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Effects of Sodium Fluoride on Calcium Homeostasis.* (29127)

CHARLES YATES, STEPHEN DOTY, AND ROY V. TALMAGE
Rice University, Houston, Texas

Previous investigations in this laboratory have been directed toward defining the parameters of plasma calcium homeostasis(1,2). The data obtained support the hypothesis that plasma calcium is maintained by two processes. The first is a basic equilibration process which appears to be a continuous attempt to bring the calcium ion into equilibrium between bone and blood. The second is a hormonal process by which a continuously secreting parathyroid gland maintains the normally observed circulating calcium values above the basic level by influencing bone resorption. Osteoclastic activity has been correlated with this second process(3), though not proven necessary for hormonal action. These studies and conclusions are based on the prevention of a drop in plasma calcium below normal levels and are not necessarily related to the problem of the existence of a second parathyroid hormone, calcitonin, which has been postulated to be concerned with prevention of hypercalcemia(4).

Whether or not the solubility of the bone crystal could be affected *in vivo* was investigated by Talmage and Kraintz(2) using acidotic animals. Such treatment increased the amount of calcium removed by peritoneal lavage in the parathyroidectomized rats. In contrast, subsequent studies showed that administration of sodium fluoride by peritoneal lavage decreased the amount of calcium removed by this procedure(5). The investigations reported here are a continuation of the NaF studies and were carried out to clarify whether or not the decreased calcium levels observed in animals receiving sodium fluoride were a consequence of a reduction in solubility of the hydroxyapatite crystal of bone. Such an effect of fluoride on bone crystal has been suggested by others(6,7).

Materials and methods. A description of the technique of peritoneal lavage and of the constituents of the buffered, isotonic lavage fluid has been given(8). In the basic procedure, a calcium-free isotonic buffered solution was introduced into the peritoneal cavity and removed after a one-hour equilibration period through a stainless steel plug sewn into a midventral incision. This procedure was repeated hourly as desired. NaF, when used, was added to the lavage fluid in amounts (0.036 g/l or 0.072 g/l) so that each animal received either 0.5 mg or 1.0 mg F^- per hour.

Holtzman rats weighing 180 to 200 g were used throughout these studies. All surgery was performed under ether anesthesia. Nephrectomy, if done, and the insertion of the stainless steel plug were carried out 18 to 20

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hours before lavage; surgical removal of the parathyroids was performed 8 hours prior to lavage.

Radiocalcium, when used for bone studies, was injected through the plug 18 hours before lavage. About 20 μc of high specific activity Ca^{45} were administered to each animal. All samples were counted on a thin-window flow counter, and corrections were made for self-absorption. For the study of the effect of fluoride on calcium distribution in soft tissue the radiocalcium was added as a component of the lavage fluid. Tissues were removed, weighed, and dissolved in 2 ml of 70% nitric acid. The solutions were filtered and brought to a constant volume, and an aliquot was planchetted for counting.

Calcium determinations were carried out on a Beckman Flame Spectrophotometer with a reversed oxyacetylene flame. Protein was precipitated with trichloroacetic acid, and NaCl and $PO_4\equiv$ were added to equalize the concentration of interfering substances in standards and samples. Fluoride concentrations were estimated by using a Dowex 1-X8 exchange resin (200-400 mesh) and a colorimetric procedure involving the bleaching of an alizarin-zirconyl nitrate complex(9).

Serum low in calcium was prepared for bone incubation studies by adding 30 g of tertiary calcium phosphate per 100 ml of serum. This mixture was chilled to 3°C for 4 hours with repeated agitation and then centrifuged at 14,000 $\times$ *g* for 30 minutes. Animals were injected with radiocalcium as described, lavaged, and their femora removed. All extraneous tissue was removed from the femora which were then split lengthwise into 4 pieces with a scalpel. These were incubated in 4 ml of low calcium serum at 37°C with constant shaking. Every 15 minutes the atmosphere of 95% oxygen-5% carbon dioxide was changed keeping the pH of the solution at 7.6.

Histological preparations of the bone and quantitative osteoclast determinations were carried out as described by Talmage and Toft(3). Hydroxyproline determinations were made using a modification(10) of the Prockop-Udenfriend method(11).

Results. A. *Effect of fluoride on calcium removal by peritoneal lavage.* The rate of calcium removal by peritoneal lavage in parathyroidectomized rats was diminished by addition of fluoride to the lavage fluid. At the lower dose (0.036 g/l) calcium values were reduced by 26%. At the larger lose (0.072 g/l) the reduction in calcium values reached 32%. In contrast, the lower dose of fluoride did not result in a reduction in calcium removal rate during the 8-hour experimental period if the parathyroid glands were functional. Only when the larger dose was used did the calcium levels gradually fall in these parathyroid-intact animals. By the end of 8 hours of continuous peritoneal lavage, these values had fallen 20% (the data are summarized in Table I).

In an experiment to determine the recovery from administered fluoride, 4 hours of lavage with fluoride (0.072 g/l) were followed by 4 hours without fluoride. In this experiment, the characteristic decrease in calcium removal into the lavage fluid produced by fluoride administration was followed by a *gradual* return to the usual parathyroidectomized level (Table IC).

B. *Effect of fluoride on hydroxyproline levels of lavage fluid.* The effects of fluoride on levels of hydroxyproline removed by peritoneal lavage were similar to those observed on calcium removal rates (Table II). Hydroxyproline removal was reduced in all parathyroidectomized animals treated with fluoride. However, if the parathyroids were functional the larger dose of fluoride (0.072 g/l) was necessary to produce a drop in the hydroxyproline values.

C. *Effect of fluoride on osteoclast numbers.* The number of osteoclasts found in the metaphysis of the femur of the rat has been correlated with endogenous parathyroid activity (3). Both nephrectomy and the technique of peritoneal lavage with a calcium-free rinse have been shown to increase the osteoclast count(3), thereby indicating increased endogenous parathyroid secretion, whereas prior parathyroidectomy negates these increases. Administration of fluoride in the peritoneal rinse further increased the osteoclast count

TABLE I. Calcium Values in mg/100 ml.

	Hours of lavage							
	1	2	3	4	5	6	7	8
A. Intact parathyroids								
1. Controls	6.2 ± .09* (40)	5.8 ± .08 (39)	5.3 ± .08 (38)	5.5 ± .08 (40)	5.5 ± .08 (39)	5.7 ± .10 (38)	5.7 ± .08 (31)	5.6 ± .08 (28)
2. NaF (36 mg/l)	6.3 ± .17 (14)	5.9 ± .17 (17)	5.3 ± .12 (17)	5.7 ± .11 (17)	5.5 ± .12 (16)	5.6 ± .12 (16)	5.4 ± .11 (16)	5.5 ± .09 (16)
3. NaF (72 mg/l)	6.0 ± .08 (29)	5.4 ± .04 (30)	4.9 ± .09 (34)	4.9 ± .09 (34)	4.6 ± .11 (31)	4.4 ± .12 (34)	4.6 ± .14 (23)	4.5 ± .19 (24)
B. Parathyroidectomized animals								
4. Controls	4.2 ± .16 (32)	4.1 ± .13 (33)	4.0 ± .12 (31)	4.0 ± .09 (32)	4.0 ± .07 (33)	4.1 ± .02 (31)	4.2 ± .09 (27)	4.1 ± .07 (27)
5. NaF (36 mg/l)	3.1 ± .11 (21)	3.0 ± .12 (22)	2.8 ± .09 (21)	3.0 ± .10 (21)	3.0 ± .08 (21)	3.1 ± .11 (20)	3.1 ± .08 (21)	3.2 ± .10 (19)
6. NaF (72 mg/l)	3.4 ± .09 (35)	3.1 ± .09 (33)	2.8 ± .09 (32)	2.7 ± .09 (32)	2.7 ± .11 (28)	2.7 ± .12 (30)	2.7 ± .14 (17)	2.8 ± .11 (18)
C. Recovery from NaF (parathyroidectomized animals)								
7. NaF (72 mg/l)	3.4 ± .17 (9)	2.8 ± .13 (9)	2.5 ± .12 (9)	2.4 ± .16† (8)	2.7 ± .19 (9)	2.9 ± .17 (9)	3.3 ± .23 (9)	3.4 ± .22 (9)

* Mean $\pm \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

† Lavage rinse with fluoride deleted; normal buffered rinse used for remainder of lavage.
Number of animals in parentheses.

TABLE II. Parathyroid Related Changes Produced by NaF Lavage.

	Hydroxyproline*		Osteoclast count†		Radioactivity‡	
	Hours 1-4	Hours 5-8	Nephx	Intact	Specific activity (c/m/mg Ca)	Total (cts/min./ml)
A. Animals with intact parathyroids						
1. Controls	2.7 ± .07 (13)	2.8 ± .06 (13)	65.0 ± 2.1	50.5 ± 3.2	1000	1000
2. NaF (36 mg/l)	2.6 ± .09 (13)	2.8 ± .05 (13)	69.0 ± 3.0	55.7 ± 2.3	—	—
3. NaF (72 mg/l)	3.1 ± .08 (13)	2.4 ± .08 (13)	77.4 ± 2.3	72.5 ± 3.3	1048	862
B. Parathyroidectomized animals						
4. Controls	1.9 ± .08 (13)	1.6 ± .08 (13)	38.5 ± 1.4	37.0 ± .7	1436	962
5. NaF (36 mg/l)	2.0 ± .10 (13)	1.1 ± .09 (13)	38.3 ± 1.1	36.5 ± 1.8	—	—
6. NaF (72 mg/l)	2.4 ± .10 (13)	1.4 ± .13 (13)	40.5 ± 2.1	38.1 ± .5	1258	588

* In μmoles $\times 10^{-2}$/ml lavage wash.
† Cells/2.6 mm^2 metaphysis of femur.
‡ Ca^{45} removed by lavage-controls normalized to 1000 for comparative purposes.

in the metaphysis of the femur and while this increase was marginal at the lower dose, the increase was highly significant when concentration of fluoride was increased to 0.072 g/l (Table II). The fact that this phenomenon was related to parathyroid function was demonstrated by the failure to produce these increases in the parathyroidectomized animals.

D. *Soft tissue distribution of radiocalcium following fluoride administration.* Radiocalcium (approximately 1 μc/animal/hr) was added to the lavage rinse concurrently with fluoride. At the end of 8 hours of peritoneal lavage, the femora, seminal vesicles, kidney, liver, a portion of the gut, and a sample of the musculature of the body wall were removed and the radioactivity determined. Although most of the radioactivity was found in the femur, there were no differences in the distribution of radiocalcium between NaF treated animals and their controls (parathyroidectomized and parathyroid-intact), indicating that the reduced calcium removal resulting from fluoride administration could not be attributed to extraosseous precipitation of calcium fluoride. The specific activity of the administered radiocalcium was sufficiently high (727 μc/mg) that the total amount of stable calcium added with the radioactivity was negligible.

Of the fluoride administered with the lavage fluid, 15% remained in the wash removed at the end of each one-hour equilibration period. The amount of fluoride found in the femur following an 8-hour NaF lavage containing 0.072 g/l NaF was approximately 0.5 mg F^-/g of bone. No differences could be detected in this concentration between control and parathyroidectomized animals.

E. *Effect of fluoride on removal of previously injected radiocalcium.* In some of the experiments, radiocalcium (20 μc) was administered to the rats 18 hours prior to onset of the lavage procedure. Such experiments have been reported previously(2) which demonstrated that the removal rate of the radioactivity in the lavage fluid was not affected by parathyroidectomy unless the Ca^{45} was administered 3 or more days before lavage. However, since the stable calcium values fell, the specific activity of the removed Ca^{45} in the lavage wash was increased in the parathyroidectomized rats. The influence of added fluoride is summarized in Table II. NaF at the larger dose caused a 14% reduction in Ca^{45} removal in animals with intact parathyroids which was increased to a 41% reduction in parathyroidectomized animals. The percentage decrease in rate of radiocalcium removal from bone in NaF treated animals

TABLE III. Radioactivity in Serum from Bone Incubation (c/m/ml).

Incubation time	Buffered rinse			NaF rinse (0.072 g/l)		
	Parathyroid-ectomized	Control	Combined	Parathyroid-ectomized	Control	Combined
30 min.	174 ± 16	206 ± 12	190 ± 11	147 ± 12	161 ± 13	154 ± 8
1 hr	236 ± 12	284 ± 23	260 ± 15	186 ± 18	209 ± 1	198 ± 9
2½ "	549 ± 30	634 ± 29	592 ± 25	428 ± 41	465 ± 24	447 ± 23
	Total calcium (mg %)					
3½ "	5.3 ± .04	5.7 ± .06	5.5 ± .09	4.5 ± .15	4.9 ± .12	4.7 ± .12

Serum before incubation = 3.3 mg %. 4 animals per group.

was of the same magnitude as that for total calcium, indicating that fluoride incorporation in bone was in the same general areas as that of the radiocalcium injected 18 hours previously.

F. *Effect of fluoride on removal of stable and radioactive calcium from bone incubated in plasma.* In these studies, radiocalcium was administered 18 hours prior to lavage, and, subsequent to the lavage, bones from these rats were prepared for incubation in plasma. The incubation was carried out for 4 hours with aliquots of plasma being removed at intervals for stable and radiocalcium analysis. The data are summarized in Table III. Values for both stable and radiocalcium of plasma incubated with bone containing fluoride were significantly lower than the control values. Differences between bones from parathyroidectomized and parathyroid-intact rats are questionable.

Discussion. The data presented here strongly suggest (1) that the effect of fluoride on calcium removal rates by peritoneal lavage results from a decreased solubility of the bone salt after incorporation of the F ion and (2) that this decrease in solubility affects the equilibration of calcium ions between bone and blood (the basic level) and, thereby, indirectly stimulates endogenous parathyroid secretion.

In a previous publication(5), the possibility was considered that the effects noted might be due to metabolic poisoning caused by fluoride. Experimental evidence was presented against this hypothesis based on the failure to find differences in oxygen consumption of tissue between the experimental groups. This evidence is strengthened by this report in which similar results were produced by NaF at a reduced level which is considered to be well below a concentration which would cause metabolic poisoning(12).

Since CaF_2 is known to be a relatively insoluble salt, the possibility was considered that the effects noted might be due to soft tissue precipitation of this salt. This was ruled out when the Ca^{45} administered with the F^- failed to show any increased concentration in any of the tissues studied as compared to rats not receiving fluoride. This would have occurred if the fluoride had precipitated the stable calcium in these tissues. As further evidence against this possibility, experiments were carried out to check the recovery after discontinuance of NaF administration. If extraosseous precipitation had occurred, one would expect an immediate return to normal calcium levels when rinse not containing NaF was administered to sodium fluoride treated animals (Table IC). The return to normal levels, however, was gradual.

In contrast, there is additional evidence to support the concept that the effects noted were caused by an interaction of the fluoride with the surface of the hydroxyapatite crystal complex(13). The most convincing data include the changes noted in plasma calcium concentration after incubation with bone slices. Values were significantly lower in those bones containing fluoride. Lower Ca^{45} plasma levels were observed even though the NaF treated rats had released less radiocalcium into the lavage fluid (Table II) and thus should have had more radioactivity in their femora.

The difference in the effect of fluoride on control and parathyroidectomized rats is explained by our previously reported concept of the calcium replacement mechanism of

parathyroid function(1). The effect of fluoride interaction with bone is primarily in those areas of bone which function to maintain a basic equilibrium between bone and fluid (14). A reduction of this basic level results in a compensatory increase in endogenous parathyroid secretion as indicated by the increased osteoclast counts. If the fluoride concentration of bone is increased sufficiently, the resultant drop in the basic equilibration level is sufficient to produce a secondary effect on the parathyroid-intact animal. This conclusion is further supported by the changes produced by fluoride in removal of hydroxyproline by peritoneal lavage. These changes parallel the calcium changes exactly, indicating that the matrix breakdown, which appears always to accompany the removal of calcium, is similarly affected by the fluoride.

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Antacid and Antiulcer Properties of the Polysaccharide Chitosan in the Rat. (29128)

IRA W. HILLYARD, JOHN DOCZI AND PAUL B. KIERNAN*
(Introduced by Martin M. Winbury)
Departments of Pharmacology and Biochemistry, Warner-Lambert Research Institute, Morris Plains, N. J.

Purified polysaccharides have been evaluated for efficacy in prevention and treatment of peptic ulceration because of their chemical and physical similarity to the mucopolysaccharides of gastric mucin(1). The most efficient (antiulcer) polysaccharide reported thus far has been a sulfated galactosan obtained from sea weed (carrageenin). Chitosan is a glucosamine polymer (poly 2-amino-2-desoxy-β-D glucose) obtained from the chitin of lobsters and crabs. Purified chitosan contains from 7 to 8% nitrogen in the form of titratable primary amino groups. This free amino group content will bind an equivalent amount of acid. *In vitro,* approximately 6 mEq of HCl are bound by 1 g of the compound. The acid binding capacity in combination with the physical properties of a mucopolysaccharide, prompted our evaluation of this material for antiulcer potency. Chitosan however, lacking sulfuric acid ester groups, did not have the antiproteolytic activity inherent in carrageenin(2,3,4,5). Chitosan sulfate was very effective in this respect, but possessed anticoagulant properties. We thus investigated a preparation obtained by coprecipitation of chitosan and Aluminum Hydroxide (Chitosan-AlOH), since it has been shown that aluminates are also significant inhibitors of peptic activity(6,7). This

* Present address: Dept. of Pharmacology, Boston Univ. School of Medicine, Boston.