $Ca^{2+}$ on PTH secretion (Llach, 1995). In keeping with this model, urinary $Ca^{2+}$

excretion was significantly reduced in the Nx animals in both 4 and 8 week groups, which is similar to that found in patients with mild to moderate chronic renal insufficiency (Healy et al, 1980; Cremer et al, 1985).

Patients treated with F in doses between 10-61 mg/day for osteoporosis have been shown to exhibit signs of hyperparathyroidism, as have patients with skeletal fluorosis. Faccini (1969) demonstrated that daily doses of F given for a week stimulates the production of PTH. Yates et al(1964) and Nicholas et al (1965) explained the observed elevation of the hormonal activity in circulation as the possible consequence of F induced reduction in serum calcium accompanying F induced depression of bone mineral solubility (Krishnamachari, 1986). This has resulted in bone changes similar to that seen in osteomalacia (Roholm, 1937). Turner et al. (1996) have demonstrated osteomalacia in renal deficient rats given 15 ppm F (equivalent to 3 ppm F drinking water in humans) in the drinking water. It would be of interest to see if high levels of F with calcium supplementation would lead to osteosclerosis in the renal deficient rat, as seen in skeletal fluorosis with high calcium in the diet.

Urine F levels were significantly reduced in the 4 week Nx animals and significantly increased in the 8 week Nx animals as compared to the sham-operated animal. This is consistent with the fact that F clearance has been shown to correlate with creatinine clearance in the kidney, such that with a decrease in creatinine clearance there is a reduction in F excretion (Schiffl and Binswanger, 1980). The renal insufficiency in the 4 week Nx animals was worse than that found in the 8 week Nx animals. The significant increase in F excretion in the 8 week Nx animals may be explained by the increased consumption of water in these animals as has been demonstrated in rats with renal insufficiency. In patients with renal insufficiency the substantial reduction in creatinine clearance, leads to an increase in plasma F levels (Parson et al., 1975, Schiffl and Binswanger, 1980).

We were initially surprised to find the high serum F levels in both the Nx and sham-operated animals in the 4 and 8 week groups. However, upon further investigation

we found the anesthetic agent used prior to cardiac puncture was a fluoridated anesthetic agent. The duration of exposure to the inhalation anesthetic agent was similar in all of the animals, and therefore, we assumed that the basal levels in the Nx and sham-operated groups were similarly increased. There was an increase in F serum levels in the Nx animals compared to the sham-operated animals in both the 4 week and 8 week group. It was only found to be significant in the 4 week group. Again this difference between the Nx group was likely due to the severity of renal failure in the 4 week group being worse than that found in the 8 week group. The increased plasma F levels are consistent with the elevated F levels found in humans with renal insufficiency. The serum F levels in patients with impaired renal function range from 21 µg/l to 41 µg/l serum F in patients with end-stage renal disease. The levels of serum F in patients with normal renal function are 9.8 µg/l. (Schiffl and Binswanger, 1980, Kimura et al., 1993).

We hypothesized that there would be a difference in the distribution of F content in bone of rats with renal insufficiency. This hypothesis was confirmed as there was a significant difference in F content in the growth plate in both the four week Nx and eight week Nx animals. Cortical bone F levels increased at a slower rate and were elevated in the eight week Nx animals. A linear relationship between the 4 and 8 week groups cannot be assumed, since the levels of renal insufficiency were not consistent. It is of significance that even with mild/moderate renal insufficiency there was an increased accumulation of F in the cortical bone and the growth plate after 8 weeks. Therefore, it can be assumed the levels of F in the moderate/ severe 4 Nx animals would increase over time.

The increase in F accumulation in the growth plate after 4 weeks was interesting since the animals did not receive supplemental F. In fact the level of F analyzed in the drinking water was $0.16 \pm 0.02$ ppm (mean $\pm$ SD), instead of the 1 ppm F expected for the fluoridated city of San Francisco. These results confirm that increased plasma F levels in the renal-deficient animal model leads to an increase in bone F levels.

The amount of F found in bone usually correlates with the amount of F in the water and diet (Legeros et al., 1982, Zipkin 1973), and is incorporated during bone formation (Grynpas and Rey, 1992). Previous studies on growing rats exposed to different levels of F for various lengths of time have shown different distributions of F in different types of bone, with the highest concentration being found in the periosteal and endosteal surfaces and lowest in the interior of the bone (Li et al., 1995, Li et al., 1993, Narita et al., 1990). However, relative F uptake into the growth plate region has not previously been determined. The result of this study show that F is more rapidly taken up in the growth plate as compared to cortical bone.

The nonlinear binding characteristics of F to bone has been shown in both rats and humans with renal insufficiency (Turner et al., 1996, Kekki et al., 1982), and is most likely due to high uptake of F into rapidly mineralizing tissues. Both the metabolic activity and mineral turnover of bone is at a maximum at the growth plate, and explains the rapid accumulation of F at the growth plate in the four week post-surgery group and the delay in accumulation of F in the cortical bone.

Our study confirms that even with 0.16 ppm F in the drinking water and 22 ppm F in the diet, there was a significant accumulation of F in the growth plate four weeks post-surgery. No histomorphometric measurements of mineralization were made, so it was difficult to predict if any disruption in mineralization had occurred. However, histomorphometric changes such as osteomalacia have been seen in renal deficient rats given 15 ppm F drinking water, while at 50 ppm F in drinking water results in osteomalacia and strength reduction after a 6 month period (Turner, 1996).

Our *in vitro* studies of the direct effect of F on chondrocyte in culture did not show any significant difference in cell proliferation or $^{35}$S incorporation in GAG in proteoglycan synthesis. Previous animal studies *in vivo* have shown no obvious changes in the proliferative or maturation zones in rats drinking 100 ppm F in water. In this cell culture GAG was measured within a 24 hr period which did not allow the cells to progress further

than the maturation zone seen in the growth plate. These results confirm what is seen histologically at the growth plate in the femur of rats given high levels of F (100 ppm F equivalent to 10μM F in the cell culture). However, changes have been shown in the lower hypertrophic zone, which was increased in length (Kameyama, 1974; Ream and Pendergrass, 1982; Mizohata and Kameyama, 1988; Harbrow et al., 1992). Our in vitro system did not allow analysis of the hypertrophic zone. Further studies are required to determine whether F has a direct effect on ALP production, a marker for the hypertrophic zone or on cell apoptosis.

In fluorotic bone (containing in excess of 5000 ppm of F), there is an increase in glycosaminoglycans production which is thought to be partially responsible for the defective calcification in skeletal fluorosis (Prince and Navia, 1983; Jha and Susheela, 1982). In our studies, GAG production in chondrocytes was decreased at 50 μM F, but was not found to be significant.

Clinical Implications

Further investigations are needed to determine, during active growth, if high F levels and disruption of calcium metabolism (elevated PTH levels) in patients with chronic renal insufficiency, interferes with normal mineralization at the growth plate and growth. This is of particular concern since the incidence of dental fluorosis has increased due to hyperfluoridation, (increased F uptake from multiple fluoridated sources) (Pendrys and Stamm, 1990), suggesting increasing levels of F in consumption. The ubiquitous presence of F in food and beverage products regardless of the degree of water fluoridation suggests that the overall F exposure in individuals with renal insufficiency should be more closely monitored.

## REFERENCES


Ampil M. and Wendland B.: Nutritional status in renal disease. Care of the renal patient. Levine D. Z. (1983),33-44

Anand J.K., Roberts J.T.: Chronic fluorine poisoning in man: a review of literature in English (1946-1989) and indications for research. Biomed and Pharmacother (1990) 44, 417-420.

Angmar-Mansson B. and Whitford G. M.: Environmental and physiological factors affecting dental fluorosis. J. Dent. Res. (1990) 69 Special, 706-713

Brunier G.: Calcium/Phosphate imbalances, aluminum toxicity, and renal osteodystrophy. ANNA (1994),171-177

Caverzasio J., and Bonjour J.: Characteristics and regulation of Pi transport in osteogenic cells for bone metabolism. Kidney Inter. (1996) 49: 975-980

Cheng P., and Bader S.: Effects of fluoride on rat cancellous bone. Bone and mineral. (1990):153-161

Coburn J. W., and Slatopolsky E.: Vitamin D, parathyroid hormone, and the renal osteodystrophies. In B.M. Brenner and F. C. Rector (Eds) The Kidney (4th edition) (1991): 2036-2120. Philadelphia: W. B. Saunders Company.

Cremer B., Lubbers E., Klooker P., Schmidt-Gayk H., Ritz E.: Calciuric response to 1-25 $(OH)_2D_3$ in early renal failure. Mineral Electrolyte Metab. (1985)11: 182-185

Dunipace A., Brizendine E., Zhang W., Wilson M., Miller L., Katz B., Warrick J., and Stookey G.: Effects of aging on animal response to chronic fluoride exposure. J.Dent. Res. (1995) 74: 358-368.

Dure-Smith B., Farley S., Linkhart S., Farley J., and Baylink D.: Calcium deficiency in Fluoride-treated osteoporotic patients despite calcium supplementation. J. Clin. Endocrine. Metab. (1996), 269-275

Ekstrand J., Spak C., and Vogek G.: Pharmacokinetics of fluoride in man and its clinical relevance. J. Dent. Res. (1990) 69 Special, 550-555.

Ekstrand J.: Relationship between fluorides in the drinking water and the plasma fluoride concentration in man. Caries Res. (1978) 12:123-127

Ekstrand J.; Ehrnebo M.; Whitford G.; Jarnberg P.: Fluoride Pharmacokinetics during acid-base balance changes in man. European J. Of clinical Pharmacology. (1980) 18: 189-194

Ericsson Y.,and Hammarstrom L. Mouse placenta transfer of F18 in comparison with $Ca^{45}$. Acta Obstet Gynecol Scand 1962;41: 144-58

Faccini J.: Review : Fluoride and bone. Calcific. Tissue Res. 1969: 1-16

Fejerskov O., Ekstrand J., and Burt B.: F in dentistry. Second Edition (1996). Munksgaard, Copenhagen. 55-87

First M. R.: Chronic renal failure: pathophysiology, clinical manifestation, and management. Garden City, N.Y. Medical Examination Pub. Co., 1982: 1-25.

Fletcher S., Jones R., Rayner H., Hordon P., Aaron J., Oldroyd B., Turney J., and Smith M.: Assessment of renal osteodystrophy in dialysis patients: use of bone alkaline phosphatase, bone mineral density and parathyroid ultrasound in comparion with bone histology. Nephron 1997, 75: 412-419

Ganong W. F.: Review of medical Physiology. Norwalk. Conn.:Appleton and Lange. 14th Edition (1989), 326-337

Gruber H and Baylink D.: The effects of fluoride on bone. Clin. Orthop. Relat. Res. (1991), 297: 264-277

Grynpas M., Simmons E., Pritzker K., Harrison J. Is fluoridated bone different from non-F bone? Cell mediated calcification and matrix vesicles. In Yousuf S. Elsevier, New York. P 409-414.

Grynpas M. Fluoride effects on bone crystal. J of bone and mineral. 1990:S169-S175.

Grynpas M., and Rey C.: The effect of fluoride treatment on bone mineral crystals in the rat. Bone. 1992: 423-429

Guyton A. C.: Textbook of medical physiology (8th edition) (1991). Philadelphia: W.B. Saunders Company

Hruska K.: Renal osteodystrophy. Baillere's Clinical Endocrinology and Metabolism. 1997. 11:165-194

Harbrow D., Robinson M., and Monsour P.: The effect of chronic fluoride administration on rat condylar cartilage. Australian Dental Journal (1992): 55-62

Healy M., Malluche H., Goldstein D., Singer F., Massry S.: Effects of long-term therapy with calcitriol in patients with moderate renal failure. Arch Intern. Med. (1980)140: 1030-1033

Ingram A., Parbtani A., Clark W., Spanner E., Huff M., Philbrick D., and Holub B.: Effects of flaxseed and flax oil diets in a rat-5/6 renal ablation model. American J. of Kidney Diseases. 1995,25: 320-329

Ishiguro K. et al.: Distribution of fluoride in cortical bone of human rib. Calcif. Tissue Int. (1993): 278-282

Ittel T., Gruber E., Heinrichs A., Handt S., Hofstadter F., and Sieberth H.: Effects of fluoride on aluminum-induced bone disease in rats with renal failure. Kidney Intern. 1992,41: 1340-1348

Kameyama Y.: Histologic and histometric study of the effect of fluoride on the rat mandibular condyl. J. Oral Pathol 1974,3: 205-216

Kimura T., Yamamoto G., Yoshitake K., and Ando T.: Ionic and nonionic fluoride levels in blood of dialyzed and undialyzed patients with renal failure and kidney transplanted patients. Jpn. J. Med. Sci. Biol., (1993) 46: 131-139

Jha M and Susheela A.: Characteristics of glycosaminoglycans from normal and fluoride treated rabbit iliac crest. Biochemical and Biophysical research communications. (1982)105:711-716

Kekki M., Lampainen E., Kauranen P., Hoikka V., Alhava E., and Pasternack A.: The nonlinear tissue-binding character of fluoride kinetics in normal and anerphric subjects. Nephron (1982), 129-134

Krishnamachari K. Skeletal fluorosis in humans: a review of recent progress in the understanding of the disease. Progress in food and nutrition science; 1987:279-314

Legeros R. Z., Singer L., Ophaug R., Quirolgico G., Thein A., and LeGeros J.: The effect of fluoride on the stability of synthetic and biological (bone mineral) apatites. Menczel J., Steinberg R., eds. Osteoporosis. New York: J. Wiley and Sons; 1982: 327-341

Li J., Nakagaki H., Kato K., Ishiguro K., Ohno N., Kameyama Y., Muki M., Ikeda M., Weatherell J., and Robinson C. Distribution profiles of fluoride in three different kinds of rat bones. Bone (1993): 835-841

Li J., Nakagaki H., Kato K., Tsuboi S., Kato S., Morita I., Ohno N., Kameyama Y., Chen R., and Robinson C. Effects of stopping fluoride administration on the distribution profiles of fluoride in three different kinds of rat bones. Calcified Tissue Int. (1995): 292-296

Llach F. and Coburn J.: Renal osteodystrophy and maintenance dialysis. (1989) In J.F. Maher (Ed), Replacement of renal function by dialysis (3rd ed.) (pp. 911-952) Dordrecht, Holland: Kluwer Academic Publishers

Llach F.: Secondary hyperparathyroidism in renal failure: The trade-off hypothesis revisited. Am. J. Kidney Dis. (1995)25: 663-679

Miller R., Philips P. The effects of age on the level and metabolism of fluoride in the bones of the fluoridated rat. J. Of Nutr. 1956; 59: 425-433

Mizohata M., and Kameyama Y.: Histological, histometrical and fluoride electrode studies of the effects of fluoride on the mandibular condyles in growing newborn rats. Acta. Anat. (Basel) 1988, 133: 134-139

Narita N., Kato K., Nakagaki H., Ohno N., Kameyama Y., and Weatherell J.: Distribution of fluoride concentration in the rat's bone. Calci. Tissue Int. (1990): 200-204

Nicholas G., Flanagan B., and Woods J.: Influences on bone biosynthetic mechanisms. In: Gaillard, PJ, ed Parathyroid gland ultrastructure, secretion and function. 1965:243-260

Neuman W., Neuman M. The chemical dynamics of bone mineral, Chicago: University of Chicago Press 1958: 75-100

Ormond D., and Miller T.: Experimental Uremia. Nephron 1980,26: 249-254

Pak C., Sakhaee K., Piziak V., Peterson R., Breslau N., Boyd P., Poindexter J., Herzog J., Heard-Sakhaee A., Haynes S., Adams-Huet B., and Reisch J.: Slow-release sodium fluoride in the management of post-menopausal osteoporosis. Ann. Inter. Med. (1994), 120: 625-632

Parsons V., Choudhury A. A., Wass J. A., and Vernon A.: Renal excretion of fluoride in renal failure and after renal transplantation. British Med Journal (1975) 1:128-130

Pendrys D. G. and Stamm J. W.: Relationship of total fluoride intake to beneficial effects and enamel fluorosis. J. Dent. Res. (1990) 69: 529

Price J. S., Oyajobi B. O., and Russell R.: The cell biology of bone growth. European J. of Clinical Nutrition (1994) 48 Supp: s131-S149

Prince C. and Navia J.: Glycosaminoglycans in rat bone due to growth and fluorosis. J. Nutr. (1983)113:1576-1582

Ream L., The effects of short-term fluoride ingestion on bone formation and resorption in the rat femur. Cell Tissue Res. (1981): 421-430

Ream L. and Pendergrass P.: Scanning electron microscopy of the femoral epiphyseal plate and metaphysis of the rat after short-term fluoride ingestion. J. Submicrosc. Cytol 1982,14: 73-80

Reyes A., Purkerson M., Karl I., and Klarh S.: Supplementation with L-Arginine ameliorates the progression of renal disease in rats with subtotal nephrectomy. Am. J. Kidney Dis. (1992) 20: 168-176

Rich C and Ensick J.: Effect of sodium fluoride on calcium metabolism of human beings. Nature.(1961), 191: 184-185

Roholm K.: Fluorine intoxication : a clinical hygienic study. Copenhagen : Nordisk Forlog, Arnold Busck, 1937

Schiffl H. H. and Binswanger U.: Human urinary fluoride excretion as influenced by renal functional impairment. Nephron (1980) 26: 69-72

Sherrard D., and Andress D.: Aluminum-related osteodystrophy. 1989, 34:307-323

Singer L. and Ophaug R.: CRC critical reviews in clinical laboratory sciences. Volume 18, issue 2: 111-140.

Srivastava R., Gill D., Moudgil A., Menon R., Thomas M., and Dandona P.: Normal ionized calcium, parathyroid hypersecretion, and elevated osteocalcin in a family with fluorosis. Metabolism (1989):120-124

Suki W. and Rouse D.: Renal transport of calcium, magnesium, and phosphorus. In B.M. Brenner and F. C. Rector (Eds) The Kidney (4th edition) (1991): 380-423. Philadelphia: W. B. Saunders Company.

Suttie J., Philips P. The effects of age on fluoride deposition in the femur of the rat. Arch Biochem. 1959; 83: 355-359

Sutton R. and Cameron E.: Renal osteodystrophy: Pathophysiology. Seminars in Nephrology, (1992)12(2), 91-100

Sutton R. and Dirks J.: Disturbances of calcium and magnesium metabolism. In B.M. Brenner and F. C. Rector (Eds) The Kidney (4th edition) (1991): pp 841-1081. Philadelphia: W. B. Saunders Company.

Takebayashi T., Iwamoto M., Jikko A., Matsumuru T., Enomoto-Iwamoto M., Myoukai F., Yamaai T., Matsumoto K., Nakamura T., Kurisu K., and Noji S.: Hepatocyte growth factor/scatter factor modulates cell motility, proliferation, and proteoglycan synthesis of chondrocytes. The J. of Cell Biology (1995) 129:1411-1419

Taves D.:Seperation of fluoride by rapid diffusion using hexamethyldisiloxane. Talanta (1968) 15: 969-974

Turner C.H., Owan I., Brizendine E.J., Zhang W., Wilson M.E., and Dunipace A.J.: High fluoride intakes cause osteomalacia and diminished bone strength in rats with renal deficiency. Bone (1996) 19: 595-601.

Whitford G. M.: The physiological and toxicological characteristics of fluoride. J. Dent. Res. (1990) 69 Special, 539-549.

Whitford G. M.: Acute and chronic fluoride toxicity. J. Dent. Res. (1992) 71: 1249

Whitford G., Pashley D. Fluoride absorption : the influence of gastric acidity. Calcified Tissue international. (1984) 36: 302-7

Whitford G. M., Biles E., and Birdsong-Whitford N.: A comparative study of fluoride pharmacokinetics in five species. J. Dent. Res. (1991) 70: 948-951

Whitford G. and Pashley D.: Fluoride reabsorption by nonionic diffusion in the distal nephron of the dog. P.S.E.B.M. (1991) 196: 178-183

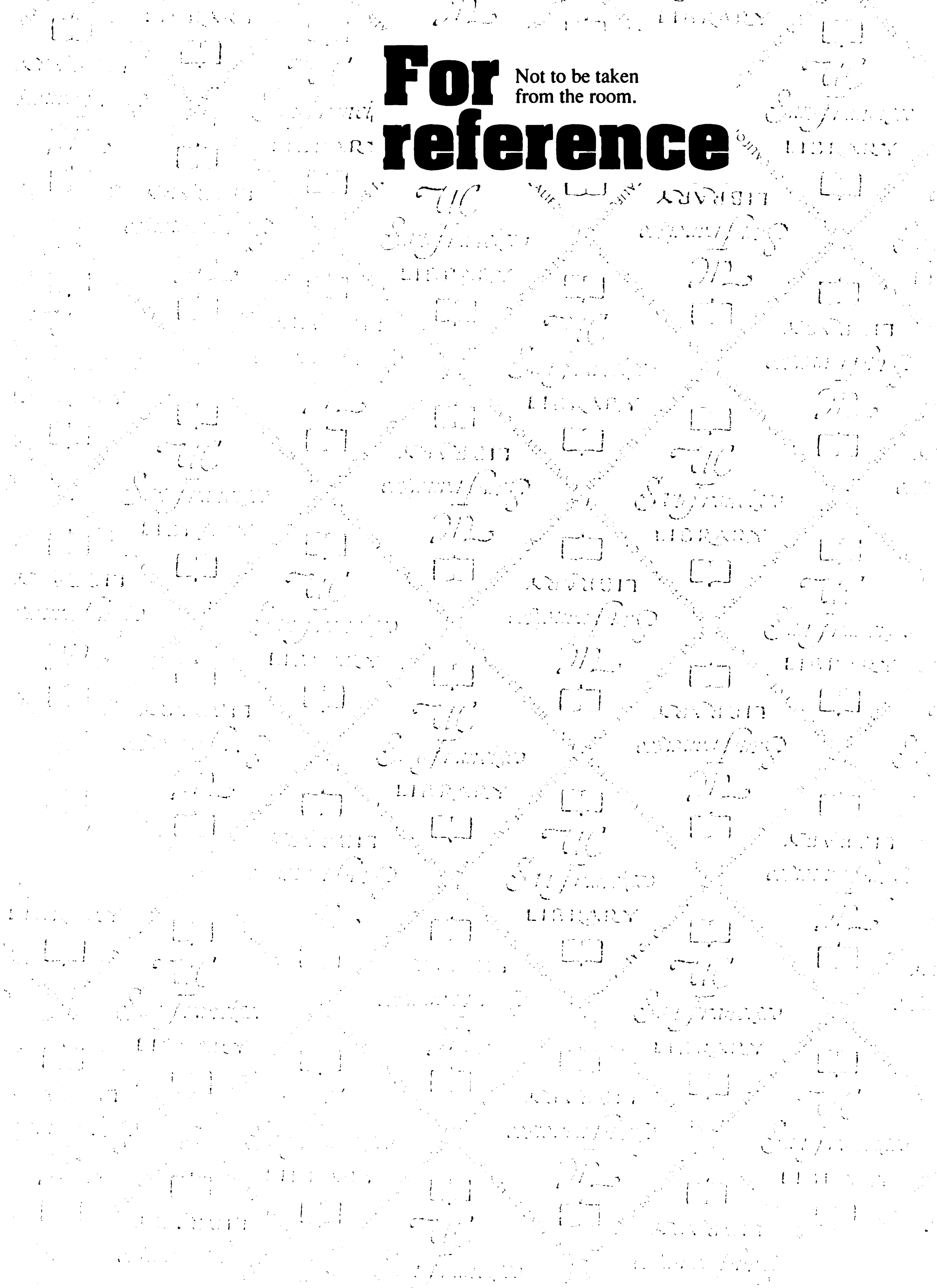
Whitford G.,and Reynolds K.: Plasma and developing enamel fluoride concentration during chronic acid-base disturbances. J. Dent. Res. (1979) 59: 2058-2065

Willinger C. C., Moschen I., Kulmer S., and Pfaller.: The effect of sodium fluoride at prophylactic and toxic doses on renal structure and function in the isolated perfused rat kidney. Toxicology (1995) 95: 55-71

Yates C., Doty S. and Talmye R.: Effects of sodium fluoride on calcium homeostasis. Proc. Soc. Exp. biol Med 1964: 1103-1108

Zipkin I., McClure F. Deposition of fluoride in the bones and teeth of the growing rat. J of Nutr. 1952; 47:611-620

Zipkin I.: Fluoride in calcified structures. Zipkin I., ed. Biological Mineralization. New York: Wiley-Interscience; 1973; 487-505



For reference
Not to be taken
from the room.