ABSTRACT

Alvin Richard Gates. THE EFFECTS OF PARATHYROID HORMONE AND OVARECTOMY ON THE CALCIUM AND FLUORIDE CONCENTRATION OF THE STAPEDIAL BONES OF FLUORIDE FED RATS. (Under the direction of Everett C. Simpson, Ph.D.) Department of Biology, April, 1978.

The influence of parathyroid hormone (PTH) and ovariectomy on stapedial calcium and fluoride levels was investigated in rats administered fluoride in the drinking water. Four groups of 51-day-old rats were placed on the following regimes: First group, control; second group, ovariectomized; third group, PTH injections daily; and the fourth group, ovariectomized and PTH injections daily. After three weeks, animals were sacrificed and the stapes were removed for analysis of calcium and fluoride content.

There was no significant difference in the stapedial calcium or fluoride concentration of the four groups of rats. The results demonstrated a positive correlation between fluoride and calcium concentration and indicated that increased fluoride accumulation in the bone is accompanied by increased calcium retention.

THE EFFECTS OF PARATHYROID HORMONE AND OVARIECTOMY ON THE CALCIUM AND FLUORIDE CONCENTRATION OF THE STAPEDIAL BONES OF FLUORIDE FED RATS

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THE EFFECTS OF PARATHYROID HORMONE AND OVARIECTOMY ON THE CALCIUM AND FLUORIDE CONCENTRATION OF THE STAPEDIAL BONES OF FLUORIDE FED RATS

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INTRODUCTION

Mineralization of bone may be influenced by several factors. Parathyroid hormone is the main controlling factor of the body involved in resorption of bone. Increased resorption possibly due to increased parathyroid hormone levels in the body or decreased incorporation of minerals may lead to osteoporosis, a bone disease characterized by deficiency in bone mineral content.

Estrogens are known to have an effect on bone metabolism. The relationship between osteoporosis and menopause, which results from loss of estrogens, was first noted by Albright (1941). Estrogens reduce bone resorption in rats (Lindquist et al., 1960; Anderson et al., 1970). Much evidence indicates that estrogens inhibit the action of parathyroid hormone and thus inhibit bone resorption.

Fluoride incorporation into long bones and teeth has been accepted for many years. Osteoporosis was linked to fluoride deficiencies in humans by Leone (1960) and Bernstein et al., (1966) by comparing the incidence of the disease in areas of low- and high-fluoride content in the drinking water. Evidence suggests that fluoride reduces bone resorption and osteoporosis.

Middle ear deafness in humans may be the result of increased resorption of the ossicular bones. This condition is often referred to as otosclerosis, otospongiosis, and localized otoporosis (Shambaugh et al., 1973). It should not be surprising to find that resorption of the ossicular bones is affected by parathyroid hormone, estrogens, and fluoride.

Injections of parathyroid hormone and ovariectomy results in significant calcium losses from stapedial bones (Pearson, unpublished results). The possibility of an interaction of PTH, endogenous estrogens, and fluoride on the ossicles has not been evaluated. The present study is designed to determine whether such an interaction exists.

LITERATURE REVIEW

The first calcium-regulating factor to be discovered was parathyroid hormone (PTH), a secretion of the parathyroid glands. The glands were associated with calcium metabolism by MacCallum and Voegtin (1909). They showed that one of the effects of parathyroid removal, tetany, could be alleviated with calcium administration. The next step was to isolate the factor of the parathyroids that affected calcium levels in the body. In 1959, the hormone, parathormone, was isolated by Aurbach (1959) and Rasmussen and Craig (1959). This isolation of the hormone has laid the groundwork for an extensive study of parathyroid hormone and its action on the body and calcium metabolism.

The three principle areas of PTH effect are the intestines, the kidneys, and the bone. The effect of parathyroid hormone on bone is of principle interest. Administration of parathyroid hormone to rats causes a rise in plasma calcium. Bone is a major source of this increased plasma calcium. Bones labeled with ^{45}Ca and subjected to parathyroid hormone showed increased levels of ^{45}Ca in the culture media (Gaillard, 1963, 1965; Raisz and Niemann, 1967). Similar results were obtained with in vivo radioisotopic studies (Elliot and Talmage, 1958). Thus parathyroid hormone has a role in calcium homeostasis and functions to stimulate bone remodeling by causing resorption of bone.

Many substances are known to inhibit PTH mediated bone resorption. Estrogens are a very important group of these substances. Albright (1941) noted the relationship between osteoporosis and post-menopause. He suggested that this osteoporosis was due to the loss of estrogens at menopause and assumed that this condition could be treated with replacement

estrogens. Davis et al. (1966) showed that estrogen treatment could prevent or retard post-menopausal osteoporosis by modifying the effect of PTH on bone metabolism. Their investigation was substantiated by later research that showed estrogens did counter the action of PTH on bone. Stilbestrol, an estrogen-like drug, blocked the action of PTH on bone in tissue cultures (Nordin et al., 1971). In vitro studies by Stern (1969) and Atkins and co-workers (1972) showed that estradiol inhibited the resorptive, demineralizing influence of PTH on radioactive bone. Estrogens have also been shown to reduce bone resorption in rats (Lindquist et al., 1960; Anderson et al., 1970).

Young et al. (1968) reported a correlation between the lack of estrogens and the activity of the parathyroids. They found post-menopausal osteoporosis to be more severe in hyperparathyroid women and less severe in hypoparathyroid women as compared to the general population. Post-menopausal women constitute the majority of patients with hyperparathyroidism (Muller, 1969; McGeown, 1969).

The high incidence of bone fractures in post-menopausal and ovariectomized women has been correlated with reduced mineral content in the bone (Dalen et al., 1974). It is suggested that the lack of estrogens releases the inhibitory action on PTH. This results in increased bone resorption and less mineralization. Heaney (1965) describes this condition as increased sensitivity of bone to parathyroid hormone in the absence of estrogens.

Menopause, whether natural or artificial, tends to increase plasma and urinary levels of calcium (Young and Nordin, 1967). This increase is due to the increased resorption of bone in the absence of estrogen.

Estrogen treatment can reduce plasma and urinary calcium levels of post-menopausal women and in post-menopausal hyperparathyroid women (Gallagher and Nordin, 1972). Estrogens have also been shown to reverse or have inhibitory effects on the development of osteoporosis that is induced biochemically or by immobilization. Thus in these cases of osteoporosis, bone resorption may be slowed by estrogen treatment (Orimo et al., 1970).

Bone and teeth were shown to contain fluoride in the early 1800's. Fluoride content was suggested to be related to dental caries in the late 1800's. This suggestion was followed by many analyses of teeth of both normal and carious conditions. Although the range of fluoride content varied with different research, one finding held true; the fluoride content of normal teeth slightly exceeded that of carious teeth (Cohen et al., 1969). Early studies indicated that fluoride incorporation into teeth in high quantities might cause a darkened, mottled appearance. Churchill (1931) proved this theory to be correct. These findings prompted extensive research to find levels of fluoride ingestion that could offer protection against dental caries yet not result in mottling. After much research the proper levels were thought to be known and Grand Rapids, Michigan in 1945 became the first city to intentionally add fluoride to its drinking water (Cohen et al., 1969).

The importance of fluoride to bone composition and structure was first observed in 1891 by Brandl and Tappeiner. They reported that fluoride ingestion in dogs caused bones to become thicker and presumably stronger. This work was soon substantiated by Rost (1907). He reported bone structural changes with large fluoride ingestion. Many subsequent tissue examinations showed that bone and teeth tended to concentrate

fluoride in a much higher degree than other tissues.

Research results reported during the 1920's and 1930's were puzzling. In many separate studies fluoride ingestion showed increased fluoride accumulation in the bone but reduced calcium accumulation. Fluoride accumulation was reported to be inversely proportional to the strength of the bone (McClure and Mitchell, 1931). It was soon demonstrated that massive dosages of fluoride caused gastrointestinal disturbances and reduced intestinal absorption of calcium and protein. Reduced intake of calcium and protein resulted in lowered bone calcium (Rich et al., 1964; Cohen and Gardner, 1964; Cohen, 1966; Cohen and Gardner, 1966).

Many studies have shown a correlation of fluoride ingestion and skeletal change in man (Cohen et al., 1969). Leone and co-workers (1955) surveyed two towns in Texas for the occurrence of osteoporosis. The incidence of osteoporosis proved lower in the town with the higher fluoride content in the drinking water. Similar research results were demonstrated by Bernstein et al.(1966). Increased calcium uptake was shown to be correlated with increasing amounts of fluoride in the drinking water. This substantiated earlier work by Hauck et al.(1933) that demonstrated calcium absorption in bone paralleling fluoride incorporation.

Although fluoride increases mineralization and thickening (Posner et al., 1963) and decreases resorption of bone, there is no clear evidence for fluoride-induced increased strength of bones (Saville, 1967; Riggins et al., 1974). However this increased mineralization does lead to increased hardness of the bone (Carlstrom, 1954; Amprino, 1958).

Such research has emphasized the possible use of fluoride in cases of bone mineral deficiency diseases. In patients with Paget's disease, a condition characterized by bone cell hyperplasia and bone demineralization, calcium uptake into bones was increased following fluoride therapy (Purves, 1962). In patients with osteoporosis, fluoride therapy caused increased uptake of calcium (Rich et al., 1964). Some success also was seen in treatment of other bone demineralizing diseases or by chemically induced demineralization (Cohen and Gardner, 1964; Cohen, 1966; Bernstein and Cohen, 1967). These workers substantiated earlier x-ray studies which showed that fluoride increased bone growth (Moller and Gudjonsson, 1932). In addition, a new technique of tetracycline labelling proved increased fluoride ingestion to increase rate of bone formation (Bernstein and Cohen, 1967).

Previous researchers have demonstrated that fluoride has an affinity for and is incorporated into bone; but, exactly how, and what its role is, remains to be determined. Its role may be to maintain optimal hardness of bone.

Neuman and fellow researchers (1950) and Megirian and Hodge (1951) were among the first to show that fluoride replaced hydroxyl radicals in the hydroxyapatite of bone. This exchange formed fluoroapatite crystals. The mechanism for increasing calcium in fluoride-treated bone is not fully understood. Decreased solubility of apatite crystals of fluoride treated bone could lead to increased retention of calcium and hardness of bones (Neuman et al., 1950; Megirian and Hodge, 1951; Issac et al., 1958; Neuman and Neuman, 1958). Hauck et al. (1933) and Talmage and Doty (1962) suggest that fluoride causes incorporation of calcium into bone thus

reducing levels of blood calcium. The reduced blood calcium stimulates the parathyroid glands to increase PTH secretion. The effect of PTH is thought to be less in the fluoride-stabilized bone. More blood calcium is furnished from absorption in the intestines and retention in the kidneys, since both of these sites are under PTH influence.

Some evidence has suggested that fluoride is a metabolic catalyst in mitochondria. Some mitochondria under actively respiring conditions may accumulate calcium as calcium phosphate and as hydroxyapatite (Greenawalt et al., 1964). This leads one to believe that bone-cell mitochondria could behave similarly. Fluoride could enhance calcium uptake by mitochondria. This calcium could be incorporated into a less soluble form, fluoroapatite (Cohen et al., 1969). Causse and Chevance (1973) believe fluoride inhibits certain enzymes which favor bone breakdown. This results in an increase in bone formation. Messer et al. (1973a, 1973b) disagree with this theory. In vivo bone studies revealed no changes in acid phosphatase activity in either resorption or remodeling of bone. Fluoroapatite crystals did however cause the bone to be less influenced by PTH.

Knowing that parathyroid hormone, estrogens, and fluoride affect bones in general; it is reasonable to assume that ossicles might be affected similarly. The osteoporotic condition, known as otosclerosis in the ossicles, may be similarly affected.

Although the term otosclerosis is widely used in the literature, Shambaugh et al. (1973) suggest the more appropriate terms of otoporosis and otospongiosis be used to describe mineral loss in the ossicles.

These terms agree with the observed physiological state. The ossicles become spongy and are less mineralized (Roberto et al., 1972).

Escat (1923) was the first to prescribe fluoride therapy for "otosclerosis." Shambaugh and Scott (1964) found that administration of sodium fluoride to some patients with otospongiosis alleviated clinical symptoms of the disease after three months. Thus it is possible that fluoride causes recalcification and inactivation of the otospongiotic area. This response is comparable to the effect of fluoride on osteoporotic long bone.

Daniel (1969) found that the incidence of "otosclerosis" in high- and low-fluoride drinking water areas varied significantly. There was a lower amount of "otosclerosis" in the high-fluoride area. Daniel et al. (1973) reported in a study of ossicular bones that more fluoride was found in "otosclerotic" bone than in normal bone especially in optimal fluoride areas. This agrees with the findings of Linck et al. (1967). This increased fluoride is due to the greater metabolic activity and vascularity of the relatively spongy bone. Thus the opportunity for fluoride to come in contact with otospongiotic bone and exchange with hydroxyl ions is increased. The stable, less soluble, fluoroapatite prevents resorption and stabilizes otospongiosis (Daniel et al, 1973).

Daniel et al. (1972) found an increase in fluoride in the rat stapes of those animals given fluoridated water. In most cases, increased fluoride was accompanied by an increase in calcium content. This substantiated early long bone studies. By using radioactive ^{85}Sr, Lithicum et al. (1973) and House and Lithicum (1974) showed that there was an increased uptake of calcium and a decreased resorption of ossicular bone

in patients treated with moderate sodium fluoride levels.

Parathyroid hormone, estrogens, and fluoride have definite roles in the resorption of bone and therefore have a role in the resorption of ossicular bones. Stabilization of bone by fluoride has been reported in the literature. Interest lies with the role of fluoride as a stabilizing factor of the calcium levels of the stapes under different experimental conditions. This investigation is concerned with the effect fluoride has on calcium content of the rat stapes under the conditions of increased PTH levels and/or the absence of estrogens.

MATERIALS AND METHODS

Female albino rats, Rattus norvegicus, of Sprague-Dawley strain were used in this investigation. The rats were housed in animal quarters with a constant temperature of $75^{\circ} \pm 2^{\circ}$F and a 14 hour day/light regime. All animals received Wayne laboratory feed.

The animals at 44 days of age were divided into control, ovariectomized, PTH injected, and ovariectomized/PTH injected groups. They will be referred to as FI, FII, FIII, FIV groups, respectively.

In a pretreatment period of one week, all groups were given distilled water for drinking. At 51 days of age, all animals received 5 ppm fluoride in the drinking water. This treatment was continued for 21 days. This concentration of fluoride in drinking water results in significant changes in calcium levels in the stapes of the rat (LeDoux, 1972).

Ovariectomy was performed as the animals were coming into sexual maturity; thus, bone formation had not been influenced by estrogens. The ovariectomized animals were put on treatment one week later. The one week recovery period also assured that sexual maturity had occurred in the remaining rats. Groups FII and FIV animals were ovariectomized at 44 days of age. This age is similar to that of rats used by Orimo et al. (1972) and Pearson (unpublished results) in their estrogen-PTH related bone studies. Those animals to be ovariectomized were anesthetized by an intraperitoneal injection of nembutal (50 mg/kg body weight). Bilaterial ovariectomy was performed by a procedure outlined by Simpson (Appendix E).

The parathyroid hormone used was a crude extract of the parathyroid gland in solution at the concentration of 1 ml extract equivalent to 248 USP units (Eli Lilly and Co.). Administration of PTH began at 51 days of age and was given daily by subcutaneous injection in the shoulder region at the rate of 30 USP units for 21 days. Other workers (Orimo et al., 1972; Person, unpublished results) had successfully used a similar amount of PTH in their studies.

The weight of each animal was recorded at 51 days of age and at the end of the 21-day-treatment period. When the treatment was terminated, all rats were sacrificed by an overdose of chloral hydrate injected intraperitoneally. Each animal was decapitated and the heads were frozen until the stapes could be removed. The stapes were removed utilizing a revised technique of LeDoux (Appendix F). Both stapes were used in order to have a sufficient amount of bone for analysis. Soft tissue was removed from the bones. The stapes were then cleaned by a technique developed by Linck et al. (1967). They were soaked in diethyl ether for 48 hours, transferred to acetone for 24 hours, and dried for 24 hours at 60°C. The stapes were then stored at room temperature until analyses for fluoride and calcium were undertaken. After storage the stapes were again dried for 24 hours at 60°C, allowed to cool and then weighed as pairs.

Fluoride and calcium analyses required separation of the two ions. a modification of Whitney-Wharton technique (1962) for fluoride analysis involved separation of fluoride ions from the bones. The bones were dissolved in perchloric acid in a sealed microdiffusion dish. The dissolved bones released hydrogen fluoride which was absorbed in the

sodium hydroxide compartment of the dish. The fluoride ion solution was reacted with zirconium SPADNS colorimetric reagent which is bleached by fluoride. The amount of fluoride was determined in each sample by reading the absorbance of the unknown against a reference solution with the Beckman DB spectrophotometer at λ max. To predict the content of the fluoride in a sample, this spectrophotometer reading was substituted into a regression equation completed from known fluoride standards (Appendix A). The remaining acid solution containing the dissolved bone was analyzed for calcium content by a revised technique of Connerty and Briggs (1966). Orthocresolphthalein complexone reagent was used to determine calcium. The absorbances of the unknown samples were read against a reference solution with the Beckman DB spectrophotometer λ max. These readings were substituted into a regression equation which was prepared using known calcium standards (Appendix B).

Amounts of calcium and fluoride were expressed in micrograms per milligram of bone. The null hypotheses were constructed stating that there was no significant difference between any groups in fluoride and calcium concentrations in the stapes of the animals studied. Paired uncorrelated t-tests and analysis of variance were used to determine significance. A correlation coefficient (r) between calcium and fluoride concentrations of each group was computed and tested for significance.

RESULTS AND DISCUSSION

The results of the analyses of the fluoride and calcium content of the stapes of this experiment is shown in Appendix C. A summary of these data follows in Table I.

Table I. Summary of the effect of ovariectomy and PTH injection on the calcium and fluoride concentration of the stapedial bones of fluoride-fed rats.*

Group	No. of rats	Treatment	F conc. µg/mg bone Mean±SE	Ca conc. µg/mg bone Mean±SE
FI	10	Control	0.341±0.076	229.0±9.55
FII	10	Ovariectomy	0.319±0.057	221.3±7.18
FIII	10	PTH 30 u daily	0.334±0.060	217.5±7.24
FIV	9	Ovariectomy + PTH 30 u daily	0.418±0.077	212.4±6.47

*Each group received 5 ppm F in drinking water.

Examination of the mean fluoride concentration in the stapedial bone (Table I) reveals that an ovariectomy/PTH injection regime may increase fluoride levels in the bones of such animals. The increased levels in this group (FIV) is probably due to the greater metabolic activity and vascularity of the bone caused by PTH action. The absence of estrogen as a PTH inhibitor increases this action (Linck et al., 1967).

The null hypothesis was constructed stating that there was no significant difference in the fluoride concentration of the four groups. The analysis of variance test chosen was a factorial design. This permitted separation and evaluation of ovariectomy and PTH injection and interaction of the two factors on the fluoride concentration in the stapes. The

calculated F scores for each factor ($F_{F1,35}$ ovar=0.169, $_{F1,35}$PTH=0.404, and $F_{F1,35}$ interaction=1.18) were less than the .05 probability level; therefore, the null hypothesis was accepted. Paired uncorrelated t-tests were also utilized to indicate if significance between any two groups existed. Even though the group means for fluoride content range from 0.319 to 0.418 µg/mg of bone, there was no significant difference.

Previous studies indicate that increased fluoride in the diet tends to stabilize the fluoride concentration in the bone(Daniel et al., 1973). The results of the present study agreed with the findings of Daniel and co-workers. However, LeDoux (1972) reported a significant difference in the fluoride concentration of the stapedial bones of adult and young rats given 5 ppm fluoride in their drinking water. This difference from other studies is thought to be due to increased bone activity of young rats leading to increased retention and concentration in bones.

Examination of the mean calcium concentrations of the stapedial bones of the four groups reveals expected trends (Table I). Animals of group FII had a lower mean concentration than did the control group animals (FI). The loss of ovarian estrogens from the animals in group FII may account for the trend of a greater loss of calcium from bone when compared to the control animals. This is in agreement with the works of Atkins (1972), Lindquist et al. (1960), and Anderson et al. (1970).

Animals given PTH injections (Group FIII) had a lower mean concentration of calcium than did the control animals. This increased PTH level in the rat is thought to lead to increased bone resorption and decreased bone calcium levels (Gaillard, 1963, 1965; Raisz and Niemann, 1967; Elliot and Talmage, 1958).

Ovariectomized/PTH injected animals had the lowest mean calcium concentration of this investigation. Increased PTH levels in the absence of estrogen would lead to greatest resorption.

To test these trends, a null hypothesis was constructed stating that there was no significant difference in calcium concentration of the four groups. The calculated F scores from the analysis of variance test for each factor ($F_{Cal,35}$ ovar=0.669, $F_{Cal,35}$PTH=1.79, and $F_{Cal,35}$interaction= 2.55) were less than the .05 probability level (Appendix D). The null hypothesis was accepted. However, the F score for the interaction of PTH and ovariectomy did approach the .10 probability level. This indicated a trend of lower calcium levels in the animals of group IV. Paired uncorrelated t-tests were also utilized to indicate if significance between any two groups existed. Each of these t-tests indicated no significant difference in concentration of calcium between any two groups.

Increased PTH levels and sensitivity of bone to PTH in the absence of estrogens significantly enhance bone resorption of calcium (Gaillard, 1963, 1965; Raisz and Neimann, 1967; Atkins, 1972; Lindquist et al., 1960; Anderson et al., 1970; and Orimo et al., 1972). It must be pointed out that the majority of such research did not include increased fluoride in the rat's diet. Messer et al. (1973a, 1973b) reported that increased fluoride in the diet of rats inhibited loss of calcium and thus tends to stabilize the calcium level of long bone. This stabilization also applies to increased sensitivity of bone of PTH in the absence of estrogens.

Pearson (unpublished results) reports that stapedial calcium loss is significantly increased by either PTH injections or by ovariectomy in rats. In the present study, there is no significant difference in calcium content of stapes when animals were given PTH injections or

ovariectomized. Pearson did not supplement the diet of the rats with fluoride as was done in the present study. These facts suggest a stabilizing effect of fluoride on the calcium content of the stapes.

Correlation coefficients were calculated for the calcium and fluoride concentration in the stapes. A true correlation existed between the calcium and fluoride concentration in the stapes within each group studied (Table II). This correlation is in agreement with studies on long bone. This study has shown that the stapes reacts to fluoride in a

Table II. Correlation Coefficient (r) for Groups (F:Ca)

Group	(r)	t value	
FI	0.830	4.21**	*sign. at .05 level
FII	0.773	3.45**	**sign. at .01 level
FIII	0.787	3.61**	***sign. at .001 level
FIV	0.631	2.15*	
All groups	0.693	4.15***	

manner similar to long bone. Additional research may demonstrate that the use of fluoride may have some therapeutic value in post-menopausal otospongiosis. This disease is due to a decreased level of estrogen and an increased sensitivity of the ossicles to endogenous PTH. Use of fluoride in drinking water may have other beneficial effects in addition to the reduction of dental caries in the young. A comparative epidemiological study of the incidence of osteoporosis in areas that have used fluoride in drinking water since the forties and fifties would be of interest. LeDoux (1972) demonstrated the increased accumulation of

fluoride in the stapes of the young rat given fluoride in the drinking water. This report shows that fluoride in the bone concentrates and stabilizes bone calcium.

SUMMARY AND CONCLUSIONS

Four groups of 51-day-old females rats were used in this study. All were given fluoride in distilled drinking water at the rate of 5 ppm. The animals were divided into a control, an ovariectomized (at 44 days of age), a PTH injected, and a PTH/ovariectomized group. They were on a 21-day treatment regime. All animals were sacrificed at the end of the regime and the stapes were removed, cleaned, and analyzed for fluoride and calcium levels. No significant differences were found between levels of fluoride or calcium in the stapes of any of the four groups. PTH has been shown repeatedly to promote bone resorption. This was not the case in this study. Fluoride has been shown to reduce the effects of PTH on long bones. The present study demonstates the stabilizing effect of fluoride on the stapes under conditions known to cause bone resorption. Additional information using microanalysis techniques such as atomic absorption, specific ion analysis, and correlating microhardness investigations would be helpful.

APPENDIX A

ISOLATION AND DETERMINATION OF MICROCONCENTRATIONS OF FLUORIDE IN BONE--A MODIFICATION OF THE WHITNEY-WHARTON TECHNIQUE (1962).

REAGENTS AND APPARATUS.

REAGENT A. Dissolve 3.16 grams of SPADNS, 4,5-dihydroxy-3-(p-sulfophenylazo)-2,7-naphtalene-disulfonic acid, trisodium salt in 550 ml of deionized water.

REAGENT B. Dissolve 0.133 grams of Zirconyl chloride octahydrate in 50.0 ml of deionized water. To this solution add 350 ml of concentrated HCl and dilute the resulting solution to 500 ml with deionized water.

REFERENCE SOLUTION. Add 50.0 ml of REAGENT A and 35.0 ml of concentrated HCl to 500 ml of deionized water. This solution is stable.

SINGLE SPECTROPHOTOMETERIC REAGENT. Mix equal volumes of reagents A and B. This solution is stable. All remaining A and B reagents can be mixed since these isolated reagents are not needed after the single spectrophotometric reagent is prepared.

STANDARD FLUORIDE SOLUTION. Dissolve 22.1 mg of Sodium fluoride in 1 liter of deionized water. This provides a solution of 10.0 micrograms F per ml of solution.

The polypropylene microdiffusion cells (size 44), the wetting agent (tergitol), which is diluted to 0.1% with deionized water, dessicator, Pasteur pipets, and silicon stopcock grease are available from Fisher Scientific Co., Raleigh, NC. The staticmaster brush, No. 1S200, was from Nuclear Products Co., El Monte, Calif.

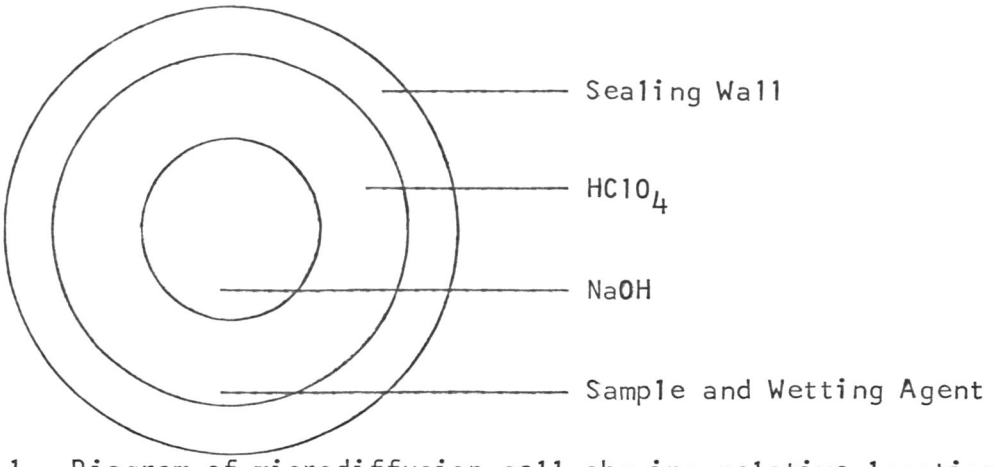

Figure 1. Diagram of microdiffusion cell showing relative locations of materials for F^- diffusion.

PROCEDURE

1. Brush the dry diffusion cells with the staticmaster brush. This removes static charges which may expel samples and possibly introduce sample into the center chamber.

2. Place a weighed bone sample into the inner annular chamber of the cell (see Figure 1).

3. Pipet 0.30 ml of 1.3N NaOH into the center chamber (see Figure 1).

4. Place 3 drops of diluted wetting agent and 0.30 ml of deionized water directly on the sample to slow down attack of the acid to be added (see Figure 1).

5. Pipet 0.50 ml of concentrated perchloric acid in the inner annular chamber away from the sample.

6. Generously lubricate the edge of the plastic top with silicone-stopcock grease and place over the diffusion cell.

7. Tilt and revolve the cells to mix contents of the inner annular chamber.

8. Place the cells in a heated dessicator at 60°C for 24 hours. This heat causes the NaOH to spread and wet the center chamber. F^- diffuses from inner annular chamber to the center chamber.

9. After 24 hours, allow the cells to cool to room temperature.

10. Remove plastic top and fill the center chamber with 0.6 ml of deionized water and empty the contents into a small bore test tube with a 3.0 ml volumetric line. Repeat this washing three times.

11. Add 0.30 ml of Zr-SPANDS single spectrophotometric reagent to the test tube and dilute with deionized water to make 3.0 ml solution.

12. Read the absorption at 590 nm with the reference solution in the reference cell.

13. Run microaliquots of the standard fluoride solution representing 0.0 µg, 1.0 µg, 2.0 µg, 3.0 µg, 4.0 µg, and 6.0 µg of F^- through the diffusion operation. The average of several runs is more representative.

14. Record the average absorbance for each amount of fluoride.

15a. Construct a standard curve [µg F^- (x) vs absorbance (y)]. From this curve an unknown sample's µg F^- (x) with a known absorbance can be determined.

15b. This alternate method is more accurate. Compute the slope of the standard line [µg F^- (x) vs absorbance (y)] using the following equation.

$$\text{slope, } b = \frac{\Sigma xy - (\Sigma x \Sigma y / N)}{\Sigma x^2 - [(\Sigma x^2)/N]}$$

Compute the µg F(x) at a given absorbance (y) by using the following equation (Schefler, 1969).

$$x_p = \frac{(y_p - \bar{y})}{b} + \bar{x}$$

APPENDIX B

DETERMINATION OF CALCIUM LEVELS BY MEANS OF ORTHOCRESOLPHTHALEIN COMPLEXONE--A MODIFICATION OF THE CONNERTY AND BRIGGS TECHNIQUE (1966).

REAGENTS.

14.8 M AMINOETHANOL-BORATE BUFFER (AEB). Add 18.0 grams of boric acid to 50.0 ml of deionized water. Mix with a magnetic stirrer. Add 25.0 ml of 2-aminoethanol to this solution while stirring. After 5 minutes, add another 25.0 ml of 2-aminoethanol. When the boric acid is completely dissolved add 400 ml of 2-aminoethanol while stirring. The resulting solution is stored in the refrigerator.

OCPC SOLUTION, 0.8 mg/ml. Add 0.080 grams of OCPC, Orthocresolphthalein complexone, to 25.0 ml of deionized water. Add 0.50 ml of 1 N potassium hydroxide and stir until OCPC is dissolved. Now add 75.0 ml of deionized water and 0.50 ml of glacial acetic acid.

FIVE PER CENT W/V SOLUTION OF 8-QUINOLINOL. Dissolve 5.0 grams of 8-quinolinol (8-hydroxyquinolin) in 100 ml of 95 per cent ethanol. This is stable for approximately one month and should be refrigerated to maintain stability.

OCPC COLOR REAGENT. Add 5.0 ml of 14.8 M ethanolamine-borate buffer and 1.5 ml of 5 per cent solution of 8-quinolinol to a 100-milliliter volumetric flask. Add 5.0 ml of OCPC solution and dilute to 100 ml with deionized water. This reagent is unstable and must be prepared fresh daily.

CALCIUM STANDARD SOLUTION. Dissolve 2.97 grams of oven-dried calcium carbonate in 60.0 ml of 1 N HCl and dilute to 1000 ml with deionized water. The resulting solution is 1 mg Ca/ml solution.

WORKING STANDARD CALCIUM SOLUTIONS. A, 0.5; B, 1.0; C, 1.5; D, 2.0; and E, 3.0 mg/100 ml are prepared which correspond to equivalent calcium concentration under test conditions of 5, 10, 15, 20, and 30 mg per 100 ml. These working standards are stored in plastic bottles.

PROCEDURE

Wash all glassware with detergent, rinse with deionized water and finally rinse with 0.5 N HCl to remove traces of calcium.

1. Fill the inner annular chamber with deionized water, collect with pasteur pipet and place in a 10.0-ml volumetric flask. Repeat this washing twice. Fill the volumetric flask to the 10.0 ml mark with deionized water.

2. Place 1.0 ml of the resulting wash in a test tube containing 10.0 ml of OCPC color reagent.

3. Measure the absorbance at 570 nm with 1.0 ml of deionized water and 10.0 ml of OCPC color reagent as the blank.

4a. Use 1.0 ml of the working standards with 10.0 ml of OCPC color reagent to establish a calibration curve [µg Ca(x) vs absorbance (y)] from which the unknowns are read.

4b. This alternate method is more accurate. Compute the slope of the standard line [µg Ca(x) vs absorbance (y)] using the following equation.

$$\text{slope, } b = \frac{\Sigma xy - (\Sigma x\ \Sigma y/N)}{\Sigma x^2 - [(\Sigma x)^2/N]}$$

Compute µg Ca(x) at a given absorbance (y) by using the following equation (Schefler, 1969).

$$x_p = \frac{(y_p - \bar{y}) + \bar{x}}{b}$$

APPENDIX C

THE EFFECT OF OVARIECTOMY AND PTH INJECTION ON THE CALCIUM AND FLUORIDE CONCENTRATION OF THE STAPEDIAL BONES OF FLUORIDE FED RATS.

Fluoride and Calcium Content of Stapedial Bones of the Group FI (Control Animals)

No.	Total Bone (mg)	Total F (in µg)	µg F / mg Bone	Total Ca (in µg)	µg Ca / mg Bone
1	0.275	0.125	0.45	65.7	239
2	0.250	0.030	0.12	45.8	189
3	0.100	0.030	0.30	24.8	248
4	0.150	0.054	0.36	38.4	256
5	0.250	0.125	0.50	61.5	246
6	0.125	0.101	0.81	33.2	266
7	0.200	0.077	0.39	42.6	213
8	0.225	0.101	0.45	56.3	250
9	0.225	0.000	0.00	45.8	203
10	0.225	0.006	0.03	40.5	180

Fluoride and Calcium Content of Stapedial Bones of the Group FII (Ovariectomized Animals)

No.	Total Bone (mg)	Total F (in µg)	µg F / mg Bone	Total Ca (in µg)	µg Ca / mg Bone
1	0.275	0.054	0.20	51.0	185
2	0.225	0.125	0.56	56.3	250
3	0.225	0.054	0.24	52.1	232
4	0.225	0.077	0.34	46.8	208
5	0.300	0.000	0.00	55.2	184
6	0.250	0.101	0.40	56.3	225
7	0.225	0.101	0.45	53.1	236
8	0.225	0.125	0.56	55.2	245
9	0.250	0.077	0.31	55.2	221
10	0.225	0.030	0.13	51.0	227

Fluoride and Calcium Content of Stapedial Bones
of the Group III (PTH Injected Animals)

No.	Total Bone (mg)	Total F (in µg)	µg F / mg Bone	Total Ca (in µg)	µg Ca / mg Bone
1	0.225	0.125	0.56	56.3	250
2	0.250	0.054	0.22	45.8	187
3	0.225	0.054	0.24	47.3	210
4	0.250	0.030	0.12	45.3	183
5	0.175	0.054	0.31	36.3	207
6	0.225	0.054	0.24	46.4	206
7	0.300	0.219	0.73	72.0	240
8	0.300	0.054	0.18	66.8	223
9	0.225	0.054	0.24	51.0	227
10	0.250	0.125	0.50	53.1	242

Fluoride and Calcium Content of Stapedial Bones
of the Group IV (PTH Injected/Ovariectomized Animals)

No.	Total Bone (mg)	Total F (in µg)	µg F / mg Bone	Total Ca (in µg)	µg Ca / mg Bone
1	0.250	0.125	0.50	52.7	211
2	0.225	0.054	0.24	44.8	199
3	0.225	0.054	0.24	49.1	218
4	0.225	0.196	0.87	56.3	250
5	0.225	0.125	0.56	44.9	200
6	-----	-----	----	----	---
7	0.225	0.054	0.24	51.0	227
8	0.200	0.125	0.63	44.3	222
9	0.225	0.054	0.24	41.6	184
10	0.225	0.054	0.24	45.3	201

APPENDIX D

STATISTICAL ANALYSIS SUMMARY

Table I. Summary of Analysis of Variance for Fluoride

Source of Variance	df.	SS	MS	F
Ovariectomized	1	.00780	.00780	0.169
PTH injection	1	.01859	.01859	0.404
Interaction	1	.05426	.05426	1.178
Error	35	1.6124	.04607	
Total	38	1.6774		

Table II. Summary of Analysis of Variance for Calcium

Source of Variance	df.	SS	MS	F
Ovariectomized	1	367.9	367.9	0.669
PTH injection	1	983.1	983.1	1.788
Interaction	1	1400.6	1400.6	2.547
Error	35	19244.0	549.8	
Total	38	21996.0		

APPENDIX E

OVARIECTOMY PROCEDURE

1. Shave a three cm diameter area on each side of the rat in the dorsolateral-lumbar region. Clean the areas with 70 per cent isopropanol. Repeat steps 2 through 6 for both sides of animals.

2. Grasp the exposed skin with forceps and make an incision through the skin approximately one cm in length. Cut through the underlying fat to expose the musculature of the abdominal wall. Now secure the abdominal wall with forceps, lift, and make a small incision to expose the underlying viscera.

3. Carefully probe the viscera with forceps for a mass of fatty tissue within which the ovary may be found. Pull this through the opening in the wall to facilitate work.

4. Tie off the uterine tube and ovary with thread and then cut away and remove this tissue.

5. Replace viscera and securely suture the abdominal wall incision with thread. Close the external skin incision using metal wound clips (Remove these one week following surgery.).

APPENDIX F

STAPEDECTOMY PROCEDURE

1. Clear all musculature from the area around the auditory bulla with forceps.

2. Carefully break away bulla using forceps. Remove sufficient bone to see the tympanic membrane.

3. Using a small scalpel or probe, cut the membrane away from the neck of the malleus.

4. Cut the tendon that holds the malleus to the wall of the bulla.

5. The malleus articulates with the incus. Break this joint and remove the malleus.

6. The incus articulates with the stapes. Break this joint and remove the incus.

7. Cut the stapedial muscle and blood vessels over the footplate of the stapes. Remove the stapes carefully.

NOTE: All work must be performed under a dissecting microscope.

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