The Effects of Fluoride on the Total- and Glycosaminoglycan–Hexosamines in Bones of Young Rats[1] (36246)

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Attempts to explain the defective calcification in osteofluorosis have emphasized the possibility that the bone matrix might be abnormal. Indirect evidence of changes in the glycoproteins or in the amount and state of polymerization of glycosaminoglycans (mucopolysaccharides) during mineralization of bones and teeth, and during deposition of fluoride has been obtained by histochemical methods: metachromatic staining, the periodic acid–Schiff reaction and autoradiography with ^{35}S (1–3). The present study provides quantitiative evidence that deposition of relatively large amounts of fluoride in bone alters the concentration of total hexosamines and the amount of glucosaminoglycan-hexosamines in certain areas of bones of young, growing rats.

Interest in the carbohydrate–protein complexes in connective tissues have grown rapidly in the last few years since micromethods were devised for their separation and identification (4, 5) but the determinations in bone and dentin are complicated by the fact that relatively small amounts of these substances are present and they must be isolated from the mineral phase. Changes in amounts of the glycosaminoglycans can be determined by analysis for hexosamines and hexuronic acid. Microseparation and identification of the specific glycosaminoglycans can be accomplished by electrophoresis or column chromatography—precipitation with cetylpyridinium chloride (CPC) and fractional elution—and determination of the hexosamines and hexuronic acid in the eluates.

The acidic glycosaminoglycans in calcified tissue identified earlier are: chondroitin-4-sulfate, chondroitin-6-sulfate, hyaluronic acid, and keratan sulfate, but some of these may have been from epiphyseal cartilage, since whole bones were used in some studies. Glycoproteins isolated from bovine cortical bone by Andrews *et al.* (6) and in teeth by Rogers (7) and Clark *et al.* (8) may also contribute to the amino sugars present.

Hjertquist and Vejlens (9) reported that subcutaneous injection of parathyroid extract in 3- to 4-month-old puppies did not cause depolymerization of the glycosaminoglycans in epiphyseal cartilage or diaphyseal bone, and did not alter the synthesis or sulfatation of chondroitin sulfate. Dulce (10) found less hexosamine, hexuronic acid, and sulfate in bones of rachitic rats than in normal animals, and these substances increased during healing (11). Bones of scorbutic guinea pigs contained more hexosamine than normal animals (12).

The present study was undertaken to determine whether fluoride had any effect on the hexosamines in bone matrix, and to obtain more detailed information on the normal pattern of glycosaminoglycans in various sites and types of bones from young, growing rats. In our earlier studies of the effects of fluoride on citrate in bone (13) and on the responses to parathyroid extract (14) or large doses of vitamin D_2 (15), we wished to obtain high deposition of fluoride in bone without severe toxic symptoms. This was accomplished by administering the fluoride (125 ppm) in the drinking water. In the present study, we wished to produce some toxic symptoms with definite alterations in bone. In order to do this, it was necessary to incorporate the fluoride in food, as well as

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water. To be sure that initial deposition was high, a massive dose was given for 2 days, which turned out to be too high and produced acute symptons. The rats were allowed to recover for 3 days and then transferred to the various treatments described below.

Materials and Methods. Animals. A large lot of weanling, male, Sprague-Dawley rats, all born on the same day were randomized by weight, housed 5/cage and fed Rockland Rat Pellets and distilled water for drinking. At 30 days of age, 5% NaF, incorporated in the Rockland pellets, was fed for 2 days. The rats were then transferred back to the regular diet, and 3 days later to either: (a) 125 ppm F in the drinking water[2]; (b) 0.05, 0.1, or 0.2% F incorporated in the pellets; or (c) fluoride in both food and water. The untreated controls received the regular Rockland diet and distilled water the entire time. The rats were sacrificed with ether after varying intervals (2 to 28 days) of treatment along with an equal number of suitable controls of the same age.

Bone sites and types. The bones were removed, rapidly stripped of flesh, and kept in Dry Ice until they were stored in the freezer. As was convenient, the bones were thawed, freed of cartilage as completely as possible, and sectioned as described previously (13) into epiphysis and metaphysis (cancellous), diaphysis (compact), and calvaria (membranous bone). The sections were cut into small pieces and extracted with occasional shaking over a period of at least 72 hr with 3 transfers of a 50:50 mixture of anhydrous ether–acetone. They were then dried for 3 hr at 105° and pulverized in 15 to 60 sec in a Wig-L-Bug[3]. Bones of 5 rats were composited for each site. The values reported are an average of two such composites; *i.e.*, 10 rats/treatment.

Analyses. Fluoride was determined by the microdiffusion method of Wharton (17).

Total hexosamines. Powdered, fat-free, dry bone (20 to 50 mg, depending on the site) was hydrolyzed in a test tube, fitted with a Teflon lined screwcap, with 2 ml of 6 *N* hydrochloric acid for 16 hr at 100°, and then centrifuged. One milliliter of the supernatant was evaporated *in vacuo* at 60° and then dissolved in 3.0 ml of distilled water. Two 1 ml aliquots were distilled according to the method of Cessi and Piliego for determination of hexosamines (18).

GAG-hexosamines. The hexosamines which correspond to the cetylpyridinium chloride precipitable acid glycosaminoglycans were determined after solubilizing the bone powder with papain in an aqueous solution of EDTA[4] according to the procedure of Hjertquist and Vejlens (9). Bone powder (20 to 50 mg) was digested in 10 ml of a mixture of 0.05 ml suspension of papain[5] in aqueous EDTA (0.05 *M*) and cysteine hydrochloride (0.005 *M*). The pH was adjusted to 7.0 with 2 *N* sodium hydroxide and checked at intervals and readjusted if necessary during the 6 hr digestion in a Dubnoff shaker at 65°. After centrifugation, aliquots of the supernatant were diluted with 8.5 vol of water to decrease the salt concentration, and 0.5 vol of a 1% (w/v) aqueous solution of cetylpyridinium chloride was added slowly. The precipitate of acid glycosaminoglycans was allowed to form overnight, recovered by centrifugation (30 min) and hydrolyzed and analyzed for hexosamines as described for the total hexosamines. The procedures used here were similar to those used by others for bones. No attempt was made to determine the optimum conditions for hydrolysis or precipitation of the glycosaminoglycans in our materials. These will be established for future studies.

Results. The rats on the high fluoride diets ate less than the controls or those ingesting 0.05% F in food or 125 ppm F in drinking water. Toxic symptoms were clearly evident at the high levels of treatment; the rats looked miserable and their weights at sac-

[2] The use of ppm F in the drinking water has been retained here instead of 0.0125% F because it corresponds with the notation used in our earlier publications and those of many others. It also serves to identify the mode of administration; namely, in drinking water (ppm) and in food (%).

[3] Crescent Dental Mfg. Co., Lyons, IL.

[4] Disodium ethylenediaminetetraacetate.

[5] Papain (345 units/ml); Worthington Biochemical Corp., Freehold, NJ.

TABLE I. Difference Between Membranous, Compact, and Cancellous Bone in the Uptake of Fluoride and in the Concentrations of Total- and Glycosaminoglycan (GAG)–Hexosamines.

Treatment[a] and bone site	Type of bone	Fluoride uptake (μg/g)[b]	Hexosamines		
			Total[c] (μg/g)	GAG[d] (μg/g)	GAG/total (%)
Untreated control					
Calvaria	Membranous	34	1543	574	37
Diaphysis	Compact	85	1875	595	32
Epiphysis	Cancellous	97	6869	3751	55
Metaphysis	Cancellous	122	6263	3000	48
0.1% F					
Calvaria		2981	1964	552	28
Diaphysis		3718	1901	602	32
Epiphysis		3730	8692	5671	65
Metaphysis		4847	7815	4252	54
0.2% F + 125 ppm F					
Calvaria		4961	1668	538	32
Diaphysis		6222	2020	587	29
Epiphysis		6815	8252	4896	59
Metaphysis		7980	5386	2694	50

[a] Treatments: 30-day-old rats received 5% NaF incorporation in Rockland Rat Pellets for 2 days; 3 days later they were transferred to the indicated treatments. To avoid confusion, the concentration of F incorporation in food is specified in %, that ingested in drinking water in ppm (equiv to 0.0125%). There were 10 rats/treatment; sacrificed at 51 days of age.

[b] All concentrations in bone are in μg/g of fat-free, dry bone.

[c] Total hexosamines after hydrolysis with 6 *N* HCl at 100° for 16 hr.

[d] GAG–hexosamines are the hexosamines corresponding to the acid glycosaminoglycans precipitated by cetylpyridinium chloride and then hydrolyzed.

rifice were 30 to 50% below those of the controls. There were two deaths from the 0.2% treatments and the remainder of the rats would not have survived much longer treatment; their bones were small, thin, and soft.

There were significant differences between the types of bones in: (i) the uptake of fluoride, as we have reported previously (13); (ii) the concentrations of total hexosamines, and the hexosamines corresponding to the CPC-precipitable acid glycosaminoglycans (GAG–hexosamines); and (iii) the ratios of GAG–hexosamines to total hexosamines (Table I).

The significant effects of fluoride observed in cancellous bone were not obtained in more fully calcified compact and membranous bone where the hexosamines are lower. Larger quantities of bone and further refinements in technique may be required to detect effects in these bone sites.

Differences in the effects of increasing levels of fluoride ingestion on fluoride deposition in epiphysis and metaphysis, and on the total- and glycosaminoglycans–hexosamines are shown in Figs. 1 and 2. Although the concentrations of hexosamines are generally higher in epiphysis than in metaphysis, this difference may not be a real one because some contamination with compact bone could not be avoided when metaphysis was sectioned from diaphysis where the hexosamines are lower (Table I). Also the peak concentrations of hexosamines observed in epiphysis at 4300 μg of fluoride/g of bone may have been passed over in metaphysis where fluoride deposition was higher (4800 μg/g). In young rats, deposition of fluoride is always higher in metaphysis during the first 4 or 5 weeks of treatment. After that time, fluoride deposition continues to increase in epiphysis

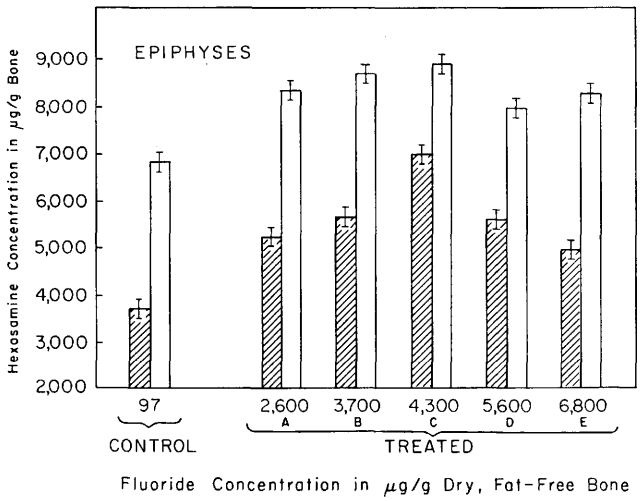

FIG. 1. Comparison of fluoride uptake and total- and glycosaminoglycan (GAG)-hexosamines in epiphyseal bone from young untreated rats and at 5 levels of fluoride treatment: (A) 125 ppm F in the drinking water; (B) 0.1% fluoride incorporated in the pelleted food; (C) 0.1% in food and 125 ppm in drinking water; (D) 0.2% in food; and (E) 0.2% in food and 125 ppm fluoride in the drinking water over a period of 20 days starting at 30 days of age. The controls received Rockland rat pellets and distilled water during the same period. (□) Total hexosamines from powdered bone hydrolyzed with 6 *N* HCl at 100° for 16 hr; (shaded area) acid glycosaminoglycans [from bone solubilized with papain in EDTA] precipitated by cetylpyridinium chloride and then hydrolyzed.

and the concentration exceeds that in metaphysis, where it tends to level off to a steady state (13).

Discussion. In untreated young rats, the total hexosamines decreased with age as has been reported by others in human (7), cattle (10), rabbit (19), guinea pig (12), and rat bone (10). The GAG–hexosamines in compact and membranous bone constituted about ⅓ of the total hexosamines and approximately 0.05 to 0.06% of the total dry weight. This agrees with the report of Hjertquist and Vejlens (9) for compact bone in puppies. In cancellous bone, the GAG–hexosamines constituted more than half of the total and approximately 0.3 to 0.5% of the dry weight (Table I).

Rapid, high deposition of fluoride (2600 to 4300 μg/g) in epiphyses of young rats given fluoride from age 30 to 51 days during rapid bone growth produced a 40–80% increase in the GAG–hexosamines (Fig. 1). Whether this increase is due to increased synthesis of the same or different glycosaminoglycans, or to depolymerization of these macromolecules is currently under investigation.

At higher concentrations of fluoride (possibly with excessive resorption and decreased calcification since the bones were small, thin, and soft) the hexosamines declined to near or slightly below normal at 8000 μg of fluoride/g of metaphyseal bone (Fig. 2). The high treatments were obviously extremely toxic and the rats were severely debilitated, so accurate evaluation of the changes in hexosamines at these levels was not possible.

This was a preliminary investigation to determine appropriate treatments for future study. The results indicate that fluoride may have a pronounced effect on the glycosaminoglycans in bone matrix. The effects are dependent upon the age of the animal, the maturity and calcification of the bone site, and the amount and rate of deposition of fluoride in the site. Further, carefully designed experiments are necessary in order to differentiate the exact effects of fluoride.

The possibility that the concentration of glycosaminoglycans might be increased at one level of fluoride intake and decreased at another level of intake is not inconsistent

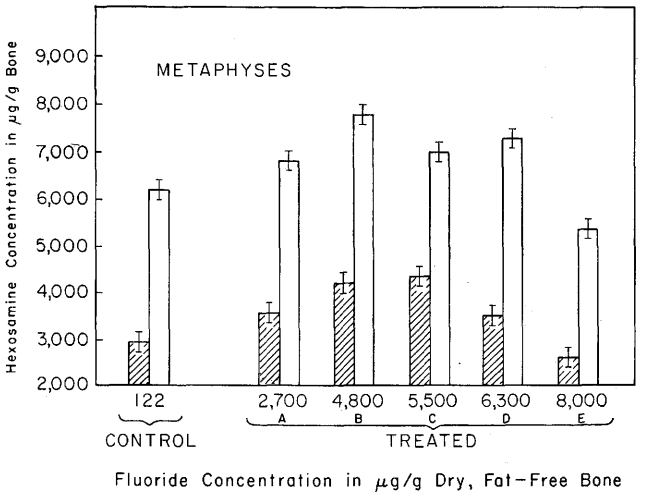

FIG. 2. Comparison of fluoride uptake and total- and glycosaminoglycan (GAG)-hexosamines in metaphyseal bone: bones are from the same rats as Fig. 1; (□) total hexosamines; (shaded area) GAG-hexosamines.

with the clinical effects of fluoride. Roholm, in his classic monograph on "Fluoride Intoxication" (20), pointed out that moderately severe exposure to fluoride caused an increase in bone growth and calcification, and the development of exostoses; while higher concentrations resulted in extensive resorption of bone, a decrease in growth and calcification, and the formation of osteoid tissue. The lesions in bone, which accompany fluoride accumulation, have been likened to osteosclerosis, osteoporosis, Paget's disease, and osteomalacia. This variability has led to confusion and frustration in studying osteofluorosis. Now, it may be that some of these differences can be explained by alterations in the glycoproteins and/or the glycosaminoglycans in bone matrix.

The specific factors, which bring about calcification, resorption, and remodelling of hard tissues, are not known. If the mucopolysaccharides in bone matrix are at the crux of these processes, as well as of the effects of fluoride on bones and teeth, as some have suspected from histochemical data, we are now in a position to thoroughly investigate and establish this.

Conclusions. Rapid, high deposition of fluoride in young rats altered the concentration of total hexosamines and the hexosamines corresponding to the acid glycosaminoglycans precipitable by cetylpyridinium chloride, in the cancellous, growing ends of the bones. There were indications that the hexosamines were increased at one level of fluoride ingestion and decreased at another, which would be in agreement with both the increase in bone growth and calcification sometimes reported in cases of osteofluorosis and the decrease in calcification and formation of osteoid reported in others. Similar effects were not obtained in more fully calcified compact and membranous bone, where the uptake of fluoride was less, and the concentration of the hexosamines was relatively low.

In bones from normal rats, the total hexosamines decreased with age. In compact and membranous bone, the hexosamines corresponding to the glycosaminoglycans constituted about one-third of the concentration of total hexosamines and approximately 0.05–0.06% of the dry weight. In cancellous bone, they constituted more than 50% of the total hexosamines and 0.3–0.5% of the dry weight.

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