Some Effects of Administration of Fluoride on Calcifying Cartilage in Rat* (33473)

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Whereas little is known about the mechanism by which cartilage becomes calcified, there is evidence (1, 2) that glycogenolysis is involved in the mineralization process; lipids have also been implicated as participants in calcification (3–5). Sodium fluoride inhibits enolase, the enzyme in the glycolytic pathway that catalyzes the conversion of 2-phosphoglyceric acid to phosphopyruvic acid. In this study, sodium fluoride was administered to young rats in order to examine the possibility of a relationship between the glycolytic pathway, lipids, and calcification. Lipids and calcium contents in the calcifying cartilage of fluoride-treated rats were measured.

Materials and Methods. Animals. Albino rats of the Sprague-Dawley strain, old enough to be weaned, were placed on a diet of Purina laboratory chow and deionized water. As a preliminary experiment had shown no difference in the calcium or lipid contents between the cartilage of male or female rats of the same size, subsequent samples were prepared without regard to sex. The costal bone and cartilage were removed immediately after decapitation and any adhering muscle tissue was scraped off. The bone and cartilage were separated with a minimum of handling and rinsed with acetone. The bones were dried overnight at about 100° and then pulverized with a mortar and pestle. The cartilage was homogenized in a Virtis "45" homogenizer and lyophilized. The bone was analyzed for calcium and fluoride; the cartilage for calcium, fluoride, and lipid.

Measurement of lipid. To each weighed sample of lyophilized cartilage was added an appropriate amount (about 2% of the weight of the cartilage) of heptadecanoic acid (Applied Science Laboratories, Inc., State College, Pa.) to serve as an internal standard. The lipids in the lyophilized cartilage samples were extracted (6), washed (6), and saponified (7). The free fatty acids were converted to methyl esters (8) and determined quantitatively by gas-liquid chromatography.

Chromatography conditions. Instrument: F and M model 400 gas chromatograph with flame ionization detector; column: 17% diethyleneglycol adipate ester on acid-washed chromosorb W (30-60 mesh) from Applied Science Laboratories, Inc., State College, Pa.; oven temperature: 160°; carrier gas: helium 30 ml/min; air: 325 ml/min; hydrogen: 20 ml/min.

Measurement of calcium and fluoride. The defatted cartilage tissue was dried overnight at 100°. A sample of dried bone or cartilage was weighed in a tared Conway diffusion cell and analyzed for fluoride according to Wharton (9). In this method, after diffusion of the fluoride the calcium of the tissue remains dissolved in 70% perchloric acid (w/w). This solution was removed and the calcium was measured with a Zeiss flame spectrophotometer (10).

Determination of optimum weight range in which to study calcification. As a preliminary experiment, coastal cartilage was removed from rats of various sizes (6–220 g) and divided into two zones—the section adjoining the coastal bone and the section connected to the sternum. The calcium in the costal bone and cartilage was measured as described above. In this way, the rate of calcification of costal cartilage of normal untreated rats was determined.

Fluoride administration. The experiment was performed three times; each time the conditions were slightly different. Several litters of young rats, born within a few hours of each other were used. Between 1 and 3 days after birth, the young rats (about 60) were

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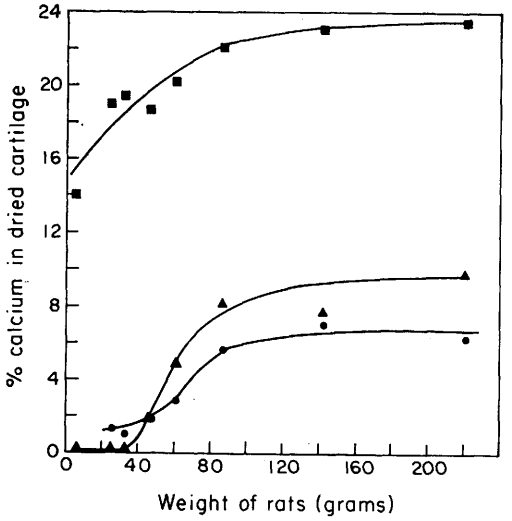

FIG. 1. Rate of calcification of costal bone, (■); costal cartilage adjoining costal bone, (●); and costal cartilage adjoining sternum, (▲); in normal untreated albino rats at different ages. Each point represents material pooled from 4–6 rats. The bone or cartilage was dried as described in the text.

divided into three or four groups. The litters were kept intact; the subdivision into groups being made within each litter, so that the growth rate would be affected only by the fluoride and not by differences in nutrition that might result from the rats in each group being nursed by different mothers. The rats in each group were weighed daily and injected with 0.1 ml of liquid/10 g of body weight (NaF concentrations 0–0.12*M*).

In a fourth experiment, the rats in each litter were divided into two groups. At fourteen days of age, injections were begun; one group received 0.1 ml/10 g of body weight of 0.1 *M* NaF; the controls received the same amount of 0.1 *M* NaCl. After 2 weeks, the rats were killed and the costal cartilage was removed. A portion of the tissue was analyzed for calcium and the remainder was reserved for histologic examination.

Results. Increase of calcium content with age. Figure 1 shows that the costal cartilage contains very little calcium at birth and does not begin to increase its calcium content significantly until the young rat weighs about 35 g (at just over 2 weeks of age). At this time, calcification occurs at a rapid rate until a body weight of about 80 g is reached (about 4 weeks of age). Therefore, to determine the effect of fluoride administration, all of the rats used were between 2 and 4 weeks old.

Effects of Fuoride on growth, costal cartilage, and costal bone. Those rats receiving fluoride showed a somewhat depressed rate of weight gain (Fig. 2) and a depressed rate of calcification of costal cartilage (Fig. 3); these effects increased with the dosage of fluoride. The costal bone was already two-thirds calcified at birth and although it stored fluoride (Fig. 4), the calcification that occurred during the period of fluoride administration was not affected. Possibly the costal bone calcifies according to a different mechanism from the costal cartilage; also the fluoride may have been administered too late to have an effect on the calcification of the costal bone. At the dosages given, the

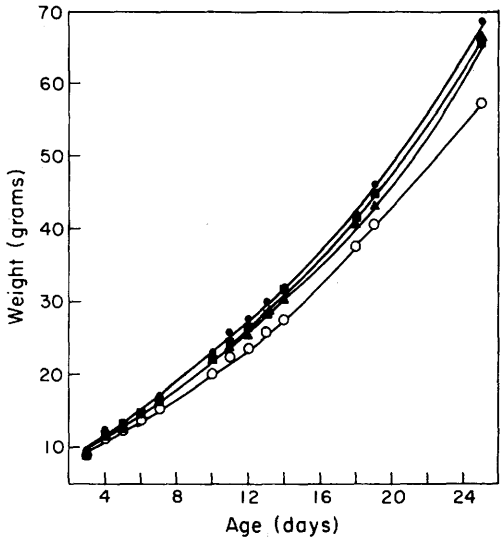

FIG. 2. Effect of NaF administration on the rate of weight gain in young albino rats. Daily injections of NaF in water (volume 0.1 ml/10g of body weight; concentration (●), 0.00 *M*; (■), 0.04 *M*; (▲), 0.08 *M*; (○), 0.12 *M*) were started at 3 days of age. At the beginning of the experiment, there were 63 young rats, but approximately 1/3 of these were sacrificed at 14, 19, and 25 days. Statistical analysis of these data showed that by 19 days of age, daily administration of 1.2 mmoles of NaF/kg of body weight lowered the average weight of the rats ($p < .05$).

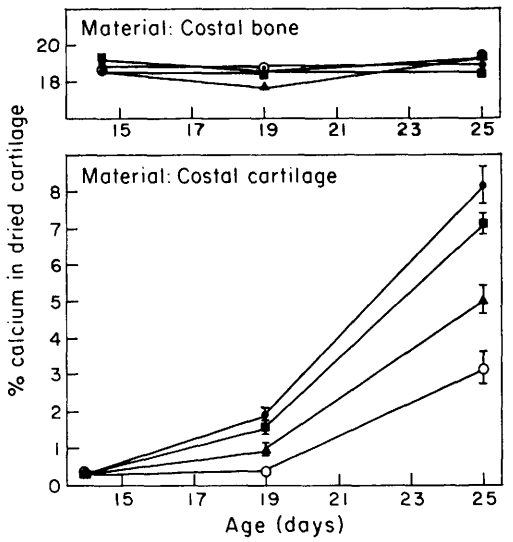

FIG. 3. Effect of NaF administration on the calcification of costal bone and cartilage of young albino rats. The rate of gain of body weight of these rats is shown in Fig. 2. Each point represents material pooled from 6 rats. Standard deviations for the percentage of calcium in the bone samples are smaller than ± 0.4% in all cases. For the cartilage samples, the standard deviations are indicated on the graph. Where no vertical line is shown, the standard deviations are less than ± 0.1%. The bone or cartilage was dried as described in the text. Daily dose of NaF (mmole/kg of body wt.) (●), 0.0; (■), 0.4; (▲), 0.8; (○), 1.2.

fluoride was not apparently toxic; the rats appeared normal and healthy. The neonatal death rate was low and equal in the various groups; after the neonatal period, deaths were very rare in all groups. The fluoride-treated rats were found to contain more fatty acids in their costal cartilage than the controls (Table I). Palmitic and oleic acids, which together accounted for over 60% of the total fatty acids, were slightly elevated ($p<0.1$) in the fluoride-treated rats. Examination of the stained tissues from the fourth experiment showed that in both the fluoride-treated and control rats, the cell structure is regular along the entire strip of costal cartilage and there is no evidence of ossification.

Discussion. This study shows that fluoride inhibits calcification of cartilage, rather than bone formation. This inhibition (Fig. 3, 4) was probably the cause of the slightly lower growth rate of the treated animals. As noted previously, the fluoride-treated rats appeared normal and healthy upon internal and external examination and the death rate was normal.

The purpose of this study was to examine the possibility of a relationship between lipids and glycolysis in the organic matrix of calcifying cartilage. There is evidence that both lipids (4, 11) and glycogen (2) accumulate in precalcifying tissue. If a linkage does exist between carbohydrate and lipid metabolism in calcifying cartilage, then one would expect that fluoride, an inhibitor in the breakdown of glycogen, might eventually inhibit the synthesis of fatty acids. The fact that more fatty acids were found in the lipids of cartilage of fluoride-treated rats suggests that the link between carbohydrate and lipid metabolism does not exist. There are several

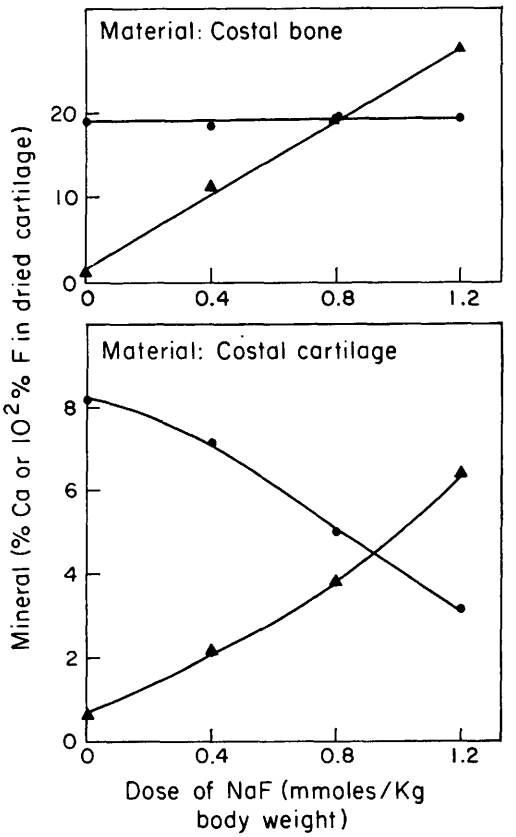

FIG. 4. Effect of increasing dosages of NaF on fluoride, (▲); and calcium (●) contents of costal bone and cartilage of rats at 25 days of age. Each point represents material pooled from 6 rats.

TABLE I. Fatty Acids in Lipids of Costal Cartilage of Rats 14–25 Days Old.[a]

Fatty acid	(mg of fatty acid/100 g of dried costal cartilage; mean ± SEM) Controls (5)[b]	Fluoride-treated (4)
Lauric	15 ± 13	13 ± 13
Myristic	116 ± 14	121 ± 41
Pentadecanoic	32 ± 3	16 ± 10
Palmitic	566 ± 41[c]	711 ± 53[c]
Palmitoleic	269 ± 32	258 ± 58
Stearic	212 ± 13	318 ± 82
Oleic	670 ± 50[d]	925 ± 85[d]
Linoleic	162 ± 25	123 ± 49
Total	2040 ± 140[c]	2490 ± 80[c]

[a] Note: Lipids were extracted from the cartilage and saponified. Total fatty acids were then determined by gas chromatography. The control rats were injected with water, the fluoride-treated rats were injected daily with 1.2–1.5 mmole of NaF/kg of body weight.

[b] Represents the number of cartilage samples analyzed. Each cartilage sample consisted of material pooled from 5–6 rats.

[c] $p < .05$.

[d] $p < .10$.

explanations for the results obtained. Travis (12) has suggested that the glycogen is drawn from lipid reserves—perhaps the inhibition of glycolysis by fluoride ion would result in an accumulation of lipid material. This is a reversal of the expected pathway in which lipids would be synthesized from carbohydrates. Another possibility is that under the influence of fluoride, breakdown of lipids might have been inhibited and this could account for the increased lipid content. Alternatively there might be equal quantities of lipid in the cartilage of fluoride-treated and untreated rats. Several authors, (3, 12, 13) have postulated a mechanism for calcification in which lipid acts as a carrier for calcium. This lipid–calcium complex may be bonded to positively charged sites on the protein–polysaccharide material in the organic matrix such as a complex between a sugar and magnesium. Fluoride could interfere with this process by competing for the lipid-binding sites. Thus, the calcification would be inhibited and the lipid would be more readily extractable by organic solvents. Although the present study demonstrates a possible linkage between the inhibition of calcification by fluoride ion and the lipid content of cartilage, the mechanism of this link must be clarified by future research.

Summary. Costal cartilage of untreated rats contained less than 1% calcium at birth. Calcium content remained constant for the first 2 weeks of life but during the third and fourth weeks it increased to 5–7% of the total dry weight. A microscopic examination of the costal cartilage of 4-week-old rats showed no evidence of bone formation. When sodium fluoride was administered daily to rats, starting soon after birth, the calcification of the costal cartilage was inhibited. The degree of inhibition was proportional to the dose of fluoride (maximum dose 1.5 mmoles/kg of body wt.). The cartilage of the fluoride-treated rats contained more total fatty acids than the cartilage of the untreated rats.

1. McLean, F. C. and Urist, M. R., *in* "Bone, An Introduction to the Physiology of Skeletal Tissue," (P. P. H. De Bruyn, ed.), p. 53. Univ. of Chicago Press, Chicago, Illinois (1955).

2. Dixon, T. F. and Perkins, H. R., *in* "The Biochemistry and Physiology of Bone," (G. H. Bourne, ed.), p. 300. Academic Press, New York (1956).

3. Fells, I. G., Nature **190**, 1012 (1961).

4. Irving, J. T. and Wuthier, R. E., Arch. Oral Biol. **5**, 323 (1961).

5. Cruess, R. L. and Clark I., Biochem. J. **96**, 262 (1965).

6. Folch, J., Lees, M., and Sloane Stanley, G. H., J. Biol. Chem. **226**, 497 (1957).

7. Böttcher, C. J. F., Woodford, F. P., Boelsma-Van Houte, E., and Van Gent, C. M., Rec. Trav. Chim. **78**, 794 (1959).

8. Radin, N. S., Hajra, A. K., and Akahori, Y., J. Lipid Res. **1**, 250 (1960).

9. Wharton, H. W., Anal. Chem. **34**, 1296 (1962).

10. MacIntyre, I., Biochem. J. **67**, 164 (1957).

11. Irving, J. T., Nature **183**, 1734 (1959).

12. Travis, D. F., *in* "Calcification in Biological Systems" (R. F. Sognnaes, ed.), p. 94. Am. Assoc. Advance. Sci. Washington D.C. (1960).

13. Howell, D. S., Arthritis Rheumat. **6**, 736 (1963).

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