ABSTRACT

Jeffrey Charles Le Doux. SOME EFFECTS OF FLUORIDE ON GUINEA PIG AND RAT STAPES. (Under the direction of Everett C. Simpson, Ph. D.) Department of Biology, January 29, 1972.

This study was undertaken in an attempt to determine if fluoride administered via the drinking water had any effect on the amount of calcium and fluoride found in the stapedes of rats and guinea pigs. The guinea pigs were basically a pilot study. They were divided into two groups, one group receiving 5 ppm sodium fluoride in their drinking water and the other group receiving distilled deionized water. Animals from each group were sacrificed and their stapedes analyzed for fluoride and calcium. The results of the pilot analyses showed that differences in fluoride levels between the two groups of guinea pigs were significant; however, the calcium levels did not differ significantly.

Two groups of 52-day old rats were placed on the same regimen as the guinea pigs, and these were bred. Ten young and the female adults (150 days) were sacrificed at weaning (21 days). The remainder were sacrificed at the end of puberty (52 days). The stapedes were removed and analyzed for calcium and fluoride content.

There was significant difference in fluoride concentration among the control groups, whereas, a highly significant difference was observed among the experimental groups.

Significant differences in calcium concentration were observed among the control groups as well as among the experimental groups. When fluoride concentration of the various control groups were compared to the various experimental groups, differences were observed between the 21-day control and the 21-day experimental groups, and between the 52-day control and the 52-day experimental groups, but no differences were observed between the 150-day control and the 150-day experimental groups. This could be accounted for since the 150-day old rats were mature when placed on their regimen and only a minimum amount of fluoride would have been incorporated into the bone.

When the control and experimental groups were compared to each other to ascertain differences in calcium concentration, differences were observed between all paired groups.

This study has shown that the administration of 5 ppm sodium fluoride via the drinking water will result in a significant increase of fluoride concentration in the rat stapedes. It also suggests that rats which receive fluoride continuously through puberty will also have a significant increase in calcium concentration in the stapedes.

SOME EFFECTS OF FLUORIDE ON GUINEA PIG AND RAT STAPES

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by

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INTRODUCTION

Recent work has indicated the increased incidence of middle ear deafness in the human known as otosclerosis to be related to the amount of fluoride in the drinking water (Daniel, 1969). It has been known for many years that fluoride is readily taken up by long bones and teeth. It would not be surprising to find that other bones may have a similar affinity to incorporate ingested fluoride, only the degree of uptake is in question. Since clinical studies are by their very nature difficult to control and with the interest stimulated by Daniel's report, it was considered appropriate to use the guinea pig and the rat under controlled conditions. This study was confined to the effects of fluoride at 5 ppm through the drinking water on the uptake of fluoride in the stapedes of the middle ear of these two species of animals.

REVIEW OF LITERATURE

Numerous studies have been reported of the effects of fluoride on bones and teeth. Some of the earliest work with fluoride was on its toxicity. It has been noted by numerous investigators that increased tolerance of fluoride is effected by dietary factors. Phillips and Hart (1935) found an extended survival time and retarded onset of weight loss in guinea pigs when large amounts of ascorbic acid were given along with toxic doses of sodium fluoride. In areas of endemic fluorosis in India, Pandit et al (1940) observed a corresponding effect in humans. Hobbs et al(1954) noted a decreased susceptibility to fluorosis in animals fed mixed pasture or freshly cut grass. Apparently ascorbic acid does not hinder absorption, as does calcium. Even when supplementary fluoride was omitted from the diet, an increase of storage of fluoride was noted in the skinned carcasses of rats (Muhler, 1958).

An increase of skeletal storage of fluorides (Miller and Phillips, 1955) and a heightened toxicity of fluorides (Phillips and Hart, 1935) was found when fat was added to the diet. It has been suggested that this is because of the increased loss of fecal calcium due to the increased production of insoluble calcium soaps, thereby depriving the animals of the known protective effect of calcium (Faccini, 1969).

Using tracer doses of radiofluorine in human subjects, Carlson et al (1960) noted that, when administered orally, the isotope entered the blood with extreme rapidity. The solubility of the compound and the presence of other ions are factors on which the absorption of fluoride from the digestive tract depends. If calcium was fed with fluoride, it was noted that there was a decreased retention of fluoride (Weddle and Muhler, 1954; Wagner and Muhler, 1960) and that the level of fluoride present in rat feces was raised (Weddle and Muhler, 1957).

Plasma carries 75% of the fluoride present in the blood (Carlson et al, 1960). This level is mainly influenced by the rate of absorption from the digestive tract, the excretion by the kidney, and the uptake by calcified tissues (Armstrong, 1961). It was noted by Singer and Armstrong (1960) that individuals from a population that used water with 0.15-2.5 ppm fluoride had plasma fluoride levels of 0.14 ppm $\pm$ 0.005 (S.E.) to 0.1 ppm $\pm$ 0.0085 (S.E.) In a community water supply with 5.5 ppm fluoride, the plasma fluoride level was significantly increased to 0.26 ppm $\pm$ 0.0124 (S.E.).

Using radiofluorine as a tracer, Wallace-Durbin (1962) observed that the concentration of fluoride in hard tissues was maximal and little remained in the soft tissues or blood four hours after ingestion. In lambs, Perkinson et al (1955)

again used radiofluorine and found that the distribution pattern of radiofluorine was similar to that of radioactive calcium, i. e., autoradiograms showed, in long bones, there was a preferential deposition of the radioisotope in the primary spongiosa beneath the growth cartilage, a generalized patchy distribution in trabeculae and a linear distribution on endosteal and periosteal surfaces. Zipkin and Scow (1956) record that uncalcified cartilage shows no affinity for radiofluorine.

Posner and his coworkers (1963) using X-ray diffraction analysis, found evidence to support Hodge's suggestion (1956) that the hydroxyl group of the bone apatite and the fluoride ion could be substituted for the other in the apatite phase because both are the same size. Furthermore, Posner and his coworkers found that the resulting ionic bond between fluorine and each of the three coplanar calcium ions in the six-fold screw axis of the apatite structure is more stable and shorter than when the hydroxyl ion occupied the same position. In addition, this substitution also leads to an increase in the bone apatite crystal size perpendicular to the c-axis (Posner et al, 1963). Posner and Zipkin (1965) suggested that a more stable mineral system is produced because of the larger crystal size of the bone apatite resultant from fluoride impregnation. This is because the rate of reaction between a crystal solid and a solution is inversely proportional to the size of the

crystals. Posner and his coworkers (1965) also suggested that since the substitution of fluoride for hydroxyl ions produces a compound less soluble in water, fluoride has a stabilizing effect on bone and also reduces the vulnerability of enamel and dentine to biochemical attack from dental caries.

Since the action of sodium fluoride has been more understood, it has been used therapeutically with some success. Sodium fluoride was used in the treatment of osteoporosis by Rich and Ensinck (1961), who knew the osteosclerotic changes that resulted from endemic and industrial fluorosis. Six patients with osteoporosis and one with Paget's disease were administered 60 mg of fluoride per day for fourteen or more weeks. Within six to eight weeks, a drop in the urinary calcium was noted, the most striking occurring in the patient with Paget's disease. The urine contained less citrate and more mucopolysaccharide than before treatment. No significant change was noted in the plasma calcium, citrate or phosphorus levels, or in renal or hepatic function.

Rich and Ivanovich (1964), in a more detailed investigation, observed that during the first 8-10 weeks of fluoride treatment, significant calcium retention was not noted. However, both calcium and phosphorus levels were retained to an increasing degree after the tenth week.

Bernstein et al (1966) also found evidence for a possible role of fluoride preventing osteoporosis. Radiologically

comparing two populations of individuals with levels of 4-5.8 ppm and 0.15-0.3 ppm fluoride in the water supply, it was found that women, aged 55-64, had a much lower incidence of collapsed vertebrae in the high fluoride area. Other studies have indirectly supported this evidence, but further work in this field is necessary.

Using a daily dosage of 60 mg fluoride, Purves (1962) treated sixteen patients with unilateral Paget's disease. Sixty mg per day was administered for two to three months, and then 20 mg per day for periods up to a year. In six out of eight patients, this treatment produced a positive calcium balance and all but two of the patients received sustained relief from bone pain after 4-6 weeks of treatment. After six to eight weeks, radioactive calcium uptake in the affected limbs approached that of the normal limb of all patients and was normal within 4-6 months in six patients tested at that time.

Bernstein and Cohen (1967) administered fluoride in doses between 10 mg/day to 61 mg/day with supplementary vitamin D therapy. They found a "modest calcium retention following fluoride therapy" and after one year or more of treatment, four of their patients showed increases in bone density on X-ray. A patient, after six months of therapy, showed signs of parathyroid hyperplasia and hyperparathyroidism. Davies et al (1968) also noted that one of their series of parathyroid adenomas had marked dental fluorosis. Thus, there exists a

possibility that fluoride could induce tertiary hyperparathyroidism.

Fluoride has a prolonged effect on cellular enzymatic systems. Its effect in osteoporosis is made difficult to interpret because of the little knowledge that is available about its pathology.

A few animal studies have been performed on the effect of fluoride on bone strength. In rats it was found that there was no difference (Naylor and Wilson, 1967). Faccini (1969) found that fluoride increased the strength of whole rabbit femora but this was due to an increase in width of the cortex and not to the quality of the bone. In fact, the elasticity and the breaking stress were significantly reduced.

Bone biopsies were performed on 15 out of 30 patients after one year of fluoride therapy and X-ray diffractions showed an increase in crystal size after therapy (Bernstein and Cohen, 1967). Biopsies proved that both resorption and formation were increased, and there were wide osteoid seams. Bernstein and Cohen (1967) also suggested that fluoride accumulates calcium in the skeleton with a less soluble apatite crystal, thereby leading to a decrease of the serum calcium, thus stimulating parathyroid activity. Upon operation of three patients, they made significant findings, i.e., that there was some hypertrophy of the parathyroid; however, as yet there is no evidence that suggests that

ordinary osteoporosis is caused by hyperparathyroidism. It is tempting to propose that this was due to fluoride therapy. Should this be the case, it can be assumed that any stabilizing effect fluoride may have on the skeleton eventually will be counterbalanced by a compensating increase in the function of the parathyroid.

Shambaugh and Scott (1964) have been concerned with a disease of the middle ear termed stapedial otosclerosis. He has also referred to this as otospongeosis progressiva because of the similarity between the otosclerotic process and the lesions of Paget's disease. In the condition of otosclerosis, which results in a conductive hearing loss, there is an ankylosis of the footplate of the stapes to the fenestra ovalis. He and others have found that by oral administration of gelatin capsules containing 10 mg of fluoride three times daily to the patients showing clinical signs of stapedial otosclerosis that the symptoms were alleviated. In a clinical study of the incidence of stapedial otosclerosis in high and low fluoride areas, Daniel (1969) noted that the incidence of this disease is significantly reduced in the high fluoride region (greater than 1.9 ppm).

This investigation is concerned with the study of any changes in the concentration of fluoride and calcium in the stapes at different stages of development. At this point, no controlled studies dealing with fluoride uptake and calcium

retention in the stapes have been found in the literature. The present study will be conducted under controlled conditions to determine if the stapedes of guinea pigs and rats are affected by fluoride in the same manner as the teeth and other bones, as noted in the literature.

MATERIALS AND METHODS

Albino guinea pigs, *Cavis porcellus*, of English stock, and albino rats, *Rattus norvegicus*, of the Holtzman strain, were used in this investigation. Both guinea pigs and rats were housed in animal quarters with a constant temperature of $75^{o} \pm 2^{o}F$, and a 14-hour light regime (5:00 a. m. to 7:00 p. m.). All animals received Purina laboratory chow *ad lib*. The females and the offspring of the control groups received distilled water throughout the study; whereas, those of the experimental groups were given water containing 5 ppm sodium fluoride, which was made up in 20-liter units weekly.

Young female guinea pigs were divided into two equal colonies and bred while on treatment. After the animals were bred, they were separated into cages in groups of two, but remained on their respective treatment. One-half of the offspring were sacrificed at birth, while the remainder of the young and their mothers were sacrificed at the termination of lactation (21 days). Since this was a pilot study, the the experimental and control groups consisted of guinea pigs of different ages.

One-third of the young rats (21 day) plus their mothers (150 day) were sacrificed at the termination of lactation, and the remainder of the young were taken at approximately late puberty (52 days). Thus, the treatment lasted for approximately one rat generation.

Sacrifice of both guinea pigs and rats was performed by giving an overdose of chloral hydrate, intraperitoneally. The age, sex, and weight of the animals were recorded and they were frozen until the ossicular chain could be removed. This chain was removed utilizing Lipscomb's technique (1969). Only one guinea pig stapes was needed for each chemical analysis; however, both stapedes of the rat were required to have sufficient quantity for a similar analysis. (See Appendix 5).

After removal, the bones were cleaned using the technique developed by Lincke et al (1967). This involved soaking the bones in diethyl ether for 48 to 72 hours, transferring to acetone for 24 hours, drying for 24 hours at 60^{o}C, and bringing to constant weight. The stapedes were then analyzed for fluoride and calcium.

The fluoride analysis involved the diffusion of hydrogen fluoride to sodium hydroxide from the sample of bone dissolved in perchloric acid in a sealed microdiffusion dish. This solution was reacted with a zirconium-SPADNS colorimetric reagent, which is bleached by the fluoride in the solution. Using a Beckman DB spectrophotometer at λ_{max}, a standard curve was constructed using one, two, three, and four microgram standards of fluoride. The concentration of fluoride was then determined in the sample by finding the optical density of the unknown solution at λ_{max} and reading the concentration

directly from the standard curve (Whitney-Wharton, 1962). (See Appendix 1 for details.)

The calcium content was analyzed by taking an aliquot of the solution containing the dissolved bone from the fluoride analysis. Again utilizing the Beckman DB and calcium standards, a standard curve was constructed. Using orthocresolphthalein complexone reagent, the optical density of the unknown samples were read and the concentration determined from the standard curve (Connerty and Briggs, 1966). (See Appendix 2 for details.)

Since this study was closely controlled, it should be possible to determine whether there is any significant difference in calcium and fluoride concentrations in the stapedes of guinea pigs and rats receiving fluoride in their drinking water.

Null hypotheses are constructed stating that there is no significant difference in the fluoride concentration and no significant difference in the calcium concentration in the stapedes of the animals studied. The statistical analyses used will involve analysis of variance and paired uncorrelated *t* tests to determine significance.

RESULTS AND DISCUSSION

After obtaining the data on the guinea pig stapedes (Table 1), analysis of variance and student t tests were applied (See Appendix 4). A null hypothesis was constructed stating that there was no significant difference in the fluoride concentration between the control and experimental groups of guinea pigs.

Table 1. Summary of the average concentration of fluoride and calcium in the stapedes of guinea pigs exposed to 5 ppm NaF compared to no NaF, in μg/mg bone.

Group	Number of Animals	Treatment	Mean weight, Fluoride	Mean weight, Calcium
I	10	5 ppm NaF in distilled, deionized water	2.27**	260.4
II	10	Distilled, deionized water	0.81	392.0

**Significant at .001 level

The calculated F score for the guinea pigs (65.00) was greater than .001, therefore, the null hypothesis was rejected. Paired uncorrelated t tests were than utilized to indicate significant differences between the two groups. The t test indicates very high significance in the amounts of fluoride found between the two groups ($t=8.98$, $d/f=1/38$, $p<.001$).

The guinea pigs of this group varied in age from 24-300 days, and despite this variation, no significant trends could

be noted. Further, it can be noted that the total weight of each stapedes varied very little as a function of age. This seems to conform to the literature which states that in animals born fully developed, e.g., the human and the guinea pig, the stapes is fully developed at birth. It is not surprising to find that sex had no apparent effect on the weight of the stapes.

A null hypothesis stating that there is no significant difference between the amounts of calcium found in the stapedes in the control and experimental groups of guinea pigs was constructed. Since the calculated F score (2.73) is less than the critical F score ($F=4.10$, $df=1/38$, $p>.05$) the null hypothesis is accepted.

As noted in the discussion of fluoride above, the guinea pigs were of mixed ages and the stapedes already mature at birth. It is well known that bone is in a state of dynamic equilibrium. This implies that only a minimal resorption and deposition of bone takes place. This would allow the substitution of the fluoride ion for the hydroxyl ion in the apatite crystal as noted in the literature (Posner, 1963), but no further calcification or addition of calcium would take place.

In order to provide adequate weight for analysis, the right and left stapes of each rat were combined. The data obtained from those analyses are summarized in Tables 2-5.

Table 2. Summary of the average concentration of fluoride and calcium in the stapedes of rats comparing different ages within each treatment.

	Control (0.0 ppm)		
	21 day	52 day	150 day
Mean F	0.51	0.67	0.96
St. Dev.	±0.90	±0.74	±0.79
Mean Ca	435.70	508.70	230.80**
St. Dev.	±104.43	± 98.94	± 23.58
	Experimental (5.0 ppm)		
	21 day	52 day	150 day
Mean F	6.29	5.11	1.42**
St. Dev.	±0.98	±1.80	±1.19
Mean Ca	613.40	742.00	427.00**
St. Dev.	± 73.17	± 85.03	± 40.62

**Significant at $p<.001$

Newborn stapedes were not used in this study since they were found to be composed entirely of cartilage (Tyndall, 1971). A null hypothesis was constructed stating that there is no significant difference among the fluoride concentrations in the stapedes of 21, 52, and 150 day old control group rats. The calculated F score (0.784) was less than the critical F score ($F=3.35$, $df=2/27$, $p>.05$), therefore, the null hypothesis was not rejected (Table 2). This is to be expected since these rats received no fluoride in their drinking water.

Analysis of variance was utilized to determine if a significant difference existed in the calcium concentrations among the stapedes of the 21, 52, and 150 day old rats within the control group. A statistically different calcium concentration among the three groups can be inferred since the calculated F score (29.30) was greater than the critical F value (9.02) at the .001 level of significance. The null hypothesis was rejected (Table 3).

Table 3. Comparison of the average calcium concentration in the stapedes of 21, 52, and 150 day old rats, in μg/mg bone.

Comparison	Control		Experimental	
21 day vs. 52 day	435.70	508.70	613.40	742.00*
21 day vs. 150 day	435.70	230.80**	613.40	427.20**
52 day vs. 150 day	508.70	230.80**	742.00	427.20**

*Significant (.05>p>.01)
**Significant (p<.001)

Paired uncorrelated t tests were utilized to determine the differences in calcium concentrations between the paired groupings within the control group. The t tests indicated non-significance between the stapedes of the 21 day and 52 day old rats (t=1.60, df=18, p>.05) but highly significant differences between the stapedes of 21 day and 150 day old rats (t=6.05, df=18, p<.001) and the 52 day and 150 day old rats (t=8.63, df=18, p<.001) in terms of calcium concentration (Table 3). The 150 day old adults were sacrificed immediately following lactation. It is generally known that gestation

and lactation does utilize the calcium in their bones as a reserve for fetal development and milk production; therefore, a lower calcium concentration could be expected.

Regarding the experimental group analysis, a null hypothesis was constructed stating that there would be no significant difference in terms of fluoride concentration among the stapedes from 21, 52, and 150 day old rats. As the calculated F score was greater than the critical F value at the .001 level of significance (Appendix 4, Table VI), the null hypothesis was rejected indicating a significant difference among these groups (Table 2). To ascertain the differences in fluoride concentrations between the paired groupings within the experimental group, uncorrelated t tests indicated non-significance between the 21 day and 52 day groups (t=1.80, df;18, $p>.05$) but highly significant differences in terms of fluoride concentration between the 21 day and the 150 day groups (t=9.94, df=18, $p<.001$) (Table 4). The 150 day old rats were already mature when placed on their regimen, which suggests that there was not a great turnover of bone, and not as much fluoride could be incorporated. No significant weight differences could be found in either experimental or control group stapedes at any age studied.

Analyses were also performed to determine the significance of any differences among the experimental group in terms of

calcium concentration. The analysis of variance performed revealed that a significant difference existed among the 21, 52, and 150 day old groups (Appendix 4, Table VI).

Table 4. Comparison of the average fluoride concentration in the stapedes of 21, 52, and 150 day old rats, in μg/mg bone.

Comparison	Control		Experimental	
21 day vs. 52 day	0.51	0.67	6.29	5.11
21 day vs. 150 day	0.51	0.96	6.29	1.42**
52 day vs. 150 day	0.67	0.96	5.11	1.42**

**Significant ($p<.001$)

Once again the highly significant F score necessitated uncorrelated *t* tests between the paired groupings within the experimental group. The *t* tests indicated a significant difference existed between the 21 day and 52 day groups ($t=3.62$, $df=18$, $p<.01$), between the 21 day and 150 day groups ($t=7.03$, $df=18$, $p<.001$), as well as between the 52 and 150 day groups ($t=10.56$, $df=18$, $p<.001$) all in terms of calcium concentration of the stapedes. This could be expected in the 21 and 52 day old rats since the experimental groups received fluoride in their water, and it was noted in the literature that fluoride will bind calcium more strongly, allowing a more dense bone, as found in the 52 day old rats. The reduction in the amount of calcium in the stapedes of the 150 day old experimental rats can be explained on the same basis as it was for the control groups of the same age.

These rats in the experimental group also had just completed lactation.

Uncorrelated *t* tests between each control group (21, 52, 150 day) and the corresponding experimental group (21, 52, 150 day) in fluoride and calcium concentrations were performed (Table 5). All *t* tests for fluoride concentration were significant beyond the .001 level except the 150 day old control group versus the 150 day old experimental group.

Table 5. Summary of the average concentration of fluoride and calcium in the stapedes of rats, in μg/mg bone.

Comparison (Control vs. Experimental	Control	Experimental
21 day vs. 21 day (F concentration)	0.51	6.29**
52 day vs. 52 day (F concentration)	0.67	5.11**
150 day vs. 150 day (F concentration)	0.96	1.42
21 day vs. 21 day (Ca concentration)	435.70	613.40**
52 day vs. 52 day (Ca concentration)	508.70	742.00**
150 day vs. 150 day (Ca concentration)	230.80	427.20**

**Significant at .001 level

When 5 ppm fluoride are added to the drinking water, there is a significant increase in the amount of both fluoride

and calcium in the stapedes of guinea pigs and rats if they have not matured prior to the addition of the fluoride to the water. If these results follow the same pattern of increasing fluoride and calcium content in the stapedes of other mammals, e.g., man, it becomes obvious that a harder, more compact stapes with a higher fluoride and calcium content could result in a reduction in the disease otosclerosis. This is consistent with Daniel's (1969) finding of a significant reduction in the incidence of otosclerosis where there is a high fluoride content in the drinking water.

SUMMARY AND CONCLUSIONS

The guinea pigs were used in this study as a preliminary project. Therefore, a consideration of that data will be presented first. The fact that there was a significant difference in the fluoride found in the experimental group as opposed to the control group indicates that fluoride was taken up by the stapedes. The calcium content did not differ significantly between the two groups. The reason for this is not clear. However, the fact that the majority of the guinea pigs used were already mature when placed on their regimens and bone formation was already complete may have reduced calcium turnover. Therefore, this indicates that only normal resorption and deposition took place, since bone is in a state of dynamic equilibrium allowing fluoride to be incorporated but no more calcium. This is also borne out in the rat studies which will be discussed next.

A brief summarization of the findings for the rat appears warranted.

1. Given no fluoride in the drinking water, the concentration of fluoride in the rat stapedes does not appear to vary significantly as a function of age.
2. Given no fluoride in the drinking water, the concentration of calcium in the rat stapedes appears to rise a little at 52 days and decrease significantly

at 150 days.

3. Given 5.0 ppm fluoride in the drinking water, the fluoride content in the rat stapedes appears to drop significantly at 150 days.

4. Given 5.0 ppm fluoride in the drinking water, there is an apparently significant decrease in the calcium content (paralleling the fluoride decrease) at 150 days.

5. Significant differences in the respective fluoride and calcium concentrations were found between the experimental and control groups at all ages except the 150 day control and the 150 day experimental in terms of fluoride concentration.

It is apparent from the findings that more research is necessary in a number of areas in order to ascertain the effect of a controlled calcium diet, various age groups other than those studied and between those studied, and the parathyroids on fluoride/calcium retention in the stapedes.

The results do show that there is a lower calcium and fluoride level at 150 days. This can be explained on the same basis as the adult guinea pig. The 150 day old rats were started on their regimens at age 48 days, which means that all bone formation was complete. Here again, fluoride was incorporated into the stapedes as the result of the

dynamic equilibrium of the bone, but no further calcification or calcium deposition could result. It should also be remembered that the adult (150 day) rats were females which had just completed lactation, which could account for a depressed calcium. All of this can be resolved with further research.

The important point is that there was a significantly greater amount of calcium found in rat stapedes which had received fluoride excepting the 150 day old rats. This indicates that more calcium is bound in the apatite crystal of the bone as a result of the presence of fluoride in the crystal.

These findings confirm the studies of Hodge, Posner, Bernstein and others done in hard tissues other than the stapedes, i.e., the femur and other long bones.

This study presents a number of avenues for further study. First, how much fluoride is effective in increasing the fluoride and calcium content of the stapedes? Second, how much involvement of the parathyroids is there in negating the binding effects of incorporated fluoride? Third, what effect does pregnancy and lactation have on the amount of fluoride incorporated in adult females; the control being hysterectomized-ovarectomized animals? Fourth, what is the overall effect of sex on the difference of fluoride uptake in both young and adult animals? Fifth, what is the effect

of estrus cycle on fluoride and calcium content of the stapes?

APPENDIX 1

ISOLATION AND DETERMINATION OF MICROGRAM AMOUNTS OF FLUORIDE IN MATERIALS CONTAINING CALCIUM AND ORTHOPHOSPHATE

Experimental

Reagents and Apparatus. REAGENT A. SPADNS 4,5-dihydroxy-3-(p-sulfophenylazo)-2,7-naphthalene-disulfonic acid, trisodium salt, Eastman Organic Chemicals, No. 7309 (3.16 grams) is dissolved in 550 ml. of deionized water.

REAGENT B. Zirconyl chloride octahydrate (0.133 gram) is dissolved in 50 ml. of deionized water. Concentrated HCl (350 ml.) is added and the resulting solution is diluted to 500 ml. with deionized water.

REFERENCE SOLUTION. Fifty milliliters of reagent A is added to 500 ml. of deionized water and 35 ml. of concentrated HCl is added. This solution is used to set the zero absorbance (100% T) of the spectrophotometer. It is stable and re-usable.

SINGLE SPECTROPHOTOMETRIC REAGENT. Equal volumes of reagents A and B are mixed. As the mixture is stable indefinitely, it is convenient to combine those solutions remaining after preparation of the reference solution.

STANDARD FLUORIDE SOLUTION. Dry, reagent grade NaF (22.1 mg.) is dissolved in 1 liter of deionized water, provides a solution containing 10.0 μg. of F^- per ml. As diffusion standards, microliter aliquots may be used without increasing the sample solution volume significantly.

The polypropylene microdiffusion cells, size 44, are available from the Aloe Scientific Co. The wetting agent, Tergitol, accompanies the diffusion cells and is diluted to 0.1% with deionized water. The heated dessicator was Harshaw No. H-18880; the Pasteur pipets were Harshaw No. H-55698; the Staticmaster brush, No. 1S200, was from Nuclear Products Co., El Monte, Calif.

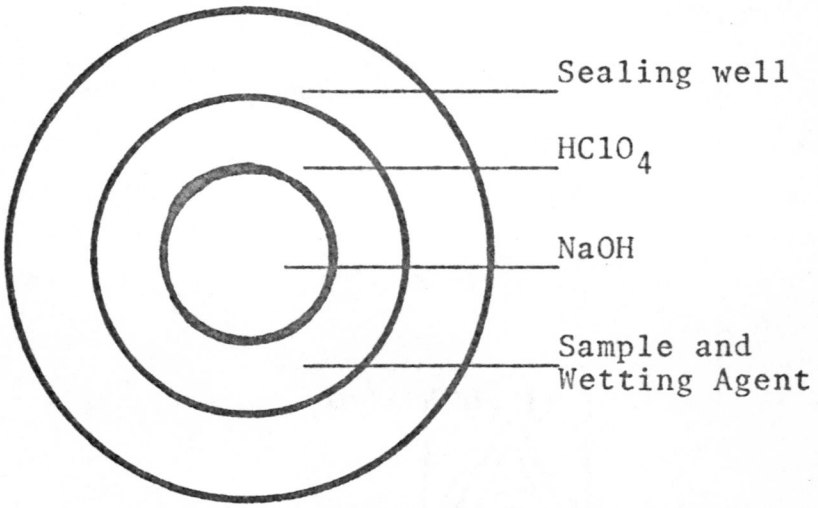

Fig. 1. Schematic diagram of microdiffusion cell showing relative locations of materials for F^- diffusion.

Procedure. Wipe the dry diffusion cells with the static-removing brush and weigh the sample containing from 0.3 to 2.5 µg. of fluoride directly into the inner annular chamber of the cell (see Figure 1). Unless removed, the

static charge is sufficient to expel some of the sample and, more seriously, to introduce sample into the inner compartment. Place 0.3 ml. of 1.3 N NaOH in the center chamber. Place 3 drops of diluted wetting agent and 0.3 ml. of water in the inner annular chamber. (With samples containing appreciable amounts of CO_3^{-2} such as teeth or bone, place the wetting agent and water directly on the solid samples to slow down the attack of the acid and reduce spattering.) Place 0.5 ml. of concentrated perchloric acid in this same chamber, but away from the sample. Liberally grease the edge of the clear plastic top with a silicone-type grease and place over the diffusion cell.

Tilt and revolve the sealed cells to mix the contents of the annular chamber thoroughly, place in the heated dessicator, and maintain at 60°C for 24 hours. Heating causes the NaOH to wet the cell and spread out uniformly, aiding the capture of diffused fluoride. After this period, cool to room temperature. Fill the inner chamber, containing the NaOH and the resulting NaF, with deionized water. Transfer the contents of this chamber by Pasteur pipet to volumetric flasks of appropriate size. Repeat this washing operation four times. For 0 to 4 μg. of fluoride, add 1.0 ml. of the Zr-SPADNS single spectrophotometric reagent and dilute the solutions to 10.0-ml. total volume. Read the absorption in 1-cm. cells at 590 mμ (or observed λ_{max}) with the reference

solution in the reference cell.

A standard curve is constructed using various concentrations of NaF which have been carried through the diffusion operation. Diffusion recovery checks can be made by adding appropriate amounts of NaOH and wetting agent to undiffused NaF standards.

The previously described, improved Zr-SPADNS method may, of course, be applied to Willard and Winter distillates. Proper spectrophotometric conditions and Zr-SPADNS requirements for various concentrations of F^- are given in Table I. The spectrophotometric method is unaffected by any $HClO_4$ that may be carried over in the distillate.

Table I. Spectrophotometric Conditions and Zr-SPADNS Requirements for Various Concentrations of F^-.

F^-, μg.	Total Volume, Ml.	Zr-SPADNS, Ml.	Reference Solution	Cells, Cm.
0-4	10	1.0	As prepared	1
0-4	25	1.0 + 6 drops concd. $HClO_4$	Dil. 1:1 with water	5
0-8	25	2.0	As prepared	1
0-8	50	2.0 + 6 drops concd. $HClO_4$	Dil 1:1 with water	5
5-40	100	10.0	As prepared	1

Taken from H. Whitney Wharton (1962). Isolation and determination of microgram amounts of fluoride in materials containing calcium and orthophosphate. Anal. Chem. 34(10):1297.

APPENDIX 2

DETERMINATION OF SERUM CALCIUM BY MEANS OF ORTHOCRESOLPHTHALEIN COMPLEXONE

Reagents

These are all made up in deionized or double distilled water.

1. Isoelectric deproteinization buffer, pH 5.2. Half fill a liter volumetric flask with water. Add 3 ml. of glacial acetic acid and 38 ml. of 1 N potassium hydroxide. Dilute to the volume mark with deionized water. This reagent may be prepared in more concentrated form and diluted accordingly to make the working reagent. The pH of the working reagent should be 5.2.

2. 14.8 M aminoethanol-borate buffer (AEB). To 50 ml. of water add 18 Gm. of Analytical Reagent (American Chemical Society) boric acid (H_3BO_3), moisten, and disperse by means of a magnetic stirrer. Continue mixing and add 25 ml. of 2-aminoethanol (Eastman No. 1597 or monoethanolamine, "Baker analyzed" reagent No. 9314). Stir for 5 min. and add another 25 ml. of aminoethanol. When the boric acid is completely dissolved, add 400 ml. of aminoethanol and mix. Store in the refrigerator. The reagent keeps for several months. When diluted 1:20, the pH should be approximately 11.0.

3. One normal HCl.

4. OCPC solution, 0.8 mg./ml. Add 80 ml. of OCPC* and 0.5 ml. of 1 N potassium hydroxide to 25 ml. of water. Stir until dissolved. Then add 75 ml. of water and 0.5 ml. of glacial acetic acid. This solution is stable for several months at room temperature.

5. Five per cent w/v solution of 8-quinolinol. Dissolve 5 Gm. of 8-quinolinol (8-hydroxyquinolin, oxine--Eastman) in 100 ml. of 95 per cent ethanol. This solution is stable for at least 1 month at room temperature, longer if refrigerated.

6. OCPC color reagent. In a 100-ml. glass-stoppered graduate, place 5 ml. of 14.8 M ethanolamine-borate buffer and add 1.5 ml. of 5 per cent solution of 8-quinolinol. Add 5 ml. of OCPC solution and dilute to 100 ml. with water. Prepare fresh daily.

7. Stock standard calcium solution, 1 mg./ml. Dissolve 2.97 Gm. of oven-dried (at 100°C.) A. R. calcium carbonate primary standard in 60 ml. of N HCl and dilute to 1000 ml. with water. Use of excess acid must be avoided.

8. Working standard calcium solutions for the direct procedure. Intermediate calcium standards containing 5, 10, 15, and 20 mg./100 ml., respectively, are first prepared.

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*Cat. No. 1367, K & K Laboratories, Inc., Plainview, N. Y. 11803

These are then diluted 1:10 with isoelectric deproteinization buffer, to prepare working standards of calcium concentration A, 0.5; B, 1.0; C, 1.5; and D, 2.0 mg./100 ml. corresponding to equivalent calcium concentrations under test conditions of 5, 10, 15, and 20 mg. per 100 ml. Store in plastic bottles.

9. Blank solution for the direct procedure. Mix 9 parts of isoelectric deproteinization buffer with 1 part of water.

10. Working standard calcium solutions for the alternate procedure. (A) 0.2 N HCl containing 1 mg. of calcium per 100 ml.; corresponding to 5 mg./100 ml. of serum calcium under test conditions. Into a 100-ml. volumetric flask place 10 ml. of 10 mg./100 ml. intermediate calcium standard and 20 ml. of N HCl. Dilute to the mark with water. (B) Solution B contains 2 mg. of calcium in 100 ml. of 0.2 N HCl. (C) Solution C contains 3 mg. of calcium in 100 ml. of 0.2 N HCl.

11. 1.33 per cent w/v ammonium oxalate solution. Dissolve 1.3 Gm. ammonium oxalate in 100 ml. of water and add 2 ml. of 1 per cent Sterox SE.*

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*Sterox SE is a nonionic polyoxyethylene thioether manufactured by the Monsanto Chemical Co., and supplied by Hartman-Leddon Co., Philadelphia, Pennsylvania.

12. Extremely pure water is desirable, as less masking agent (8-quinolinol) will be required in the color reagent. We use water which has been deionized by means of a mixed bed resin, such as Barnstead Red Cap No. 0808.

Direct Procedure

Wash all glassware, including glass stoppers, with a detergent, rinse with deionized water, and then treat with 0.5 N HCl to remove any traces of calcium.

Use Pyrex glass-stoppered volumetric flasks, glass-stoppered test tubes (the stoppers must be "decalcified"), special centrifuge tubes with fine tip, and 125- by 16-mm. Pyrex test tubes, fitted with rubber stoppers. The serum and color reagent are conveniently measured with a model 114 Fisher Dilumat. Absorbance measurements are made with a Beckman model B spectrophotometer.

Add 1 ml. of serum to a 100-ml. volumetric flask and pour in 9 ml. of isoelectric deproteinization buffer. Mix and transfer the contents to a 125- by 16-mm. test tube. Insert a rubber stopper and place in a near-boiling water bath (90 to 95°C.) for 3 min. Centrifuge while still hot, to sediment the precipitated protein. In a glass-stoppered test tube, mix 1 ml. of the centrifugate with 10 ml. of OCPC color reagent No. 6 and measure the absorbance at 570 mμ against the reagent blank, which is prepared by

combining 1 ml. of blank solution reagent No. 9 with 10 ml. of color reagent. Standards representing equivalent calcium concentrations of 5, 10, and 15 mg./100 ml. (Reagents No. 8A, 8B, and 8C) are prepared in a similar manner. The concentration of the unknowns are read from a calibration curve.

Taken from Connerty, Harold V., M. D., and Anglis R. Briggs, B. S. (1966). Determination of serum calcium by means of orthocresolphthalein complexone. The American Journal of Clinical Pathology 45(3):290-296.

APPENDIX 3

RAW DATA

Guinea Pig

Treatment--NaF

Bone--Stapes

No.	Age (Days)	Sex	Total Bone (in mg)		Total F (in μg)	μg F / mg Bone	Total Ca (in μg)	μg Ca / mg Bone
1	200	F	R	0.40	0.74	1.85	40	100
			L	0.40	1.53	3.82	416	1040
2	200	F	R	0.60	0.65	1.08	140	233
			L	0.475	1.02	2.15	227	478
3	200	F	R	0.40	0.32	0.80	50	125
			L	0.90	1.60	1.78	290	322
4	200	F	R	0.45	0.28	0.62	80	178
			L	0.45	0.73	1.62	140	311
5	200	F	R	0.80	2.14	2.68	270	338
			L	0.60	1.50	2.50	185	308
6	200	F	R	0.45	0.45	1.00	78	175
			L	0.45	1.30	2.89	124	275
7	200	M	R	0.75	0.57	0.76	90	120
			L	0.50	1.60	3.20	123	246
8	300	F	R	0.40	1.30	3.25	0	0
			L	0.35	1.73	4.94	125	357
9	65	F	R	0.45	1.68	3.73	50	111
			L	0.40	1.60	4.00	85	212
10	65	M	R	0.45	0.00	0.00	35	77
			L	0.40	1.10	2.75	84	210

Guinea Pig

Treatment--Distilled Water Controls

Bone--Stapes

No.	Age (Days)	Sex	Total Bone (in mg)		Total F (in µg)	µg F / mg Bone	Total Ca (in µg)	µg Ca / mg Bone
1	200	F	R	0.425	0.30	0.70	580	1365
			L	0.425	2.23	5.54	475	1117
2	24	F	R	0.70	0.65	0.93	380	543
			L	0.70	1.20	1.70	325	464
3	30	F	R	0.44	0.43	0.98	85	193
			L	0.44	0.00	0.00	215	488
4	200	M	R	0.65	0.45	0.69	175	269
			L	0.55	0.00	0.00	230	418
5	200	F	R	0.40	0.70	1.75	70	175
			L	0.45	0.00	0.00	220	488
6	200	F	R	0.54	0.07	0.13	100	185
			L	0.54	0.00	0.00	218	404
7	200	F	R	0.60	0.93	1.55	177	295
			L	0.60	0.00	0.00	120	200
8	200	F	R	0.44	0.50	1.25	80	182
	200	F	L	0.475	0.00	0.00	40	145
9	150	M	R	0.45	0.06	0.13	119	265
			L	0.45	0.00	0.00	119	265
10	150	F	R	0.45	0.25	0.56	82	182
			L	0.48	0.17	0.34	100	208

Rat

Treatment--NaF

Age--21 days

Bone--Stapes

No.	Sex	Total Bone (mg)	Total F (in µg)	µg F / mg Bone	Total Ca (in µg)	µg Ca / mg Bone
1	M	0.30	2.13	7.10	190	633
2	M	0.30	2.26	7.53	155	517
3	F	0.30	1.73	5.77	185	617
4	M	0.30	1.45	4.83	146	483
5	F	0.30	2.12	7.07	188	627
6	F	0.30	1.87	6.23	204	680
7	F	0.30	1.68	5.60	180	600
8	F	0.30	1.90	6.33	175	583
9	F	0.30	1.50	5.00	200	667
10	M	0.30	2.25	7.50	218	727

Rat

Treatment--Distilled Water Control

Age--21 Days

Bone--Stapes

No.	Sex	Total Bone (mg)	Total F (in µg)	µg F / mg Bone	Total Ca (in µg)	µg Ca / mg Bone
1	F	0.30	0.88	2.93	130	433
2	F	0.30	0.00	0.00	75	250
3	M	0.30	0.02	0.07	150	500
4	M	0.30	0.00	0.00	160	533
5	M	0.30	0.26	0.86	140	467
6	M	0.30	0.00	0.00	155	517
7	M	0.30	0.20	0.67	149	497
8	M	0.30	0.00	0.00	74	247
9	F	0.30	0.16	0.53	127	423
10	M	0.30	0.02	0.07	137	490

Rat

Treatment--NaF

Age--52 days

Bone--Stapes

No.	Sex	Total Bone (mg)	Total F (in µg)	µg F / mg Bone	Total Ca (in µg)	µg Ca / mg Bone
1	M	0.40	1.60	4.00	260	650
2	F	0.40	1.67	4.18	267	668
3	M	0.30	1.85	6.17	265	883
4	M	0.35	1.93	5.51	232	663
5	F	0.30	2.85	9.50	267	890
6	F	0.35	1.60	4.57	245	700
7	F	0.30	1.58	5.27	232	773
8	F	0.30	1.60	5.33	220	733
9	F	0.30	1.00	3.33	220	733
10	F	0.30	1.00	3.33	218	727

Rat

Treatment--Distilled Water Control

Age--52 days

Bone--Stapes

No.	Sex	Total Bone (mg)	Total F (in µg)	µg F / mg Bone	Total Ca (in µg)	µg Ca / mg Bone
1	M	0.30	0.30	1.00	180	600
2	M	0.30	0.66	2.20	200	667
3	F	0.30	0.00	0.00	110	367
4	M	0.30	0.37	1.23	160	533
5	M	0.40	0.26	0.85	160	400
6	M	0.30	0.00	0.00	138	460
7	M	0.35	0.05	0.14	95	400
8	M	0.30	0.37	1.23	165	550
9	F	0.30	0.03	0.10	175	587
10	F	0.30	0.00	0.00	157	523

Rat

Treatment--NaF

Age--150 days

Bone--Stapes

No.	Sex	Total Bone (mg)	Total F (in µg)	µg F / mg Bone	Total Ca (in µg)	µg Ca / mg Bone
1	F	0.30	0.57	1.90	150	500
2	F	0.30	0.58	1.93	130	425
3	F	0.25	0.00	0.00	120	408
4	F	0.30	0.51	1.70	127	423
5	F	0.30	0.07	0.23	121	503
6	F	0.25	0.00	0.00	115	406
7	F	0.30	0.22	0.73	120	400
8	F	0.30	0.50	1.67	120	400
9	F	0.30	0.68	2.27	125	417
10	F	0.30	1.13	3.77	117	390

Rat

Treatment--Distilled Water Control

Age--150 days

Bone--Stapes

No.	Sex	Total Bone (mg)	Total F (in µg)	µg F / mg Bone	Total Ca (in µg)	µg Ca / mg Bone
1	F	0.375	0.30	0.80	63	210
2	F	0.30	0.49	1.63	68	227
3	F	0.30	0.00	0.00	65	217
4	F	0.30	0.30	1.00	64	213
5	F	0.30	0.50	1.67	75	280
6	F	0.30	0.25	0.83	70	267
7	F	0.30	0.15	0.50	69	230
8	F	0.30	0.78	2.60	68	227
9	F	0.30	0.09	0.30	66	220
10	F	0.30	0.10	0.33	65	217

APPENDIX 4

STATISTICAL ANALYSIS

Table I

Summary of Statistical Analysis

of the Guinea Pig Data for Fluoride

Experimental Mean = 2.27 mg
Control Mean = 0.80 mg

Source of Variance	df	SS	MS	F	t
Between	1	110.55	110.55	65.0*	8.98*
Within	38	64.73	1.70		
Total	39	175.28	4.50		

*Significant at the .001 level

Table II

Summary of Statistical Analysis

of the Guinea Pig Data for Calcium

Experimental Mean = 275.9 mg
Control Mean = 387.5 mg

Source of Variance	df	SS	MS	F
Between	1	124,548	124,548.0	2.73
Within	38	1,608,822	45,625.5	
Total	39	1,733,370	44,445.4	

**Nonsignificant

Table III

Summary of Analysis of Variance

of Mean F in the 21, 52, and 150 Day Old

Control Group Rat Stapedes

Source of Variance	df	SS	MS	F
Between	2	1.053	0.526	0.784
Within	27	18.146	0.672	
Total	29	19.199	0.662	

Table IV

Summary of Analysis of Variance of

Mean Ca in the 21, 52, and 150 Day Old

Control Group Rat Stapedes

Source of Variance	df	SS	MS	F
Between	2	415,138.06	207,569.03	29.30**
Within	27	191,273.80	7,084.21	
Total	29	606,411.86	20,910.75	

**Significant at .001 level

Table V

Summary of Analysis of Variance

of Mean F in the 21, 52, and 150 Day Old

Experimental Group Rat Stapedes

Source of Variance	df	SS	MS	F
Between	2	129.478	64.739	34.29**
Within	27	50.968	1.887	
Total	29	180.446	6.222	

**Significant at .001 level

Table VI

Summary of Analysis of Variance

of Mean Ca in the 21, 52, and 150 Day Old

Experimental Group Rat Stapedes

Source of Variance	df	SS	MS	F
Between	2	501,029.20	250,514.60	52.79**
Within	27	128,128.00	4,745.48	
Total	29	629,157.20	21,695.07	

**Significant at .001 level

APPENDIX 5

SURGICAL PROCEDURE

After sacrifice using an overdose of chloral hydrate injected intraperitoneally, the following guidelines were used to gain access to the stapes of either the guinea pig or rat.

1. Remove the skin from the head.
2. Clear all musculature from the area around the auditory bulla with a curette.
3. Remove the mandible.
4. Carefully enter the bulla using a Rongeur or, in an older animal, a cutting disc.
5. Remove sufficient bone to visualize the tympannic membrane.
6. Using a myringotomy scalpel, carefully cut the membrane away from the neck of the malleus.
7. Cut the tendon holding the malleus to the wall of the bulla. This tendon is behind the malleus as it is viewed.
8. The malleus can be lifted out by breaking the malleo-incudal joint. In the guinea pig the malleus and incus are fused and the inco-stapedial joint is broken.
9. In the rat the incus is now removed by simply lifting it out by breaking the inco-stapedial joint.

10. The stapes in the rat comes out easily once the stapedius muscle is cut and the blood vessels over the footplate are cut with the myringotomy scalpel.
11. In the guinea pig a small shelf of bone over the footplate must be broken before the stapes can be removed intact.

All work must be done under a surgical or dissecting microscope.

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