Effects of Fluoride on Developing Bones in Tissue Culture. (19431)

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Among the significant contributions to the study of calcification in osteogenesis are two observations by Gutman and his colleagues (1,2): first, calcifying cartilage contains a phosphorylative glycogenolytic enzyme system which is capable of synthesizing potential substrates for bone phosphatase; second, if the synthesis of substrates is interfered with by sodium fluoride, calcification can be completely inhibited.

The first observation was based on a study of digests of minced epiphyseal cartilage; the second on a study of slices of epiphyseal cartilage incubated for a brief time in solutions of known chemical composition. Since the technics employed do not permit of tissue survival, the present work was undertaken to determine if NaF can inhibit the calcification process in growing bones without influencing the growth of tissue. An affirmative answer could be offered as additional evidence to disprove the current concept that glucose-1-phosphate is a substrate for bone phosphatase(3).

Methods. Metatarsals and fibulas from 9- to 10-day-old chick embryos were chosen for study because they can be grown *in vitro* with ease and because chemical analysis of embryos in this age range yield glycogen and the intermediate products of its phosphorylation(4). Pairs of corresponding bones were isolated and grown for 4 to 5 days upon the surface of clotted media consisting basically of equal parts of blood plasma and embryonic extract. One member of each pair of bones was grown on media containing NaF, the other member was grown on media free of NaF as a control. Before placing the isolated bones on the clotted media they were washed in embryonic extract which, in the case of those to be grown on fluoride, contained NaF at a concentration equal to twice that of the clotted media. Twelve pairs of bones were studied at each of the following concentrations of NaF: 1-20,000, 1-10,000, 1-5000, 1-1000, 1-500 and 1-200 M. Metatarsals were used for the 1-20,000 and 1-10,000 M experiments. For all others we used fibulas. Because of the possibility of undesirable osmotic effects the medium for the controls contained sufficient NaCl to compensate for the amount of NaF added to the medium for the experimental bones. The increase in length of the bones was determined from measurements of the overall lengths recorded at the start and at the end of the observation period. The amount of calcification was determined by measuring the length of the zones of mineralization visualized by staining the bones with alizarin red S at the end of the experiment(5). The amount of connective tissue growth was estimated by observing the extent of the fibroblastic halo. Finally, a record of the general appearance of each preparation was kept.

Results. Most of the pertinent data for 72 pairs of bones is summarized in Table I. Each value represents the average measurement for 12 bones. By comparing the various measurements of the bones grown on NaF with the measurements of the controls it can be seen that at concentrations of 1-20,000, 1-10,000, 1-5000 and 1-1000 M the NaF did not exert an effect upon either the increase in length of the bones or upon the degree of

TABLE I. See Text for Explanation.

Conc. of NaF	Overall length, mm Start	End	Zone of mineralization, mm
	Sodium fluoride		
1-20000 M	4.72	6.08	1.60
1-10000	4.29	5.75	1.60
1- 5000	3.83	4.51	1.38
1- 1000	3.67	4.24	1.25
1- 500	3.48	4.12	1.00
1- 200	3.47	3.98	.87
	Controls		
1-20000	4.75	6.01	1.60
1-10000	4.29	5.74	1.61
1- 5000	3.85	4.49	1.40
1- 1000	3.65	4.19	1.25
1- 500	3.48	4.14	1.30
1- 200	3.48	4.16	1.21

calcification, as determined by the length of the zone of mineralization. Only one difference was observed betwen the fluoride treated bones and their controls in these weaker concentrations. In many of the bones grown on fluoride the area of calcification was distinctly darker and more sharply defined than in the controls. This difference became apparent as early as the second day of the experiment and sometimes became more pronounced as time elapsed.

At 1-500 M NaF there was still no difference in the growth of the fluoride treated bones and their controls. The treated bones had grown 0.64 mm in length (3.48 to 4.12 mm) while the controls had grown 0.66 mm in length (3.48 to 4.14 mm). In contrast to this however, a marked difference in the degree of calcification between the fluoride treated and control bones appeared. Thus, the length of the zone of mineralization in the fluoride treated bones was only 1.00 mm while in the corresponding controls it was 1.30 mm.

At the highest concentration of NaF studied, namely 1-200 M the first indication of inhibition in growth was obtained. The fluoride treated bones grew only 0.51 mm in length (3.47 to 3.98 mm) while the controls showed a growth increase of 0.68 mm (3.48 to 4.16 mm). Accompanying this, there was a marked difference between the length of the zone of mineralization in the fluorine treated (0.87 mm) and control (1.21 mm) bones.

No obvious difference in the size of the fibroblastic halo was observed between the control and the fluoride treated bones until the concentration of NaF had reached 1-200 M. At this level the fluoride had a retarding influence on the development of the connective tissue halo.

Discussion. Before analyzing the results it should be understood that the metatarsals and fibulas of 9- to 10-day-old chick embryos are "bones" in the cartilage miniature stage. The "overall" length of the "bones" given in Table I are really a practical indication of the amount of cartilage which is present at the beginning and at the end of the experiment. The calcification which occurs involves only cartilage. The so-called zone of mineralization is the area of calcified cartilage which is visualized by staining with alizarin red S. In addition, metatarsal bones at this age are actually longer than are fibulas. This explains why the various measurements are greater at 1-20,000 and 1-10,000 M NaF than they are in the higher concentrations. Metatarsals were used at the two weakest dilutions.

The results themselves demonstrate that NaF, at a concentration which does not disturb growth, can seriously interfere with the calcification process. This confirms and extends the observations of previous workers (1,2,6) and emphasizes the importance of phosphorylative glycogenolysis in relationship to calcification. Sodium fluoride is known to inhibit enolase, the enzyme immediately involved in the production of phospho-pyruvate. Since calcification was stopped by the fluoride, the conclusion seems justified that phosphorylation of glycogen probably progresses to the phosphopyruvate stage before a suitable substrate for bone phosphatase is produced.

These findings are not in agreement with the current idea that glucose-1-phosphate is a substrate for bone phosphatase(3). Phosphorylase, the enzyme involved in the production of glucose-1-phosphate is not inhibited by NaF. In this connection we tried, without success, to interfere with calcification by using phloridzin and phloretin (known inhibitors of phosphorylase). Both compounds are practically insoluble in our media and are too toxic to permit of study in concentrations which could be expected to inhibit phosphorylase while not killing the bones.

Another possible factor in the stoppage of calcification by NaF must be considered. Since the fluoride might compete with the phosphate for calcium, perhaps fluoride acts by binding calcium. Thus, even concentrations of NaF which have no apparent effect upon growth or calcification cause a reaction within the zone of mineralization which renders it visibly different from the same zone in the controls. Finally it is probably significant that whereas Robison and Rosenheim (6) and Gutman, Warrick and Gutman(2) had been able to inhibit calcification in 1-

10,000 M NaF, we found it necessary to increase the fluoride concentration to 1-500 M. This 20-fold increase should be favorable to the binding of calcium by fluoride. The increase was necessary because the bones were grown on the surface of a clot. Between the bone and the clot, diffusion is the sole means of exchanging materials.

Summary. In embryonic bones growing in tissue culture the process of calcification of cartilage can be inhibited by NaF without influencing growth. To achieve this effect the concentration of fluoride had to be 20 times greater than that which will produce inhibition of calcification in test-tube experiments. The inhibition in the present experiments is probably due to at least two things: 1st, interference with the production of phospho-pyruvate as a substrate for bone phosphatase; 2nd, some degree of binding of calcium by fluoride.

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Use of a Chelating Agent for Accelerating Excretion of Radio-Iron (19432)

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(Introduced by J. H. Lawrence.)
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The ability of certain organic compounds to form strong non-ionic water-soluble combinations with metal ions suggests the possibility that these compounds might be useful for removing metal ions from the body. In this respect, Kety(1) has shown that use of citric acid may play a role in the removal of lead from bone by such a mechanism. Foreman and Hamilton(2) demonstrated that ethylenediamine tetra-acetic acid (EDTA) will chelate plutonium and yttrium *in vivo* and thereby greatly accelerate their excretion from the body.

This report deals with the application of this principle for hastening the excretion of radio-iron. Interest in accelerating excretion of iron is stimulated by the possibility of removing the excessive quantities of iron accumulated with hemochromatosis and transfusion hemosiderosis and for possible use as a tool for studying iron metabolism. "Fe-3 Specific"† is the compound chosen for this study. It has a very strong affinity for iron, particularly in the alkaline pH range(3). (It is used commonly for scavenging iron traces from solutions.) It was selected because it has, in addition, suitable characteristics for *in vivo* use, *i.e.,* it is relatively non-toxic, forms serum soluble chelates, is poorly catabolized, and is readily excreted through the kidney.

Methods. The effect of "Fe-3 Specific" on iron excretion was studied in a series of three experiments, using iron 59, *i.e.,* a) when iron 59 was given intravenously and "Fe-3 Specific administered intraperitoneally, b) when iron 59 was given intravenously and "Fe-3 Specific" by mouth, and c) when both iron 59 and "Fe-3 Specific" were given orally. In addition, the concentration of iron 59 in erythrocytes was studied. Adult male Slonaker

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† A commercial chelating agent manufactured by Bersworth Chemical Co., Framingham, Mass. Its structure is not revealed.