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# FLUORIDE

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ABSTRACTS



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## EDITORIAL

## FLUORIDE AND OSTEOPOROSIS

In 1949 Black, Kleiner and Bolker (1) administered 80 mg of NaF 4 times daily to cancer patients and to children with leukemia and cancer, for the purpose of demonstrating the "apparent lack of toxicity" of relatively large doses of fluoride over prolonged periods of time. Although the authors reported considerable symptomatic relief they were obliged to administer amphogel, an aluminum compound to counteract toxic symptoms involving mainly the gastrointestinal tract. In 1960, Leone et al. (2) compared X-ray findings on osteoporosis in natural fluoride areas of Bartlett (8 ppm) and Cameron (0.4 ppm) Texas with those in Framingham, Massachusetts (0.04 ppm). They concluded that the large number of cases of osteoporosis observed in Framingham with practically no fluoride in drinking water might be associated with the prolonged use of water containing "insufficient" fluoride. In this statistical study the authors did not consider such variants as the presence in drinking water of bone-forming minerals other than fluoride. They made no attempt to evaluate fluoride intake through food and drugs and through polluted air, nor to weigh the impact of other causes of osteoporosis.

Treatment with massive doses of fluoride was resumed in 1961 by Rich and Ensinck (3). They administered 50 to 150 mg NaF (1 mg per kg body weight) to six patients with osteoporosis and to one with Paget's disease, for 5 to 75 weeks. In almost all cases a drop in fecal and urinary output of calcium occurred within 6 to 8 weeks, indicative of a positive calcium balance.

Numerous articles have appeared since with varying results: At the Harvard Medical School, de Deuxchaisnes and Krane (4) administered 66 mg NaF (30 mg F) to five patients with Paget's disease. Three reported subjective improvement, especially relief of bone pain; two experienced epigastric discomfort and severe back pain.

In 1965, Reuter and Siebermann administered 37 to 100 mg to 31 persons with osteoporosis for a period of 21 days to more than 3 years (5). Analysis of the new bone showed distinctly less calcification than original bone. In one of their patients with osteoporosis, the treatment produced osteomalacia. After 40 weeks of treatment, the authors noted a seventeenfold increase in osteoblastic activity, but also a marked retardation of osteoid mineralization.

Statistical studies by Bernstein, et al. in 1966 (6) seemed to corroborate the theory that fluoride is beneficial in osteoporosis. Yet, Nordin (7) Head of the British Medical Research Council's Metabolism Unit in Leeds pointed to the many fallacies inherent to such statistics. In comparing unselected postmortem iliac crests from Hartlepool (high in natural fluoride i.e. 1.9 ppm) with those from Leeds with virtually no fluoride ($< 0.5$ ppm) (8) the British M.R.C. found a considerable difference in bone fluoride levels between the two

towns but no statistically significant difference in numbers or degree of osteoporosis. Elements other than fluoride in the water supplies and fluoride intake through sources other than water, which the authors disregarded, play an important role. Furthermore, doubt has been cast upon the proper selection of cities which were designated by Bernstein et al. as using high and low fluoride waters. For instance, Grafton, was classified in the category of "low fluoride" (0.1 to 0.3 ppm), but water from one well had contained 3.5 ppm (9); at a subsequent analysis, pooled water from 4 wells revealed 2.8 ppm (10). In 1959, however, according to the U.S. Public Health Service (11) Grafton's water contained 0.9 ppm. In the towns designated "high in fluoride" (4 to 4.5 ppm) the fluoride content of the wells actually ranged from 1.2 to 2.2 ppm (11).

What appears to be one of the most extensive studies concerned with decalcifying bone disease, a double blind survey (12) involving 150 patients with multiple myeloma, was undertaken by a group of 29 clinicians to determine whether or not sodium fluoride therapy could possibly influence the clinical course of this malignant bone disease. Survival in the three treatment groups was practically identical in the NaF treated and in the untreated patients. Neither beneifical nor harmful effects were demonstrated during an observation period of 53 to 70 months. Side effects occurred with almost equal frequency both in the experimental groups and in the controls.

Considerable light was shed on the subject by Hendrickson et al. (13) who induced osteoporosis by feeding a low calcium, high-protein diet for 42 weeks to beagle dogs. Thereupon they added fluoride supplements at levels which corresponded to 1.8, 6.0, 20.6, 68.2 mg/day in the diet of a human of average weight. Bone radiography, specific gravity, bending and tension tests, and ash-per-volume revealed no effect of fluoride on the degree of osteoporosis. However, they reported a significant decrease in mineral mass with increased dietary fluoride. They confirmed that the newly formed osteoid tissue is poorly mineralized, a fact which may lead to the development of osteomalacia.

The question of hyperparathyroidsim resulting from fluoride therapy was raised by Faccini (14). In young experimental rabbits and sheep who received 200 ppm of NaF in drinking water for 4 to 8 weeks, overactivity of the parathyroid gland was noted (14). The level of circulating parathyroid hormone was 5 times higher than resting and control levels. Secondary hyperparathyroidism might thus lead to increased bone resorption. Interestingly in growing rabbits, fluoride reduced the resorption of bone which contained it with a resultant increase in the resorption of normal nonfluoride-containing bone (15). In fluoride-fed kittens, the accelerated bone resorption was prevented (16) when the diet was supplemented by a high intake of calcium, and the newly-formed bone was morphologically normal.

Thus it appears that both osteomalacia and secondary hyperparathyroidism encountered in previous studies were due to a combination of fluoride

and a low calcium intake: In other words, the calcium was insufficient to mineralize the newly formed bone tissue. For this reason fluoride, to be effective with respect to minerlization of bone substance, must be associated with other minerals especially calcium and vitamin D (17).

The question of long-term systemic poisoning from large amounts of fluoride, however, has not been resolved. Hyperparathyroidism and osteomalacia are not the only untoward side effects. During five years' experience with this treatment Rich (18) encountered arthritis, gastric and intestinal disorders. In 1964, a case of irreversible blindness due to optic neuritis in one eye had been recorded (19) presumably due to treatment of osteoporosis with massive doses of fluoride. Previously a warning concerning the possible toxicity of large doses of fluoride had been issued by Shapiro, of the National Institute of Arthritis and Metabolic Diseases (20) who pointed to the experimental nature of the treatment and to many unanswered questions. Duffey et al. (21) observed unusual giant cells in the bone marrow in three patients who received 16 to 150 mg of sodium fluoride daily during periods of 1 to 36 months for treatment of osteoporosis. These cells are suggestive of mutagenic or carcinogenic activity of the fluorine ion.

It appears therefore that fluoride treatment of osteoporosis and other demineralizing bone diseases should be viewed with skepticism because of its questionable efficacy and the possibility of serious side effects.

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# FLUORIDE POLLUTION IN MONTANA

by

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SUMMARY: In 1970 an intensive study of the effect of airborne fluorides on vegetation was initiated in the vicinity of an aluminum production plant in northwestern Montana. Fluorides greater than control levels (10 ppm) were found in vegetation over a region of 214,000 acres and visual fluoride injury to conifers occurred in an area comprising 69,000 acres. Histological reactions characteristic of elevated fluoride levels occurred in conifer needle tissue, including hypertrophy of parenchymatous tissue. Forest insects were found to accumulate fluorides. Analyses of predaceous insects for fluoride indicated that fluorides are likely to be carried through the food chain.

Even though the company reduced fluoride emissions by 67 percent between 1970 and 1971, data collected in 1971 indicated that vegetation in Glacier National Park, 7 air miles distant from the source, was still accumulating abnormal amounts of fluoride. This paper is a summary of a detailed report (1) released in 1970.

## Introduction

The Anaconda Aluminum Company, located in northwestern Montana near the City of Columbia Falls, began operation in 1955. Initially the physical facility had only two potlines, but in 1968 it was expanded to five. Following the expansions, we noted necrotic foliage on vegetation over a large area, including trees and shrubs in Columbia Falls and on Teakettle, Mountain, east of the aluminum company.

According to a company report the smelter was emitting 7,500 pounds of fluoride per day in early 1970; by August 1970 emissions were reduced to 5,000 pounds per day, and by May 1971 they were down to 2,500 pounds per day.

It was obvious that a detailed evaluation was necessary to accurately assess the problem. It has been documented (2, 3, 4) that fluoride fumes emitted as waste from aluminum plants causes injury to vegetation proximal to those sources. A study was designed to determine whether or not emissions from the smelter accounted for the above-mentioned injury.

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From the United States Forest Service, Missoula, Montana.

Specifically, our objectives were:

1. to identify the most probable cause of plant injury in forested lands near the aluminum company,

2. to identify the causal source,

3. to determine the area affected,

4. to determine whether or not insects accumulate fluorides.

## Materials and Methods - 1970 Studies

At 77 locations in the vicinity of the aluminum factory, plants were collected during June and July and again during September and October 1970. The collection plots were established on radial lines originating at the company and extending into surrounding forested areas (Fig. 1). Some plots occurred in Glacier National Park. Representatives of shrubs, conifers, forbs, and grasses on each plot were collected. Based on preliminary information we felt justified in stratifying and grouping data based on vegetation type. We did not detect any real differences in fluoride accumulations per unit time between species within these groups. Therefore several different species were sampled within any particular vegetation type and these species varied from plot to plot. Control plants were collected from six plots upwind of and remote to the aluminum plant.

All vegetation samples were brought to our laboratory in Missoula for analysis. Foliage was stripped from branches and sorted by year of origin, 1969 or 1970. One hundred needles were then drawn from each sort and observed for visible fluoride injury. The ratio P of injured to healthy was recorded. Next, the length of necrotic tissue and total length of the needle was measured on 10 affected needles, providing they were available. The ratio R of length of injury to total length of needle was recorded. The product PR is an estimate of the proportion of a given year's foliage visually injured by fluorides and was called Injury Index. A sub-sample of this was sent to the Wisconsin Alumni Research Foundation for analysis of available fluoride (unwashed, included particulate and gaseous). Values were given in ppm fluoride (parts per million) on a dry weight basis.

Sixty conifer needles deemed "burned" by fluorides were sectioned at 9 microns and examined histologically for evidence of internal tissue damage. Two mm. sections from the "transition zone" (that portion between the green and burned tissue) were extracted and sectioned through routine paraffin methods.

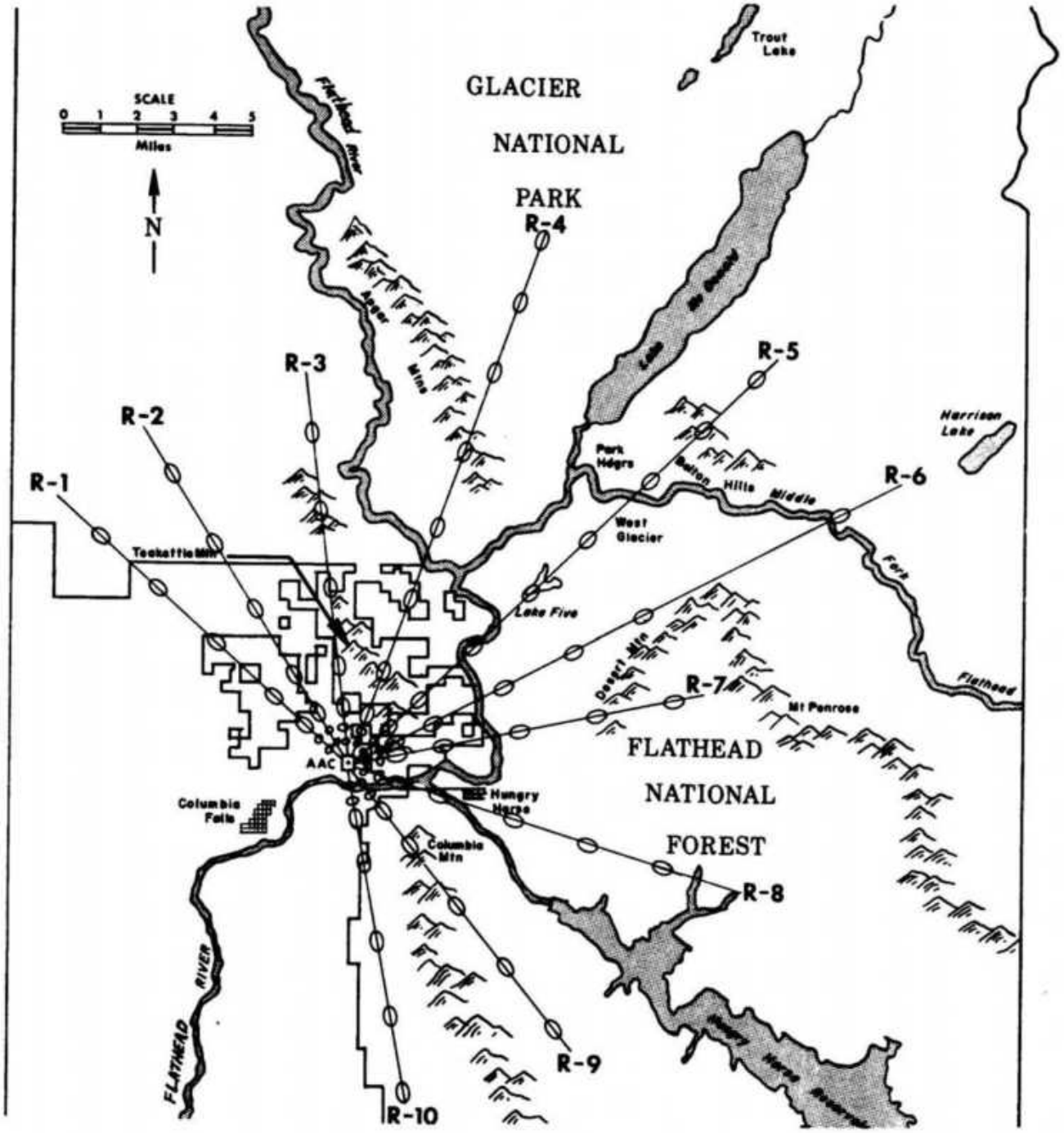
Collections of forest insects were made in June, August, and October of 1970. They were collected only within one-half mile of the aluminum company, without reference to the vegetational plots. The species included were foliage feeders, cambial feeders, pollinators, and predators. All insects were sent to Wisoonsin Alumni Research Foundation (W. A. R. F.) for chemical analysis.

## 1971 Studies

During August of 1971, 15 of the established plots were resampled to

Fig. 1

Fluoride Study Area




AAC = Anaconda Aluminum Company. Note sampling radii and plot locations (ovals).

monitor for continuing fluoride injury. Vegetation and insects were collected in the same manner as in 1970. A specific ion fluoride electrode in our laboratory was used to analyze for fluoride content.

## Results - 1970 Studies

Because the June-July and September-October collections yielded very similar results (Table 1a and 1b), only the September-October data is used here. Statistical analysis of control data showed the average fluoride concentration in all types of forest vegetation in the area to be between 6 and 10 ppm, regardless of age. The injury index of control samples was found to be less than 0.006. Thus 10 ppm of fluoride was established and injury index (I. I.) of 0.006 as standard control values.

Based on about 650 different chemical analyses, extremely high fluoride concentrations averaging from 400 to 600 ppm were found in all vegetation close to the aluminum company, whereas in distant plots the levels were low ranging from 10 to 20 ppm. The injury to 1969 conifer needles was significantly (95 percent level) correlated ($r = .2805$) to fluoride concentration. Ninety-six percent of 237 samples showing fluoride-type injury had fluoride concentrations greater than 10 ppm.

By interpreting the plot data we were able to develop a general map of the pollution extent in the area (Fig. 2). On each radius we determined the distance at which the following average fluoride concentrations in vegetation occurred*: 10, 15, 20, 30, 60, 100, 300, and 600 ppm. Similar concentrations were then connected by lines, which were termed "isopols." Thus, for example, the 20 isopol indicates the area in which vegetation contains 20 ppm fluoride or greater.

Table 2 shows the acreages included by each isopol. Vegetation on about 214,000 acres of forested land could be considered polluted by fluorides, approximately 72,000 acres of which was in Glacier National Park. Generally, fluoride-type injury to conifers was found within the 30 isopol. This included about 69,000 acres total, 9,600 of which were in Glacier National Park.

Within conifer tissue showing injury by fluorides a distinct syndrome was noted which was associated with excessive foliar concentrations of fluorides (Fig. 3). Phloem and transfusion parenchyma as well as albuminous cells had hypertrophied extensively, crushing and causing collapse of transfusion tracheids and phloem elements.

Enlarged nuclei were associated with the hypertrophied cells, and chlorophyll destruction was extensive. A normal needle is shown in Figure 4. Our

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*This average included analyses from all types of vegetation sampled and is considered valid because the same vegetation types were sampled from plot to plot.

TABLE 1a

Tabulation of Radial and Control Data - First Sampling

| Plot # | Average Fluoride Content (ppm) Shrubs | Conifers 1969 | Conifers 1970 | Herbs | Grasses | Grand Average | Injury Index Average I. I. | Injury Index High I. I. |
|---|---|---|---|---|---|---|---|---|
| Control #1-6 | 4.77 - 11.4** | 3.5 - 10 | 3 - 11 | 5 - 12 | 1.3 - 16 | 4.79 - 10.36 | 0 - .007 | 0 - .014 |
| R1-P1 - 7* | 3 - 108.5 | 5.8 - 300 | 9 - 40.8 | 12.5 - 188 | 2.5 - 70 | 6.8 - 122.36 | 0 - .305 | 0 - .442 |
| R2-P1 - 7 | 3.6 - 112.7 | 5.5 - 143.5 | 2.3 - 20 | 5.5 - 93.8 | 2.5 - 83.3 | 3.7 - 91.21 | 0 - .196 | 0 - .528 |
| R3-P1 - 7 | 10 - 1166.6 | 7 - 637 | 8 - 229 | 3.3 - 875.5 | 2.1 - 775 | 8.2 - 1004.3 | 0 - .136 | 0 - .334 |
| R4-P1 - 10 | 8 - 778 | 8.93 - 681.5 | 4 - 116.5 | 5.7 - 628 | 5.8 - 234 | 6.88 - 604.14 | 0 - .150 | 0 - .5 |
| R5-P1 - 10 | 11.05 - 1719 | 10.1 - 341 | 4.1 - 68.6 | 8.28 - 1038 | 5.5 - 600 | 7.46 - 1181.5 | 0 - .228 | 0 - .58 |
| R6-P1 - 10 | 7.5 - 1125.3 | 13.5 - 1950 | 6 - 33 | 11 - 431 | 24.5 - 581 | 11.75 - 877.6 | 0 - .202 | 0 - .442 |
| R7-P1 - 7 | 4.8 - 1073 | 10 - 168 | 4.5 - 22.3 | 7 - 600 | 20.5 - 338 | 10.87 - 871.7 | 0 - .118 | 0 - .299 |
| R8-P1 - 7 | 11.8 - 399.8 | 14.2 - 119.8 | 9.2 - 175 | 13.3 - 235 | 8 - 110 | 12.6 - 409.8 | 0 - .176 | 0 - .4 |
| R9-P1 - 7 | 5.6 - 108.7 | 7.8 - 110 | 10 - 39.5 | 6.5 - 51.5 | 5 - 41 | 7.72 - 70.97 | 0 - .026 | 0 - .026 |
| R10-P1 - 7 | 6.8 - 76.5 | 9.2 - 133 | 3.5 - 42.5 | 10 - 45 | 6.5 - 38.5 | 7.54 - 66.3 | 0 - .070 | 0 - .097 |

* R=Radius P=Plot

** Fluoride content, ppm, dry weight basis. These values represent the range of fluoride concentrations found in all plots on the radius. Lower values were associated with plots distal from the fluoride source.

TABLE 1b

Tabulation of Radial and Control Data - Second Sampling

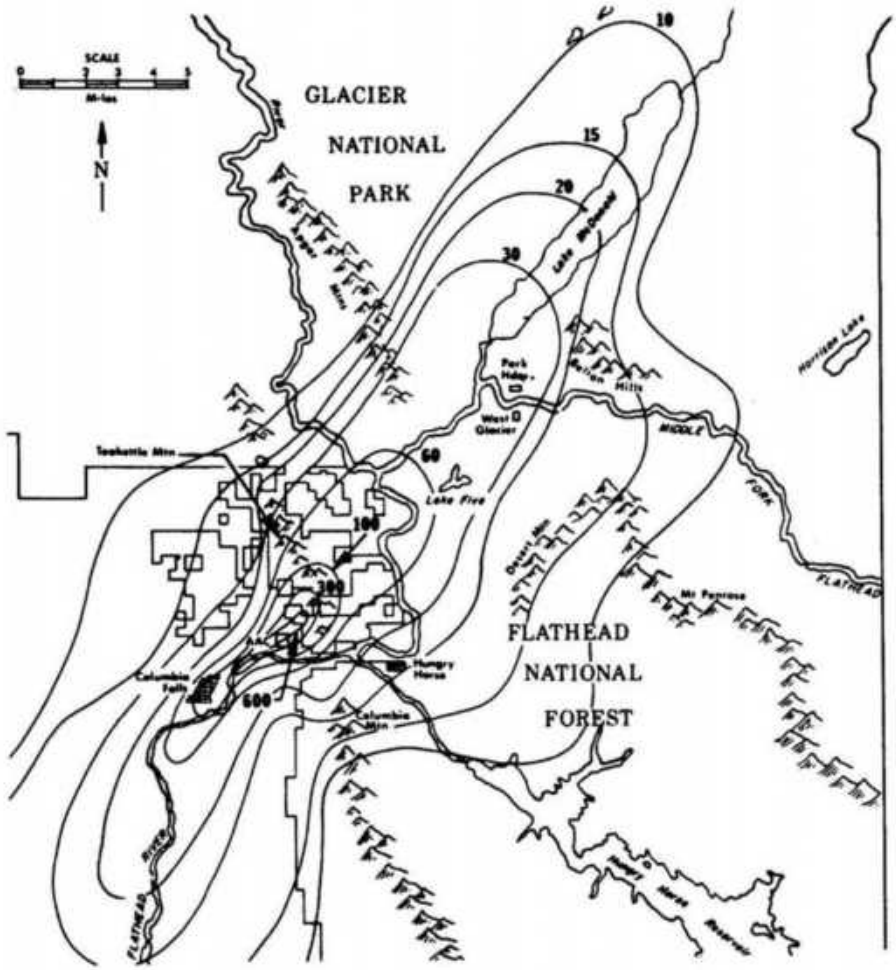
| | Average Fluoride Content (ppm) | | | | | | Injury Index | |
|---|---|---|---|---|---|---|---|---|
| | | Conifers | | | | Grand | Average | High |
| Plot # | Shrubs | 1969 | 1970 | Herbs | Grasses | Average | I. I. | I. I. |
| Control #1-6 | 5.7 - 15.8** | 5.9 - 11.3 | 4.5 - 5.8 | 6.5 - 17 | 5.5 - 17 | 6.46 - 9.74 | 0 - 0 | 0 - 0 |
| R1-P1 - 7* | 9 - 323 | 4.5 - 338 | 5.5 - 115 | 32.0 - 310 | 5 - 139 | 5.9 - 258 | 0 - .079 | 0 - .143 |
| R2-P1 - 7 | 9 - 147.5 | 8.5 - 189 | 5.5 - 64.7 | 8.8 - 146 | 4.5 - 93.5 | 7.84 - 111 | 0 - .211 | 0 - .279 |
| R3-P1 - 7 | 7.3 - 1194 | 11.8 - 496.5 | 7.5 - 367.8 | 5.5 - 794 | 4.5 - 600 | 8.2 - 754.7 | 0 - .313 | 0 - .628 |
| R4-P1 - 10 | 10.5 - 1244 | 6.2 - 390.3 | 6.1 - 123.2 | 5.5 - 1250 | 4.3 - 469 | 6.3 - 903.2 | 0 - .208 | 0 - .495 |
| R5-P2 - 10 | 12.8 - 1300 | 11.9 - 537.5 | 10 - 80.3 | 9 - 875 | 15.5 - 508 | 11.6 - 918.7 | 0 - .132 | 0 - .25 |
| R6-P1 - 10 | 14.8 - 1889 | 18.5 - 1728 | 9.3 - 775 | 14 - 3000 | 21 - 488 | 17.2 - 1831 | 0 - .182 | 0 - .291 |
| R7-P1 - 7 | 23 - 1194 | 14 - 1825 | 10.2 - 413 | 17.2 - 700 | 7.5 - 375 | 14.3 - 1120 | .002 - .289 | .002 - .567 |
| R8-P1 - 7 | 16.2 - 812.5 | 14 - 906 | 8.5 - 306 | 14.5 - 750 | 12.5 - 131 | 12.7 - 619.7 | 0 - .042 | 0 - .067 |
| R9-P1 - 7 | 9 - 250.5 | 4 - 168 | 4.27 - 76 | 8.5 - 198 | 5.5 - 132 | 5.79 - 171.7 | 0 - 0 | 0 - 0 |
| R10-P1 - 7 | 9.5 - 185.5 | 4.7 - 140 | 4.4 - 62 | 7 - 200 | 8 - 76 | 5.74 - 141.5 | 0 - .03 | 0 - .03 |

*R=Radius P=Plot

** Fluoride content, ppm, dry weight basis

Fig. 2

Isopols of Fluoride Pollution at Columbia Falls, Montana




69,120 acres are included within the 30 isopol.

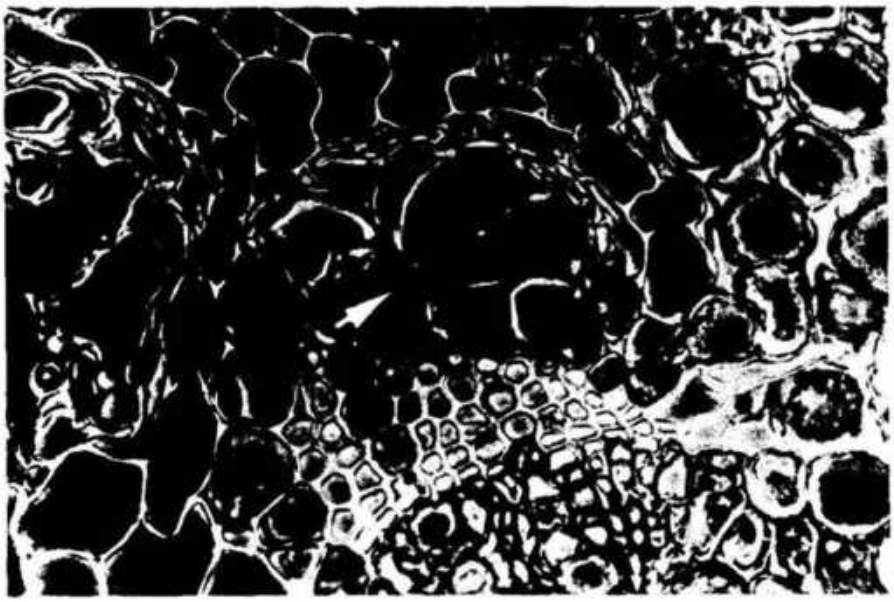
TABLE 2

Area Polluted by Fluorides

| Isopol | All lands Acres within | Glacier Park Acres within | Isopol | All lands Acres within | Glacier Park Acres within |
|---|---|---|---|---|---|
| 10 | 213,760 | 71,670 | 60 | 19,840 | 371 |
| 15 | 174,080 | 46,080 | 100 | 7,040 | - |
| 20 | 122,240 | 20,480 | 300 | 1,280 | - |
| 30 | 69,120 | 9,600 | 600 | 640 | - |

Fig. 3

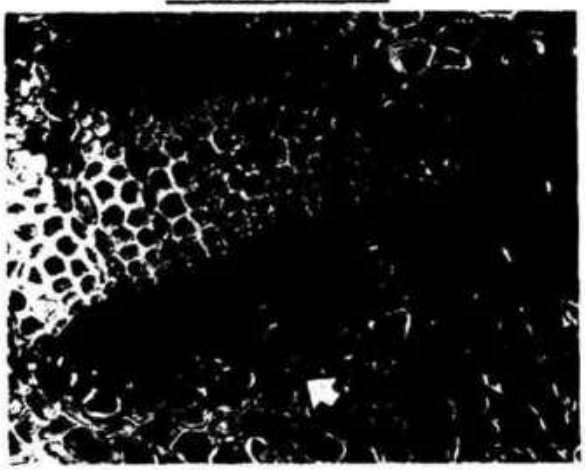
Histology of Fluoride Injury in Pine Needle



Hypertrophied transfusion parenchyma ( ↑ ) crushing and causing collapse of adjacent transfusion tracheids. This disease syndrome in conifer needles was associated with excessive foliar concentrations of fluorides. Lodgepole pine. X350.

Fig. 4

Normal Control



Transfusion parenchyma ( ↑ ) not hypertrophied. Lodgepole pine X300

TABLE 3

Fluoride Accumulation in Insects

| Insect | Date Collected | PPM* Fluoride |
|---|---|---|
| **Pollinators:** | | |
| Bumblebee - Bombus sp. | 8/12/70 | 406.0 |
| Bumblebee - Bombus sp. | 6/1/70 | 194.0 |
| Sphinx moth - Hemaris sp. | 6/1/70 | 394.0 |
| Honey bee - Apis mellifera | 6/1/70 | 221.0 |
| Skipper butterfly - Erynnis | 8/12/70 | 146.0 |
| Wood nymph butterfly - Cercyonis sp. | 8/12/70 | 58.0 |
| **Foliage feeders:** | | |
| Weevils - Mixed curculionids | 6/1/70 | 48.6 |
| Grasshoppers | 8/12/70 | 31.0 |
| Larch Casebearer - Coleophora laricella | 6/1/70 | 25.5 |
| Cicada - Cicadidae | 6/1/70 | 21.3 |
| **Cambium feeders:** | | |
| Engraver beetles - Ips sp. | 10/9/70 | 52.5 |
| Flathead beetle - Mixed buprestids | 6/1/70 | 20.0 |
| Red turpentine beetle - Dendroctonus valens LeConte | 6/1/70 | 11.5 |
| Douglas-fir beetle - Dendroctonus pseudotsugae Hopk. | 10/9/70 | 9.4 |
| Flatheaded beetle larvae-Mixed buprestids | 10/9/70 | 8.5 |
| **Predators:** | | |
| Ants | 6/1/70 | 170.0 |
| Ostomids - Temnochila sp. | 6/1/70 | 53.4 |
| Damsel flies - Argia sp. | 6/1/70 | 21.7 |
| Longlegged fly - Medeterus sp. | 10/9/70 | 10.2 |
| Ostomid larvae | 10/9/70 | 6.1 |
| **Miscellaneous Insects:** | | |
| Long horned beetles - Mixed Cerambycids | 8/12/70 | 47.5 |
| Click beetles - Mixed elaterids | 6/1/70 | 36.0 |
| Black Scavanger - Cerambycid | 6/1/70 | 18.8 |
| Larch casebearer | 6/1/70 | 16.5 |
| Bark beetle - Ips sp. | 10/9/70 | 11.5 |
| Honey bees | 6/1/70 | 10.5 |
| Damselflies | 6/1/70 | 9.2 |
| Grasshoppers | 8/12/70 | 7.5 |
| Bumblebees | 6/1/70 | 7.5 |
| Bark beetles - Dendroctonus valens | 6/1/70 | 4.8 |
| Flathead beetles | 6/1/70 | 3.5 |

*PPM = parts per million by dry weight

observations are supported by Solberg and Adams (5) and by Gordon (6).

We found that insects also accumulated fluorides. Generally, at least twice as much fluoride was found in test samples as in corresponding control samples (Table 3). Foliage feeders had from 21.3 to 48.6 ppm, cambial feeders from 8.5 to 52.5, pollinators from 58.0 to 406, and adult predators from 21.7 to 170.

## 1971 Studies

Data collected in August 1970 indicated generally that fluoride concentration in vegetation was reduced 40 to 50 percent in the study area.* Injury also was reduced by about the same amount. However, concentrations of fluoride in insects did not change significantly except in foliage feeders in which fluoride levels increased to 255 ppm.

## Discussion

Significant correlation of elevated fluoride concentrations in 1969 conifer tissue to visible injury indicated fluorides from the Anaconda Aluminum Company were the most likely cause of plant injury in the area. The systematic locatio of plots enabled us to establish approximate acreages of polluted lands. Chemical analyses of insect tissue indicated that insects in the study area had accumulated fluorides.

Although specific data was not collected, we noticed apparent differences in fluoride susceptibility in terms of visual expression of burn symptoms by plants. Western white pine (Pinus monticola Dougl.), and lodgepole pine (Pinus controta Dougl.), were quite sensitive and were being selected in the forest community. This is an unnatural change and represents a trend toward reduced biological diversity in the area.

Damselflies and ostomids, two types of predator insects which were collected, are 100 percent predatory throughout their lives. Fluoride accumulated by these insects must have come from the insects on which they fed, indicating that fluoride is likely to be passed along via the food chain.

We do not know the effect of fluorides on the pollinator complex of insects. Many plants are dependent on these insects for seed production. If the pollinators are being adversely affected, then the plant ecology in the area would also be affected. This probelm certainly warrants intensive study.

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*For complete data, see "Monitoring Fluoride Pollution in Flathead National Forest and Glacier National Park," by Clinton E. Carlson. U.S. Forest Service, Missoula, Montana, 1972.

Although our 1971 data indicated that fluoride concentrations in vegetation had decreased commensurate with the emission reductions reported by the Anaconda Company, nevertheless vegetation in the Glacier National Park was still accumulating abnormal amounts of fluorides in 1971. Even if the company further reduces emissions to meet the Montana State standard of 864 pounds per day, abnormal fluoride accumulation by vegetation as well as visual fluoride injury to plants is likely to continue to occur in Glacier National Park within a radius of 4 to 5 miles around the aluminum company.

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# CLINICAL AND HISTOCHEMICAL EXAMINATIONS OF THE NASAL MUCOSA IN ALUMINUM WORKERS

by

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SUMMARY: The authors examined the nasal mucosa in 130 workers of an aluminum plant exposed to hydrogen fluoride (HF). In 30% of these workers, chronic inflammatory changes were observed in the nasal mucosa, either hypertrophic or atrophic rhinitis, biopsy specimens of septal mucosa were examined histologically. In the patients with hypertrophic rhinitis, numerous inflammatory infiltrates were observed consisting of mononuclear cells. The blood vessels were dilated and extravasations of erythrocytes were noted. The connective tissue stroma showed evidence of edema and hyperactivity of the sero-mucous glands. In the atrophic mucosa, fibrosis and hyalinization of connective tissue stroma was seen associated with moderate inflammatory infiltrates and evidence of hypoactivity of the glands.

Upper respiratory diseases due to harmful chemicals can appear in workers in various industries. Kmita (1) described atrophic changes in the tunica mucosa of the upper respiratory tract arising from gasoline fumes. Lesions of different degrees of intensity in the nasal mucosa depend largely upon the duration of exposure. Durska-Zakrzewska (2) examined 350 workers in the salina in Wapno, Poland, and confirmed that the inhalation of salt present in the air caused not only chemical irritation but secondary infection as well. Rhinitis was encountered in 50% of the patients examined whereas in 3% the nasal septum was perforated. Pathological changes in the upper and lower respiratory tract were noted by Osietrow (3) in workers of the rubber industry. Eolian and Eramian (4) reported the effect of lead, cyanides and fluoride upon the upper respiratory tract in metallurgic industry workers. They found that fluoride causes scars in the respiratory mucosa and that it also accounts for chronic rhinitis, mostly of a hypertrophic character. Gaseous fluoride compounds in the form of vapors have a nonspecific irritating action on the mucous membranes of the respiratory tract. The morphological changes resemble those caused by other chemical substances (5).

Since production of aluminum started in the plants in Maliniec near Konin, Poland, a substantial number of patients with diseases of the upper

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respiratory tract have been observed in the laryngological consulting units.

The electrolytic process in the production of aluminum creates conditions harmful to health. The work in the electrolytic pots is a source of gaseous fluoride compounds, the inhalation of which produces corrosion on the tunica mucosa of the upper respiratory tract. The data from the toxicological laboratory, which systematically measures fluoride concentrations in the atmosphere of electrolytic halls, show that the constant concentration of HF often considerably exceeds 0.0005 mg/liter - the MAC according to Polish standards.

Upon performing laryngological examinations on 130 workers we observed hypertrophic rhinitis in the upper respiratory tract which subsequently developed into the atrophic form in both nasal cavities.

Catarrhal changes in the upper respiratory tract in 30% of the workers were most severe in subjects who had been working from one to two years. Chronic hypertrophic changes in the tunica mucosa of the nose were present in 14% of the patients examined. After one or two years of work, the mucous membranes of the nose became atrophic. This percentage increased gradually until, after ten years of work, 70% of the workers were affected. The atrophic lesions were benign in character. The nasal mucosa was thinned and pale with decreased mucous secretion. Six percent of the patients examined exhibited small erosions in the form of superficial defects of the mucosa in the anterior portion of the septum. Superficial and deeper ulcerations of the septal mucosa were present in 1.2% of the patients. These ulcers were covered by fibrin and tended to bleed easily but the septal cartilage was never exposed.

In order to confirm the histopathological changes in the tunica mucosa, biopsy tissue specimens especially from the workers who had visible changes in the nasal mucosa were taken from the septal mucosa and fixed in 10% formaldehyde. Histological investigations were performed in 35 workers. The biopsy specimens were embedded in paraffin and stained with hematoxylin and eosin. Reactions with periodic acid and Schiff reagent (PAS) and with toluidine blue and with alcian blue were also carried out. Distinct hypertrophic changes were seen in 22 cases and atrophy in 13 cases.

The principal portion of the epithelium exhibited ciliae and numerous goblet cells. Besides ciliary epithelium, the metaplastic epithelium and squamous cells showed cysts with PAS positive staining in many cases. The basement membranes were mostly thickened but their continuity was preserved. Connective tissue stroma contained numerous wide capillary vessels with signs of congestion. Outside the capillary vessels there was a moderate number of erythrocytes (Fig. 1). Infiltrations, consisting of plasma cells and mononuclear cells associated with edema, were frequently situated around the capillary vessels (Fig. 2). In only a few vessels was organized clotting seen (Fig. 3). Sero-mucous glands, characterized

Hyperplastic Rhinitis Due to Flydust from Aluminum Factory

Fig. 1

Pericapillary Infiltration with Blood Cells

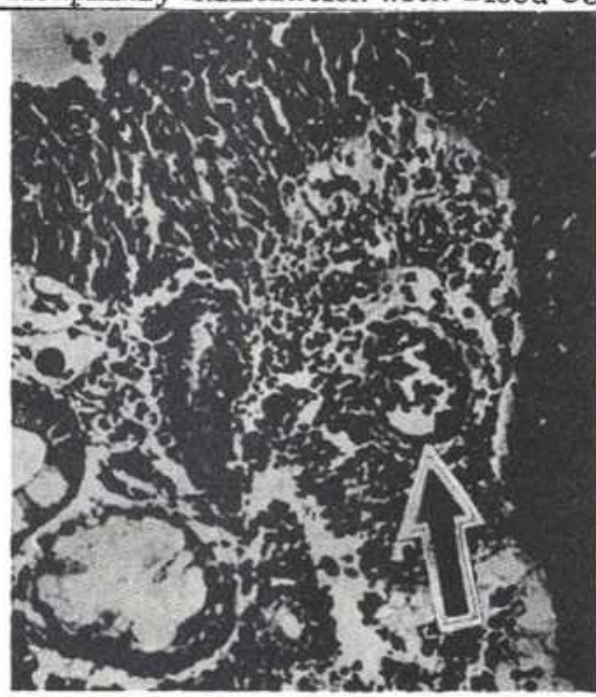


Fig. 2

Pericapillary Infiltration with Plasma Cells and Mononuclear Cells

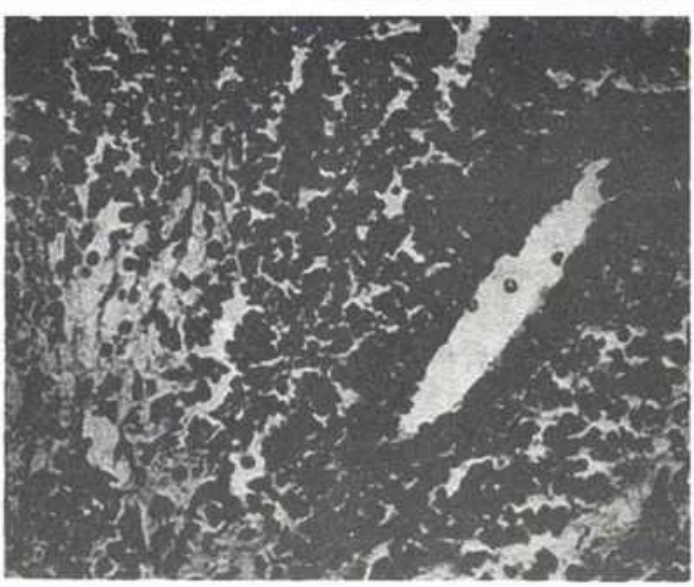
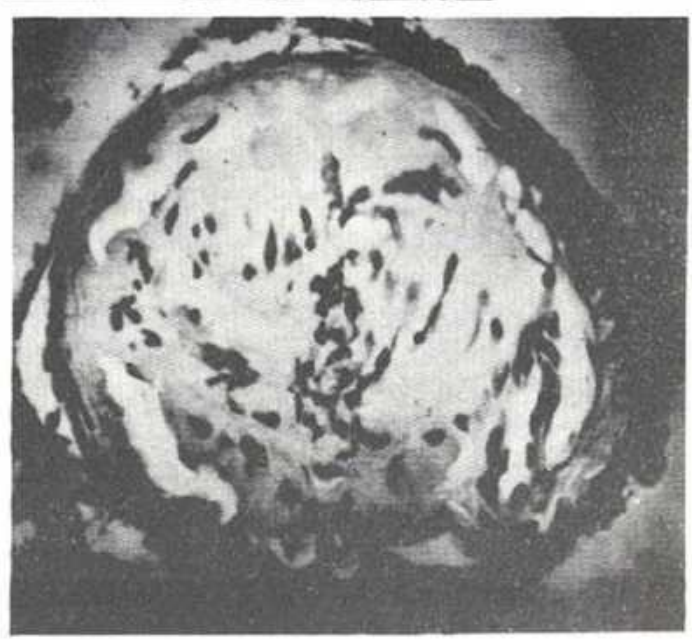


Fig. 3

Capillary Blood Vessels Containing Organized Blood Clot



by hyperfunction, were seen in the specimens from 22 cases which were investigated.

In the remaining cases, the microscopic picture was different. The connective tissue stroma exhibited fibrosis and hyalinization. The cellular infiltration in the stroma of connective tissue was not extensive and the sero-mucous glands showed the morphological features of hypoactivity. Basement membranes and connective tissue stained faintly pink and violet with the toluidine blue stain. In the reaction with alcian blue, the basement membranes stained slightly blue. The connective tissue stroma did not stain. In the goblet cells of glands, the PAS reaction with the Schiff reagent was strongly positive in the patients who showed hypertrophic changes but the same reaction was only faintly positive in those with atrophic rhinitis.

## Discussion

Except for a discussion about the general effect of HF upon the tunica mucosa, we did not find studies of histological examinations of the upper respiratory tract in the available literature on industrial fluorosis (6, 7, 8, 9, 10). Most authors are interested in bone changes (4, 11, 12, 13, 14, 15, 16) and the effect of HF upon the dentition and the tunica mucosa of the mouth (17).

On the basis of our observations, we conclude that changes characteristic of rhinitis occur several months after exposure to hydrogen fluoride. Prolonged exposure to fluoride compounds can cause a transition of the normal mucosa into hypertrophic lesions. After two years of exposure atrophic changes begin to appear. The latter condition was also observed by Russian authors (3) in employees of aluminum plants.

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# FURTHER OBSERVATIONS ON ENDEMIC FLUORIDE-INDUCED OSTEOPATHIES IN CHILDREN

by

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SUMMARY: Fluoro-osteopathy has been described in four children aged 6 or above. These observations contrast with the concept that skeletal fluorosis cannot develop prior to 10 to 20 years of high fluoride intake. The pathogenesis and the mechanism underlying the causation of this disease is discussed on the basis of the occurrence of skeletal fluorosis in growing children. Since skeletal fluorosis is a preventable disorder, it is important to either recognize it or consider it in the differential diagnosis of each bone and joint disease in a child residing in an endemic area.

In spite of the common occurrence of dental fluorosis in children residing in endemic fluoride areas, no attempt has been made to investigate the incidence and severity of skeletal fluorosis in these cases. All reports are confined to studies in adults. The only published data is that by Teotia et al. (1) who described endemic skeletal fluorosis in three children ranging in age from 11-13 years. One of the three cases had crippling fluorosis which improved markedly on allow calcium diet. In the current communication, the authors present further observations on endemic fluorosis in children.

## Materials and Methods

Because of practical difficulties, random investigations were undertaken of only those children willing to be examined in the hospital. All 16 children (11 male and 5 female) between 6 and 15 years of age were subjected to investigations for establishing the diagnosis of skeletal fluorosis. All belonged to the poor socio-economic strata and had been residing since birth in an endemic fluorosis area, in the district of Rai Bareli, Uttar Pradesh, India. For the survey all cases were given X-rays of the skeleton, particularly of the spine, pelvis and bones of the forearms.

Laboratory investigations included serum calcium, serum phosphorus, serum magnesium, alkaline phosphatase, blood urea, creatinine clearance, chemical analysis of bone ash for calcium, phosphorus, magnesium and fluoride. Histological studies of undecalcified and decalcified sections of the biopsied iliac crest bone were done. The fluoride content of the bone, drinking water and urine samples was measured spectrophotometrically, with the use

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of the zirconium dye complex as detailed by Megregian ( 2 ).

## Observations and Results

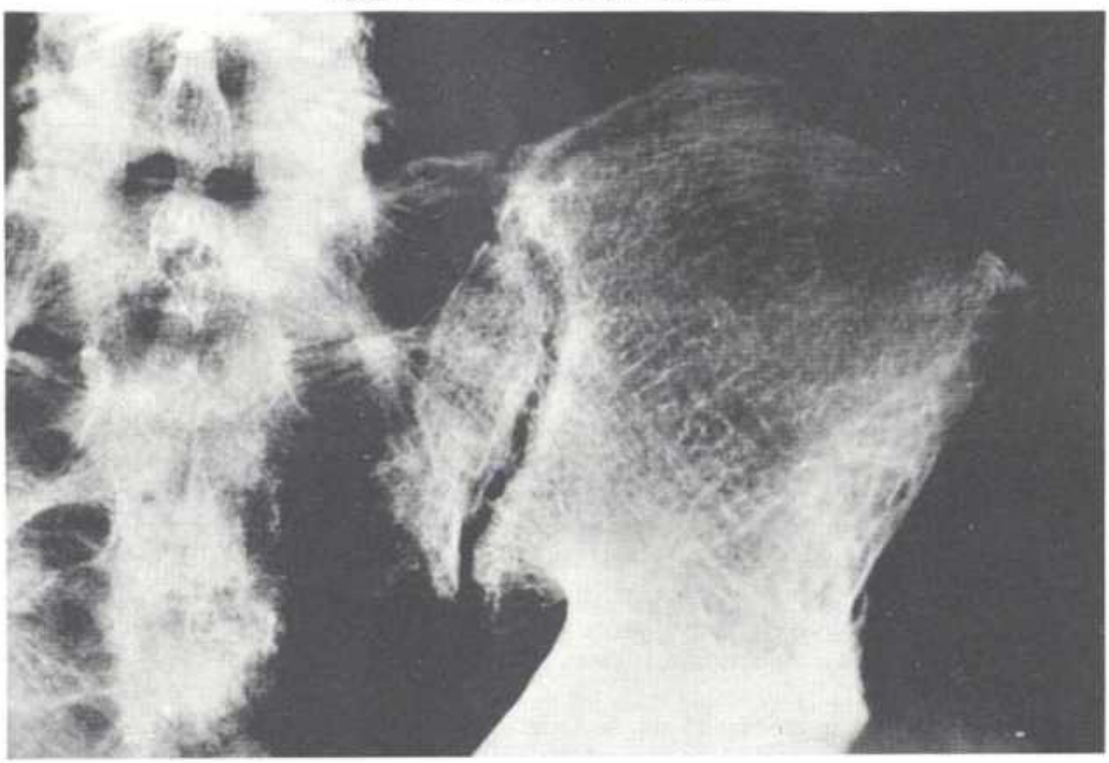
The results of the study are presented in Tables 1 to 4 and Figures 1 to 6.

## Skeletal Radiographs

The radiological changes were those of a diffuse increase in density of most of the skeleton, particularly the spine, pelvis and thorax with coarse trabeculations in the knees and elbows (Fig. 1). Two patients showed

Fig. 1

Pelvic Radiograph Showing Osteosclerosis, Coarse Cystic Trabeculations



calcification of the interosseous membrane of the forearms (Fig. 2) and excessive and irregular deposition of bone around the foramen magnum (Fig. 3). Dental radiographs revealed thinning and interruption of the lamina dura in one child.

## Histopathological Data

Histology of the undecalcified sections of the bone obtained from the

Fig. 2

Radiograph Showing Calcification of Interosseous Membrane of the Forearms as a Diagnostic Feature

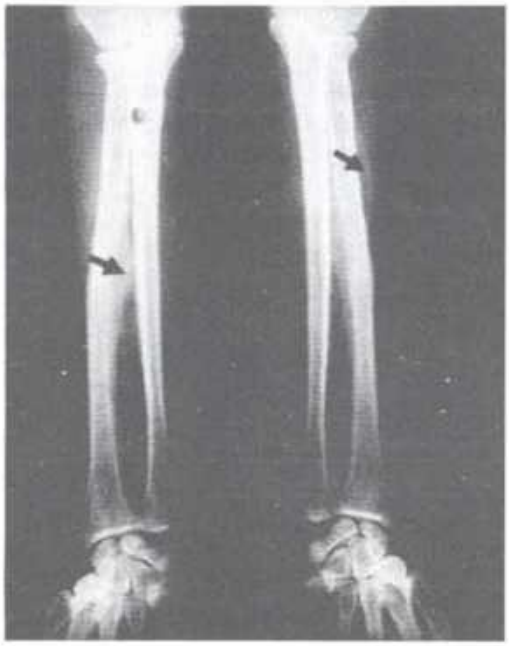
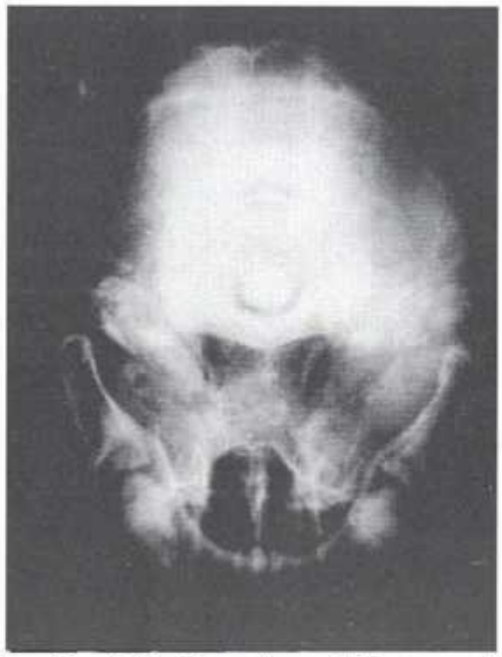


Fig. 3

X-Ray of the Skull



Marked Sclerosis of the Bone Around Foramen Magnum

Fig. 4

Histopathological Picture of Iliac Crest Bone Biopsy (Undecalcified)

Showing markedly thickened trabeculae containing excess calcium.

Fig. 5

Histopathological Picture of the Iliac Crest Bone Biopsy (Decalcified)

Poorly formed Haversian Systems, disordered lamellar orientation and absence of nuclei in some of the osteocyte lacunae, suggesting impaired viability of bone.

iliac crest by open biopsy showed thick trabeculae which contained an excess of calcium (Fig. 4). Decalcified sections revealed poorly formed Haversian system, disordered lamellar orientation of the compact bone and absence of nuclei in some of the osteocyte lacunae (Fig. 5).

## Fluoride in Water Supply

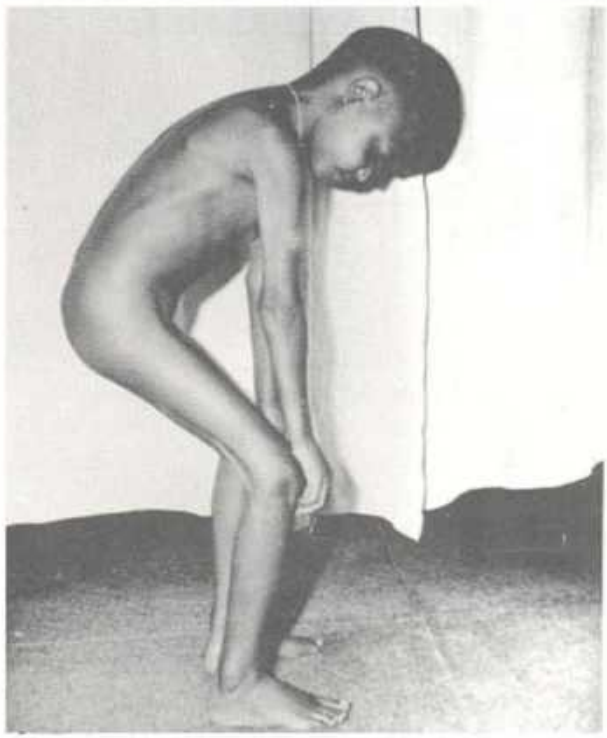
Twenty samples of drinking water were analyzed from four wells located at different places in the endemic area. They contained 10.35 to 13.5 ppm of fluoride.

All children were symptomatic (Table 1). The usual clinical features were generalized stiffness, back pain, inability to close the fist and flexion deformities of spine and joints (Fig. 6). Laboratory investigations

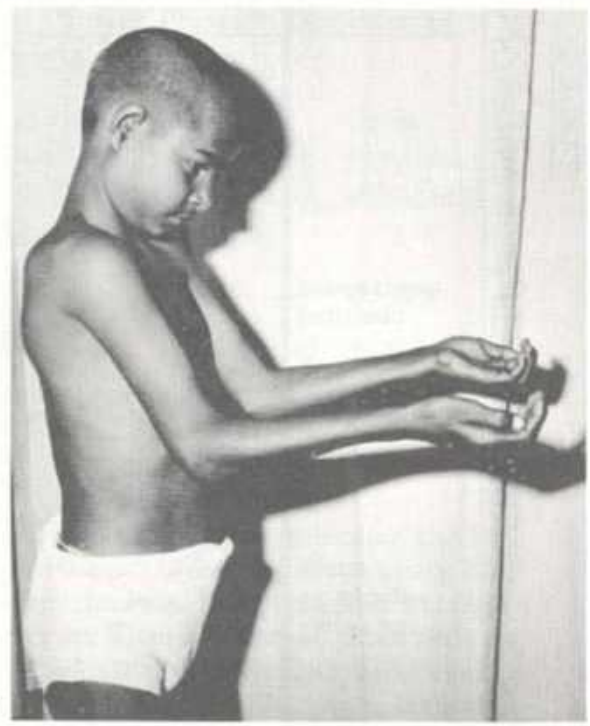
Fig. 6

A Child With Endemic Skeletal Fluorosis Showing Flexion Deformity of the Cervical Spine and Inability to Close the Fists

Before



After



showed a rise in serum alkaline phosphatase (Table 2), low urinary excretion of calcium, increased urinary excretion of phosphorus and fluoride with retention of calcium in each patient (Table 3). The usual radiological changes were those of osteosclerosis, namely periosteal bone formation and ossification of the in-

TABLE 1

SYMPTOMATOLOGY IN FOUR CHILDREN WITH SKELETAL FLUOROSIS

| Case No. | Sex | Age (Yrs) | Duration of symptoms | Walking | Skeletal pain and tenderness | Spine | Joints | Muscular | Gastro-intestinal | Chest | Mottled discoloration of teeth |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 14 | 11 | - | - | Stiffness Cervical flexion | Pains | Inability to close fists | Consti-pation | - | Brown |
| *2 | M | 6 | 2 | Difficulty | - | Backache Stiffness | Pains Bowing of legs | Inability to close fists | Consti-pation | - | Yellowish brown |
| 3 | F | 15 | 4½ | Inability | Severe | Backache Stiffness Thoracic kyphosis | Pains Stiffness Flexion at knees | Inability to close fists | Consti-pation | Fixation of chest | Brown |
| 4 | M | 11 | 2 | Limping | - | Stiffness Cervical flexion | Stiffness Painful move-ments | Inability to close fists | - | - | Yellowish brown |

*Patient had associated Rickets.

TABLE 2

LABORATORY INVESTIGATIONS IN PATIENTS STUDIED

| Patient No. | SERUM | | | | Fluoride in drinking water (ppm) |
|---|---|---|---|---|---|
| | Calcium* (mg%) | Phosphorus (mg%) | Alkaline Phosphatase (K. A. Units) | Magnesium (mEq/Lit) | |
| 1 | 10.0 | 4.3 | 32 | 2.0 | 10.4 - 13.5 |
| 2 | 9.8 | 3.5 | 25 | 1.90 | |
| 3 | 10.2 | 3.8 | 37 | 2.1 | |
| 4** | 10.8 | 3.0 | 55 | 1.80 | |

*Normal Ca 9.0 - 11.0 mg/100 ml.

**Radiological findings suggested hyperparathyroidism presumably secondary to the fluorosis.

TABLE 3

Calcium Balances (In Six Day Period) and Urinary Fluoride Excretion in Four Children With Skeletal Fluorosis

| Case No. | Calcium intake (mg/day) | Urine calcium (mg/day) | Faecal calcium (mg/day) | Calcium balance (mg/day) | Urine fluoride (ppm/day) |
|---|---|---|---|---|---|
| 1 | 860 | 28 | 590 | +242 | 3.5 |
| 2 | 890 | 15 | 620 | +235 | 2.4 |
| 3 | 860 | 40 | 495 | +325 | 3.8 |
| 4 | 860 | 60 | 560 | +240 | 3.0 |

terosseous membrane. The roughening and accentuation of muscular and ligamentous attachments, particularly of the ischial spine and iliac crests may be present before the development of osteosclerosis. Histological studies of the undecalcified sections of the iliac crest biopsies showed thick and hypercalcified bone trabeculae. The decalcified sections revealed disordered lamellar orientation and absence of nuclei in some of the osteocyte lacunae, suggestive of impaired viability of bone. The excess content of calcium, fluoride and magnesium in the bone ash indicated a close relationship between bone apatite crystal and the composition of the fluids (blood serum) in which crystal formation takes place (Table 4).

TABLE 4

Chemical Composition of Bone Obtained from the Iliac Crest of the Patients Studied (Per 100 g Dry Fat Free Bone)

| Case No. | Calcium (g) | Phosphorus (g) | Magnesium (g) | Fluoride (g) |
|---|---|---|---|---|
| *Normal Patients | 11.0 | 5.05 | 105 | 28.5 |
| 1 | 12.5 | 5.2 | 110 | 472 |
| 2 | 11.4 | 4.9 | 106 | 325 |
| 3 | 12.2 | 5.8 | 112 | 280 |
| 4 | 11.6 | 5.0 | 105 | 285 |

*Persons from non-fluoride area.

## Discussion

Our study does not support the published reports that for a definite picture of skeletal fluorosis to develop, 10 to 40 years' residence in an endemic area is necessary. In our opinion, growing children with active bone metabolism, if exposed to high fluoride intake, are more prone to develop skeletal fluorosis than adults. As bone ages and becomes more or less stabilized in the remodelling of its Haversian systems, less fluoride may be deposited. We observed that individuals residing in an endemic area since birth develop more severe skeletal fluorosis than those who have moved into the endemic zone after 17 to 18 years of age when bone growth has ceased.

Our results further indicate that fluoride is not deposited uniformly throughout the skeleton. It may be related to the rate of growth, the degree of vascularization and the physical stress in various parts of the same bone. More fluoride is deposited in the epiphyseal line, and in the epiphyses than in the diaphyseal portion of the bone. A similar observation has been reported by Zipkin and Scow (3). Thus fluoride appears to have an affinity for deposition at the sites of active physiological calcifications. The increase in the percentage of fluoride, like that of calcium in the skeleton, occurs progressively during growth. Apposition of new bone on the trabecular surfaces probably accounts for the increase of density radiologically.

On the basis of our work we recommend that all children residing in an endemic zone regardless of whether symptoms or dental mottling are manifested should be screened for skeletal disease, because early diagnosis will help to prevent the crippling stage of the disease.

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## URINARY FLUORIDE ELIMINATION AND FLUORIDE DEPOSITION IN BONES AND TEETH OF THE RATS AFTER INHALATION

by

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SUMMARY: Rats in the experiment inhaled fluoride in concentrations 9.4 - 11.7 µg per liter of air for a period of five months. The animals were divided into four groups, three exposed and one control. The first experimental group was exposed 90 hours, the second 180 and the third 270 to hydrogen fluoride in the inhalation chamber.

After inhalation of 9.4 - 11.7 µg F/l of air, rapid absorption of fluoride in the organism took place. This was indicated by increased elimination of fluorides in the urine, the occurrence of the characteristic changes in the dental enamel and elevation of fluoride in bones and teeth, without radiographic changes. The changes following administration of fluoride by inhalation are the same as those due to oral administration. Fluoride absorbed by the lung was eliminated rapidly from the organism; the amount present in the urine depended upon the duration of exposure. About one third of inhaled fluoride was eliminated in the urine. Fluoride deposition in bones and teeth increases regardless of the duration of intoxication. No increase in fragility of bones was noted in relation to their fluoride content

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Information concerning the effect of fluoride compounds on the organism is concerned predominantly with their oral administration or with fluoride intake through water; few data are available on the effect of inhalation of fluoride compounds. For this reason we investigated absorption and deposition of fluorides into the skeleton following inhalation of hydrofluoric acid vapors and its elimination through the urine.

## Method

The experiment was carried out with 40 Wistar male rats divided into three experimental and one control group. The animals were exposed to vapors of hydrofluoric acid daily for three hour periods in the inhalation chamber. The first experimental group (E I) was exposed for a total of 90 hours, the second one (E II) for 180 hours, and the third (E III) for 270 hours. The concentrations of fluoride in the air of the inhalation chamber ranged from 9.4 to 11.7 µg F/l; the temperature was 26.0°C in the chamber and 24.5°C in the room where the inhalation chamber was installed. The animals were placed in metabolic cages in which it was possible to separate the urine, so that its fluoride content could be determined. The animals were sacrificed at the termination of the exposure time. The femurs and tibias were X-rayed and chemically analyzed for their fluoride content. Radiographs and photographs were taken of the teeth which were studied histologically and their fluoride content was determined.

## Results

In the above-mentioned experimental arrangement the general state of the exposed animals was good with regard to their vitality. The third experimental group showed an increased loss of hair. The weight variations in individuals were considerable. The rats in the control group attained a higher weight (300-400 g) than the animals in the third experimental group (265-390 g), even though their feed consumption and their age were the same.

At concentrations of 9.4 - 11.7 µg F/l in the inhaled air the signs of absorption were determined after a short exposure on the basis of urinary fluoride excretion (Table 1). The analysis of our data shows that the urinary fluoride excretion was substantially higher in the first and the second experimental groups than in the control animals. A more significant elimination was observed in the third group. This supported our opinion that, at the beginning of the exposure the absorption of fluoride into the organism was higher and, some time later, increased elimination of fluoride occurred.

The average amounts of fluoride found in the rats are presented in Table 2. The fluoride content of bones increased with the duration of the intoxication. The fragility of all bones of rats was investigated by bowing during the static loading. We could not confirm an increase in fragility of bones in relation to their increased fluoride content. In radiographs of bone no osteosclerotic or osteoporotic changes were found.

TABLE 1

The Average Amount of Fluoride in Urine of Rats

| Group | n | F x (mg/l) |
|---|---|---|
| Control | 5 | 0.50 |
| E I | 5 | 5.65 |
| E II | 5 | 8.55 |
| E III | 5 | 21.55 |

TABLE 2

The Average Amount of Fluoride in Bones of Rats

| Group | n | F x (mg/100 g) |
|---|---|---|
| Control | 10 | 67.7 |
| E I | 10 | 122.7 |
| E II | 10 | 261.0 |
| E III | 10 | 367.7 |

The teeth showed typical signs of fluoride absorption. The changes in dental enamel of lower and upper incisors were noticeable even in the first experimental group. At first, the yellow-brown coloration became white and white stripes were seen. In the third experimental group uneven cut-edges of teeth were observed and the dark spots on the upper incisors as well as the dark grooves on all lower incisors deepened. These readily visible changes appeared in all exposed animals with varied intensity.

The histological investigation of the incisors of the control animals showed a normal structure. The changes in incisors of the exposed group were defined as calcification of dentin appearing in the form of typical stripes; the number of stripes varied. From the histological picture it was evident that the fluorine ion had a harmful effect on dental tissues. In bones as well as in teeth, the fluoride content of the exposed and the control group varied considerably (Table 3).

Our results indicated that rapid absorption of fluoride in the organism took place following inhalation of 9.4 - 11.7 ug F/l of air as confirmed by an increased elimination of fluoride through the urine, by the occurrence of characteristic changes of dental enamel and by an increasing amount of fluoride in bones and teeth, without producing radiographic changes. The experi-

TABLE 3

The Average Amount of Fluoride in Teeth of Rats

| Group | n | F x (mg/100 g) |
|---|---|---|
| Control | 5 | 48.6 |
| E I | 5 | 173.5 |
| E II | 5 | 194.7 |
| E III | 5 | 216.9 |

ments proved that the changes following administration of fluoride compounds by inhalation are the same as those due to oral administration of fluoride. According to our calculations about a third of the total fluoride amount taken in by inhalation was eliminated by urine.

## EFFECT OF SODIUM FLUORIDE ON THE ENZYMATIC HYDROLYSIS OF HEXAPHOSPHATE OF MYO-INOSITOL IN THE GERMINATION OF VICIA FABA L.

by

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SUMMARY: In this study the effect of various concentrations of NaF in the substrate on the level of some phosphates during the germination of Vicia faba seeds was investigated. Changes of the phosphorus content of hexaphosphate of myo-inositol, nucleic acids, 2-phosphoglyceric acid, P-esters of monosaccharides and free inorganic orthophosphate were studied. The fluoride ion caused a significant decrease in enzymatic hydrolysis of hexaphos-

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phate of myo-inositol during the course of seed germination and a gradual decrease of phosphorus content of nucleic acids, 3-phosphoglyceric acid, P-esters of monosaccharides and free inorganic orthophosphate. The current results indicate that the inhibition of the enzymatic hydrolysis of hexaphosphate of myo-inositol by the fluorine ion constitutes an important factor in influencing the phosphorus metabolism in the initial phase of ontogenesis.

Variations in tolerance to fluoride in the substrate is a striking phenomenon in the germination of seeds. Accroding to Navara et al. (1, 2, 3) seeds of some species may lose their germinating ability even at a low concentration of fluoride in the substrate. During germination the seeds depend upon endogenous nutrition present mostly in the endosperm tissue. Basic substances for biochemical processes are released by enzymatic hydrolysis. One of such substances with a high phosphorus content is hexaphosphate of myo-inositol (HPMI) which serves as a reserve phosphorus compound in the seeds and as the precursor of some biochemical reactions (4-7).

Peers (8) and Chang (9) stated that the fluoride ion inhibits the enzymatic hydrolysis of HPMI in the germination of wheat and maize kernels. This substance of the seeds contributes from 5 - 80% of the total organic phosphorus. The inhibition of its enzymatic hydrolysis by fluoride may be a limiting factor in the phosphorus metabolism during the initial phases of ontogenesis.

In this investigation, changes of enzymatic hydrolysis of HMPI during the germination of Vicia faba seeds under the influence of various fluoride concentrations in the substrate were studied. At the same time changes in the content of free inorganic orthophosphate, 3-phosphoglyceric acid (3-PGA), P-esters of monosaccharides and nucleic acids were followed. The effect of fluoride on the breakdown of HPMI in the germinating seeds, by application of various fluoride concentrations in the form of NaF in the solution was also studied. Vicia faba seeds belong to a group of plants which are very susceptible to fluoride. Therefore they were chosen for this experiment.

## Materials and Methods

Seeds of uniform size were washed in distilled water. The control seeds were left to swell in distilled water and the experimental seeds in solution of NaF at concentrations of 1, 10, 50, 100 mg F/ liter. The pH values fluctuated between 5.2 and 5.4. The seeds were placed with embryo toward the filter paper. The controls were wetted with distilled water, the experimental specimens with their corresponding NaF concentrations. The seeds were germinated in Jacobson's bed at 25°C. At 0, 24, 48, 78 and 96 hour intervals after treatment, germinating seeds were selected visually,

according to the length of the germinating tips, from all treatments and at each of the time intervals. The selected germinated seeds were immediately frozen with compressed $CO_2$ at -70°C and kept in this condition until the extraction.

Individual samples were homogenized and extracted with 0.5 M $HClO_4$ at 0°C. The extraction and homogenization was repeated three times with 50 ml 0.5 M $HClO_4$ at 10,000 rpm. The extract was then centrifuged at 2,000 g. The supernatant was filtrated through $S_2$ glass filter and the suspension was extracted again by the use of the same procedure. The cold extracts of equal volume were analyzed for the content of HPMI phosphorus, 3-PGA, P-esters of monosaccharides and free inorganic orthophosphate.

The cold extracts were fractionated by coagulation with a saturated solution of $BaCl_2$ at various pH values according to the method used by Kulaev, Belozerskij and Ostrovskij (10). Values of the total fraction content were followed as orthophosphate after mineralization with 0.2 ml 70% $HClO_4$ at 150-180°C. Values of unstable phosphate as orthophosphate were obtained after 10 min.of hydrolysis with 1% n HCl at 100°C.

## Results and Discussion

Hexaphosphate ester of myo-inositol, phytin, found in various plant organs is very important in the phosphorus metabolism in germination. The highest content was found in the seeds of plants forming from 50 to 80% of the total organic phosphorus (11, 12). During the germination HPMI dephosphorylated with the aid of the enzyme myo-inositol hexaphosphate of phosphohydrolase, phytase, releases free inorganic orthophosphate and MI. The process of the enzymatic hydrolysis of HPMI with phytase of various seed species has been studied by many investigators (8, 9, 12, 13, 14, and 15). Enzymatic hydrolysis passes gradually through penta, tetra, tri, di and monophosphate of MI. Gradual enzymatic hydrolysis was proved chromatographically by Kulaev, Valichanov and Belozerski (16) and Sobolev (12). In addition to the previous compounds, Kulaev found 3-PGA after 16 hours' incubation of the enzymatic extract of cotton seeds with sodium salt of HPMI. The compound HPMI forms calcium-magnesium salt in the plant tissues and thus releases calcium and magnesium ions associated with the enzymatic hydrolysis.

Asseva (4) reported that during the germination of wheat seeds approximately 50 percent of the HPMI phosphorus was transported to the inorganic component and the rest to various organic substances, especially nucleic acids. The importance of this substance was recognized by the fact that the dephosphorylated product myo-inositol serves as precursor of biosynthesis of pentosyl and uronosyl units of polysaccharides of the plant cell walls (5, 6, 17). This resulted in the importance of HPMI for many bio-chemical processes of plants and especially its function in releasing free orthophosphate, MI or 3-PGA by enzymatic hydrolysis. The enzymatic hydrolysis

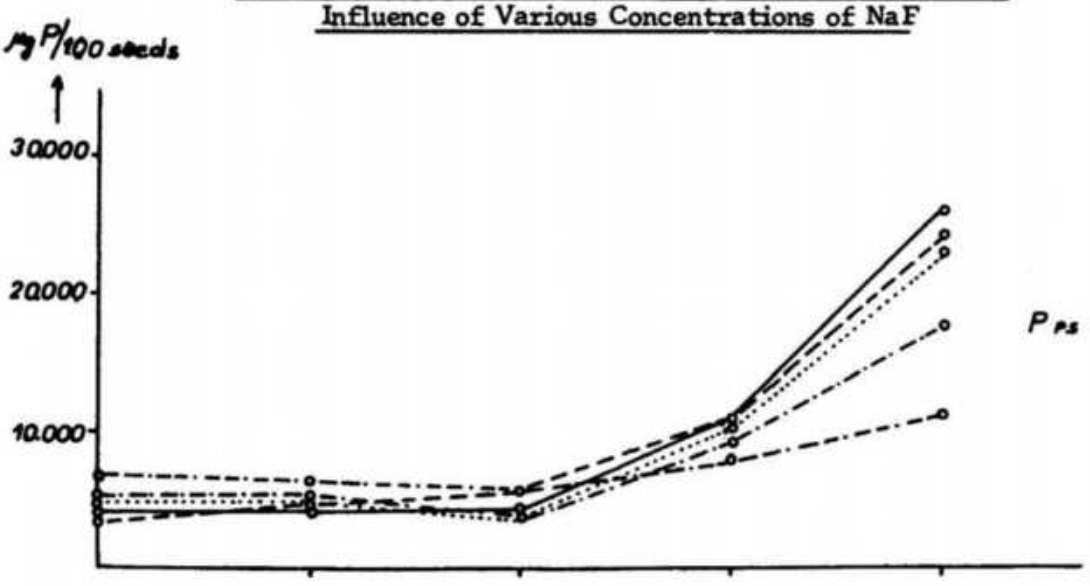
passes relatively quickly in germinating seeds; it lasts from 8 to 10 days under optimum conditions. According to Zuev and Golubeva (18), the HPMI synthesis does not take part in this stage of ontogenesis.

Chang (9) investigated the influence of various inorganic salts upon the enzymatic hydrolysis of sodium salt of HPMI by phytase from the endosperm of germinating maize kernels both in vitro and in vivo. Incubation in vitro induced a stimulating effect of $CaCl_2$ with a concentration of 0.02 m mol (25%) and significantly inhibited the effect of NaF with the same concentration (63%) compared with the control. Michaelis' constant for this enzymatic hydrolysis per $K_m$ was $.91 \times 10^{-4}$ M. The optimum value of the enzymatic activity is at 50°C and a pH of 5.6.

In our work, changes of enzymatic hydrolysis of HPMI in germinating Vicia faba seeds were investigated under the influence of various concentrations of NaF in the substrate. At the same time changes of the content of phosphorus of nucleic acids, 3-PGA, P-esters of monosaccharides and free inorganic orthophosphate were studied. The seeds contained 75% of the total organic phosphorus in the form of HPMI. The germination of the seeds was negatively affected even by low concentrations of fluoride in the substrate (Fig. 1). The lowest concentration of 1 mg F/liter substrate decreased the germination by 7% and the highest concentration of 100 mg F/liter by 72% after 4 days.

Fig. 1

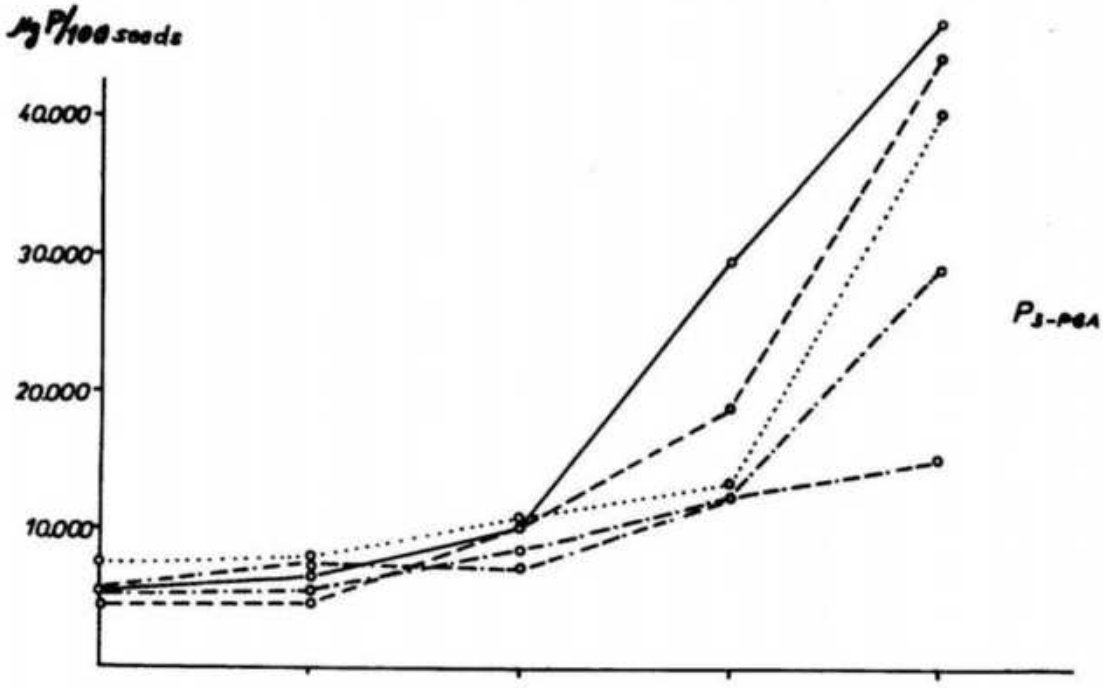
The Course of Germination of Vicia faba Seeds Under the Influence of Various Concentrations of NaF



In the initial phase the hydrolysis rate of HPMI of germinating seeds was slow (Fig. 2). On the second day a rapid increase of enzymatic activity of phytase occurred. After 4 days of germination the phosphorus content of

Fig. 2

Effect of Various NaF Concentrations in the Substance Upon the Phosphorus Content in Hexaphosphate Myo-Inositol and Free Inorganic Phosphorus During Germination



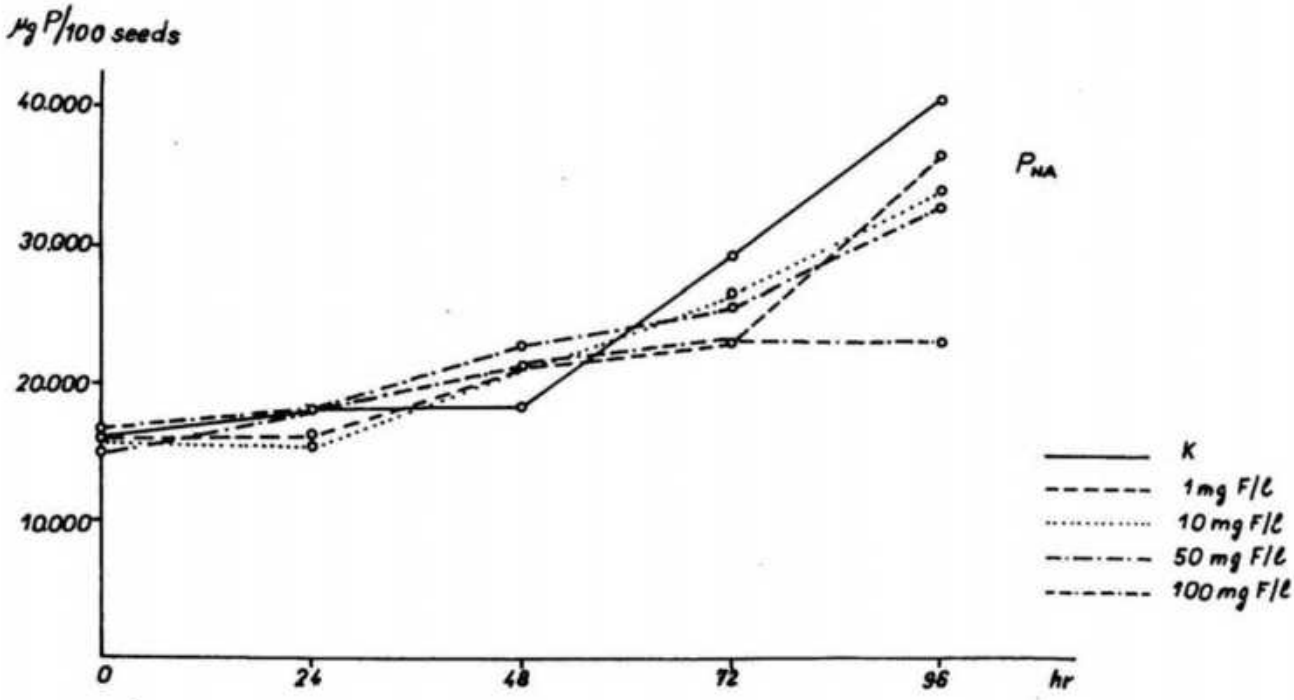

HPMI decreased to 28% of the original content. The presence of increasing fluoride concentrations in the substrate caused a decrease in hydrolysis compared to the control value. In the case of the highest concentration, i.e., 100 mg F/liter after 4 days of germination 64% of the original phosphorus content of HPMI was found. In other words, this concentration resulted in a twofold decrease of the enzymatic hydrolysis of HPMI. With the hydrolysis of HPMI, the content of the free orthophosphate in the control increased fourfold. However, after 4 days of germination in the presence of 100 mg F/liter substrate, the content of the free orthophosphate increased only slightly.

Figures 2 and 3 show that the free inorganic orthophosphate, as a product of enzymatic hydrolysis of HPMI, was partially metabolized. This caused a proportional increase in the phosphorus content of nucleic acids, P-esters of monosaccharides and 3-PGA during the germination. The increasing NaF content of the substrate caused a decline in the rate of the enzymatic HPMI hydrolysis and thus a reduction in the content of some phosphorylated substances whose synthesis depended upon the phosphate donor.

Chang (9) investigated in his work the NaF effect on the phytase from endosperm tissue of germinating maize kernels. He found a 54% decrease in enzymatic HPMI hydrolysis with 10 mmol NaF in the incubated

Fig. 3

Effect of Various NaF Concentrations in Substance Upon the Phosphorus Content in Nucleic Acids, in 3-Phosphoglyceric Acid and in P-Esters of Monosaccharides During Germination




mixture in vitro. When he used the enzymatic extract isolated from maize kernels germinated on the NaF substrate (10 mmol), Chang observed a decrease of 10% hydrolysis as compared with the control. This difference might be explained on the basis that the activity of phytase regenerates without the presence of NaF in the incubated mixture.

To date the inhibition mechanism of the HPMI hydrolysis by fluoride is unknown. It is not clear whether the inactivation of phytase was due to a complex reaction as in the case of enolase or phosphoglucomutase (19, 20) or to an increase of Michaelis' constant caused by formation of a calcium-magnesium complex of the HPMI salt with fluoride.

The free orthophosphate which originated by enzymatic hydrolysis during germination was utilized especially in nucleic acid synthesis (4, 11, 21). A decrease in nucleic acid synthesis was observed in the presence of increasing NaF concentrations in the substrate of germinating Vicia faba seeds (Fig. 3). Chang (9, 22) was able to determine from his work, that the presence of NaF in the substrate reduces the prolongation rate and the rate of cell multiplication of the root tips of germinating maize kernels. Every negative interference with the RNA metabolism may be reflected in the reduction in the growth rate. Inhibition of the enzymatic HPMI hydrolysis may affect the growth rate either in decreasing the synthesis of nucleic acid or decreasing the content of the free myo-inositol. Myo-inositol serves as precursor of uronosyl and pentosyl units of polysaccharides of cell walls in plants (5, 6, 17).

With the enzymatic hydrolysis of HPMI, an increase in 3-PGA content was observed (Fig. 3). This result is in agreement with the observations of others (16, 23). In the presence of fluoride in the substrate a decrease in 3-PGA content accompanied by a decrease in HPMI dephosphorylation was also observed. These findings indirectly verify the hypothesis, that 3-PGA may be the product of the fission of partially dephosphorylated HPMI in two molecules of 3-PGA (16).

It may be inferred from the current results that the inhibition of the enzymatic hydrolysis for the hexaphosphate of myo-inositol by fluoride might influence a number of biochemical processes.

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## BIOCHEMICAL AND BIOPHYSICAL INVESTIGATION INTO GROWTH AND AGING OF CORN SEEDLINGS TREATED WITH FLUORIDE

by

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SUMMARY: The current series of data provides evidence for the interpretation that fluoride-induced growth retardation and aging are processes controlled by changes directly related to protein formation and by changes at the site of protein synthesis, respectively. These changes are decreases in the content of total and ribosomal RNA, alteration of ribosomal components, and a shift in ribosomal distribution from polysomes to smaller particles. The factors responsible for these findings are the accumulation of ATP, the activation of ribonuclease activity in roots, and the inhibition of phytase activity in endosperm-scutellar tissues.

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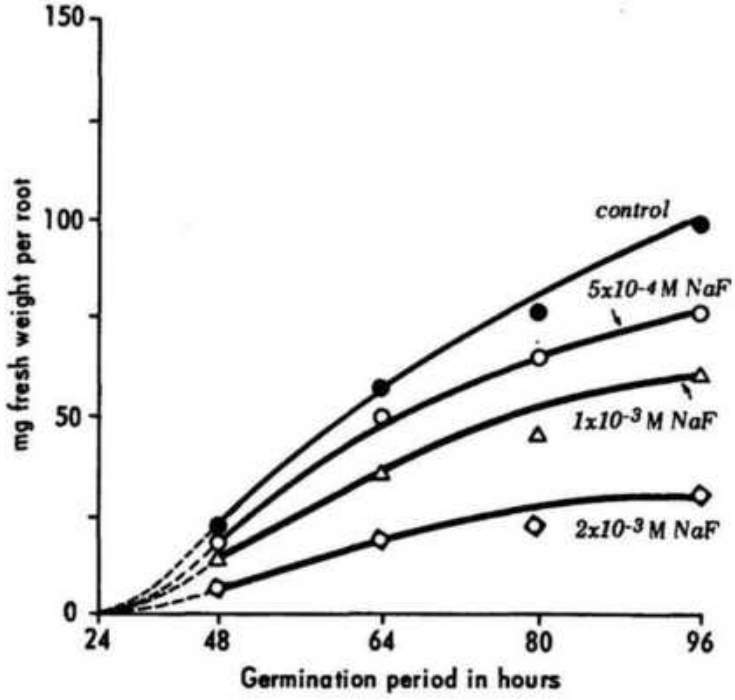
Presented at the Fourth Annual Conference of I. S. F. R., The Hague, 10/24-27/71.

In 1966, the author (1) demonstrated that fluoride induces growth inhibition of corn seedling roots. For determination of growth rate, corn seeds were treated with 0.01 M sodium fluoride for various lengths of time following which the seedling roots were grown in distilled water until they reached a standard size of 12 $\pm$ 3 mm. This size was divided by the time required for each experimental group to reach the standard root length. A similar observation of growth inhibition (2) was obtained from treatment of germinating corn seedling roots with $5 \times 10^{-4}$ M to $2 \times 10^{-3}$ M sodium fluoride (Figure 1). The conditions in the first experiments (1) inhibited the growth rate by one-third to one-half of the control level. By contrast, the conditions in the second experiments (2) suppressed the growth rate to a range of one-third to two-thirds of the control value. Treatment of corn seeds with a relatively high concentration of fluoride was required to suppress germination and growth, possibly because of the diffusion barrier of cutinized seed coat.

Since the growth rate was determined by measuring the increase in the length of the seedling root, the rate should be a function of both the rate of cell elongation and the rate of cell multiplication. For the determination of the two types of growth rates, histological slides of longitudinal sections of root tissue were prepared and stained with safranin and fast green. The cell length and cell number of cortical cells were measured and counted, respectively, with an ocular micrometer in a microscope. Both the elongation rate and cell multiplication rate of seedling roots were found to be reduced by fluoride (1). The rate of cell

Fig. 1

Effect of Fluoride on Fresh Weight of Corn Seedling Roots (2)



multiplication, however, was inhibited more than the rate of cell elongation after a short period of fluoride treatment. As the duration of fluoride treatment was prolonged, the two growth types showed similar levels of growth reduction. In addition, the number of mitotic figures of the root samples decreased with the fluoride treatment (Table 1). These data support the finding that fluoride reduces the rate of cell multiplication.

Further investigations of fluoride-inhibited cell elongation revealed that it is highest at 3-mm distance from the root tip and decreases in a basipetal direction in both control and treated roots. The reduction in cellular elongation rate is initiated by fluoride about 3-mm distant from root tip. This reduction rate parallels the control elongation rate during the later developmental stages of seedling roots (Figure 2). The rate of cell elongation when inhibited does not recover during the subsequent developmental period.

TABLE 1

Effect of Fluoride on Rates of Cell Elongation and Cell Multiplication in Corn Seedling Roots

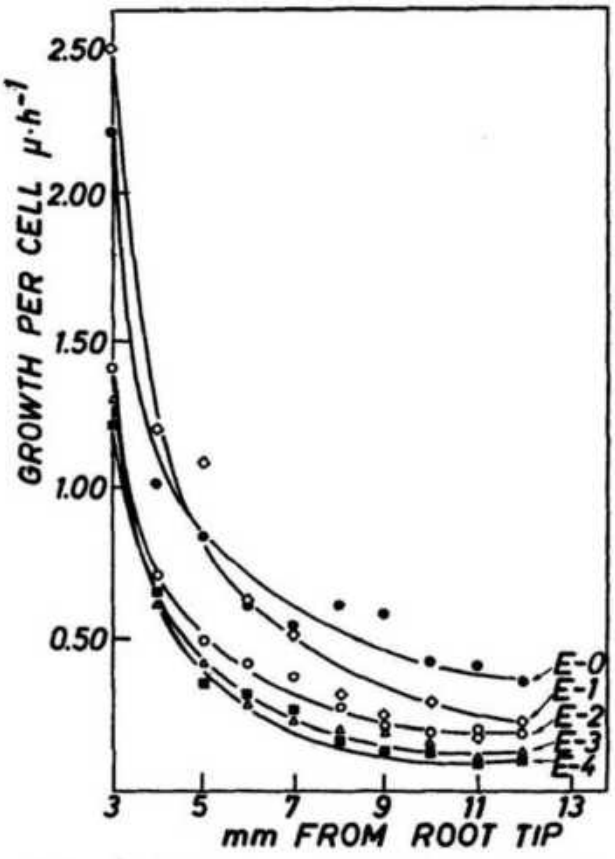
| Experiment | Rate of cell elongation | | Rate of cell multiplication | | | |
|---|---|---|---|---|---|---|
| | Mean cortical cell length of root (12 mm) per hour | | Number of cortical cells per longitudinal section (12-mm root) and hour | | Number of mitotic figures/125 $\mu^2\pi$ of root tips (3 mm) | |
| | μ | % | Number | % | Number | % |
| E-0* ..... | 0.763 | 100 | 114 | 100 | 60 | 100 |
| E-1 ..... | 0.599 | 78.5 | 65 | 57.0 | 45 | 75 |
| E-2 ..... | 0.463 | 60.7 | 56 | 49.1 | 33 | 55 |
| E-3 ..... | 0.314 | 41.1 | 46 | 40.1 | 28 | 46 |
| E-4 ..... | 0.299 | 39.3 | 43 | 37.8 | 25 | 43 |

| * Exp. no. | Pretreatment, h (with 0.01 M NaF) | Germination, h |
|---|---|---|
| E-0 ..... | none | 36 |
| E-1 ..... | 1.5 | 45 |
| E-2 ..... | 3 | 53 |
| E-3 ..... | 5 | 61 |
| E-4 ..... | 7 | 70 |

Mitotic figures were counted at 5 125 $\mu^2\pi$ areas of 3-mm root tips and the number was divided by 5. No distinction was made between cells in various stages of mitosis (1).

Fig. 2

Mean Rate of Growth Elongation At Various Developmental Stages of 9-mm Root Segments Between 3 mm and 12 mm Seedling Root




Each cell length in four rows of cortical cells of longitudinal root section was measured successively in a basipetal direction. Averages of 10 to 15 cell lengths were made at 10 equally divided successive developmental stages of root segments in a basipetal direction. Each averaged value was then divided by the cumulative time factors (in hours) required to reach each of 10 developmental stages. For explanation of E-0 to E-4, see Table 1 (1).

Neidhardt and Magasanik (3) stated that the growth rate is generally controlled by the rate of protein synthesis. RNA content appears to play a vital role in enzyme protein synthesis (4). Therefore, the influence of fluoride on the RNA content of corn seedling roots was investigated.

The author (1) found that the growth rates of seedling roots germinated

from the corn seeds treated with fluoride for various time periods are related to the total RNA content of the 3-mm root tips (Table 2). In order to determine whether or not this relationship is a reflection of the cell number of the root tips, the content of RNA was analyzed on a cell basis. For these analyses, the determination of cell volume was conducted by macerating the root-tip tissue in 5% chromic acid and by counting the cell number by means of a hemacytometer. The total RNA content per root-tip cell was found to correlate with growth rate per cell (Table 3). This finding was further elucidated by subsequent analyses of ribosomal RNA content of roots of fluoride-treated corn seedlings (5), since the ribosomal RNA represents the bulk of, and a constant proportion of,

TABLE 2

Effect of Fluoride on Growth Rates of Seedling Roots and Nucleic Acid Contents of 3-mm Root Tips in Corn (1).

| Treatment | RNA pg/3-mm root tip | Growth mm/hour |
|---|---|---|
| Water (Control)... | 21.5 | 0.36 |
| NaF, 0.01 M | | |
| 1.5 hour ..... | 17.7 | 0.28 |
| 3.0 hour ..... | 16.4 | 0.23 |
| 5.0 hour ..... | 15.2 | 0.20 |
| 7.0 hour ..... | 13.6 | 0.17 |

TABLE 3

Effect of Fluoride (0.01 M NaF) on Growth Rate and RNA Per Cell of 3-mm Corn Seedling Root Tips

| Experiment number | Cell number per 3-mm root tip | Growth rate mm/h/cell | RNA pg/cell |
|---|---|---|---|
| E-0 ..... | $150 \times 10^3$ | $2.85 \times 10^{-6}$ | $1.416 \times 10^{-4}$ |
| E-1 ..... | $140 \times 10^3$ | $2.07 \times 10^{-6}$ | $1.263 \times 10^{-4}$ |
| E-2 ..... | $184 \times 10^3$ | $1.37 \times 10^{-6}$ | $0.887 \times 10^{-4}$ |
| E-3 ..... | $195 \times 10^3$ | $1.07 \times 10^{-6}$ | $0.779 \times 10^{-4}$ |
| E-4 ..... | $191 \times 10^3$ | $0.94 \times 10^{-6}$ | $0.690 \times 10^{-4}$ |

Growth rates per hour and cell were calculated by dividing seedling root growth rates per hour (Table 2) by 3-mm root tip cell volumes (1). For explanation of E-0 to E-4, see Table 1.

the total RNA content of the cell (4). Corn roots treated with various concentrations of fluoride ($5 \times 10^{-4}$ to $4 \times 10^{-3}$ M) were found to contain decreased amounts of ribosomal RNA in relation to fluoride concentrations (Table 4). Therefore, the data shown in tables 2 and 3 are assumed to be the reflection of ribosomal RNA content.

Bonner (6) indicated that ribosomes are free in some types of meristematic cells and become associated with membranes as the cells elongate and mature. Chang (5) demonstrated the operation of such system in corn seedling roots. The ratios of free to bound ribosomes were nearly 5.0 and about 0.90 in the tissue segments at distances of 0-25 mm and 25-100 mm, respectively, from the root tip. According to Key et al. (7) the bound ribosomes are metabolized during the elongation process in corn roots. Therefore, investigation of the influence of fluoride on free and bound ribosomes in corn seedling roots was undertaken.

Fluoride ($5 \times 10^{-4}$ to $4 \times 10^{-3}$ M) was found to reduce the amounts of both free and bound ribosomes in corn seedling roots (5). However, fluoride does not modify the ratio of free to bound ribosomes (Table 5). The current data imply

TABLE 4

Amount of Ribosomal RNA From Control and Fluoride-Treated Corn Roots (5).

| NaF M | Ribosomal RNA | |
|---|---|---|
| | mg/g of fresh weight | % of control |
| 0 ..... | 2.01 | 100 |
| $5 \times 10^{-4}$ ..... | 1.48 | 74 |
| $1 \times 10^{-3}$ ..... | 1.23 | 61 |
| $2 \times 10^{-3}$ ..... | 1.13 | 56 |
| $4 \times 10^{-3}$ ..... | 1.09 | 54 |

TABLE 5

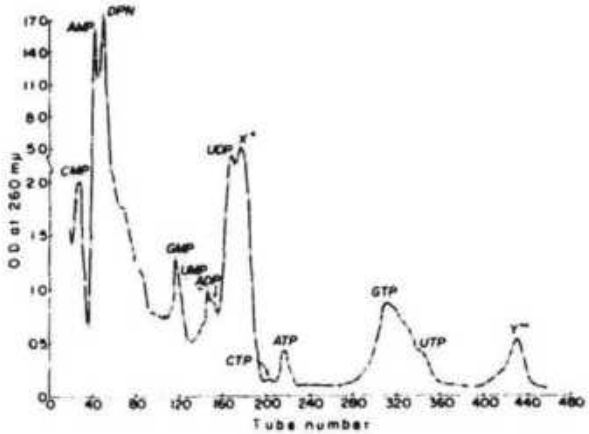
Content of Free and Bound Ribosomes From Control and Fluoride-Treated Corn Roots

| NaF M | Free ribosomes | | Bound ribosomes | | Free ribosomes/bound ribosomes |
|---|---|---|---|---|---|
| | mg/g fresh weight | % of control | mg/g fresh weight | % of control | % |
| 0 ..... | 4.18 | 100 | 0.77 | 100 | 84/16 |
| $5 \times 10^{-4}$ ..... | 3.09 | 74 | 0.69 | 89 | 82/18 |
| $1 \times 10^{-3}$ ..... | 2.85 | 68 | 0.56 | 72 | 84/16 |
| $2 \times 10^{-3}$ ..... | 2.73 | 65 | 0.51 | 66 | 84/16 |
| $4 \times 10^{-3}$ ..... | 2.85 | 68 | 0.48 | 63 | 86/14 |

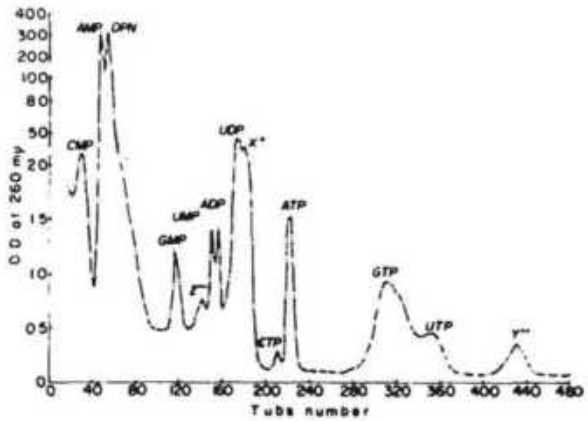
Fig. 3

Elution Chromatogram of Acid-Soluble Nucleotides Extracted From Corn Seedlings Roots (19 g Fresh Weight) After 80 Hours of Germination

Control Corn Seedling Roots




2 x $10^{-3}$ M Fluoride-Treated Corn Seedling Roots




* X - No spot was observed after paper chromatographic development of the concentrated sample with Pabst Solvent III. The peak is assumed to be some unknown phenolic compounds.

** Y - The $R_F$ value of this compound was found to be 0.26 by paper chromatographic analysis with Pabst Solvent III. This $R_F$ value is the same as the $R_F$ value (0.25) of an unknown UV absorbing compound isolated from corn (WF9 x M14) seedling roots by Cherry and Hageman (1960) (2).

*** Z - "trailing shoulder" of GMP based on paper chromatographic analysis (2).

that the decreased content of bound ribosomes is not caused by blockage of conversion of free ribosomes to bound ribosomes, since no accumulation of the free ribosomes occurs. However, the content of bound ribosomes may possibly be restricted by the free ribosomes simultaneously suppressed by fluoride, since ATP accumulates in corn seedling roots treated with fluoride (2) (Figure 3). Fluoride also decreases RNA to protein ratios in free and bound ribosomes (5). The latter are influenced more than the former (Table 6). This finding would explain the previously observed inhibition of elongation growth in corn seedling roots treated with fluoride (Table 1).

In order to better understand the decreases in RNA to protein ratios in free and bound ribosomes (Table 6), the effect of fluoride on the structural compositions of ribosomes was investigated. Fluoride did not modify the base compositions of the ribosomal RNA (Figure 4). By contrast, fluoride does influence the

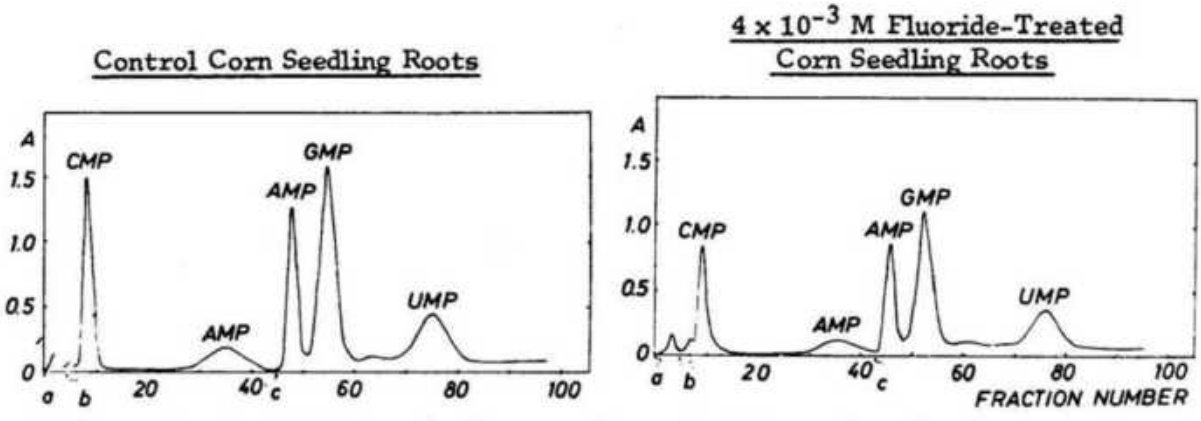
TABLE 6

Relative Ratios of RNA to Protein in Free and Bound Ribosomes Isolated From Control and Fluoride-Treated Corn Roots (5)

| NaF M | Free ribosomes RNA/protein | Free ribosomes % ratio | Bound ribosomes RNA/protein | Bound ribosomes % ratio |
|---|---|---|---|---|
| 0 ..... | 1.09 | 100 | 1.11 | 100 |
| $5 \times 10^{-4}$ ..... | 0.95 | 87 | 0.86 | 78 |
| $1 \times 10^{-3}$ ..... | 0.93 | 85 | 0.76 | 69 |
| $2 \times 10^{-3}$ ..... | 0.87 | 79 | 0.74 | 67 |
| $4 \times 10^{-3}$ ..... | 0.79 | 72 | 0.57 | 51 |

Fig. 4

Chromatography of Alkaline Hydrolysate of Ribosomes From Corn Seedling Roots

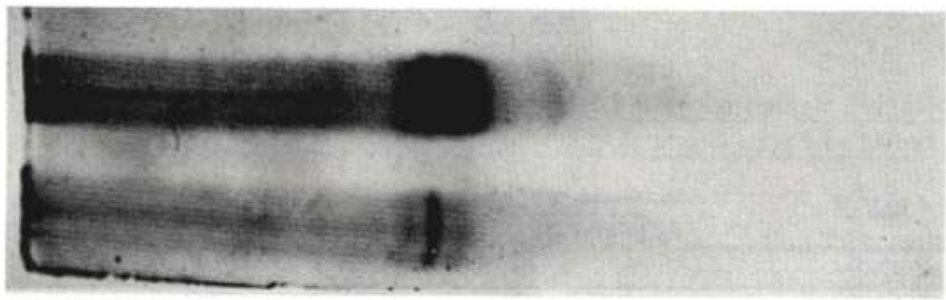



Dowex-1 x 8, 200-400 mesh, formate form, 1 x 25 cm of resin column, 3.5 ml per fraction, deionized water used as reference for measuring absorbancy (A) a=$H_2O$, b=0.23 N HCOOH, c=4.0 HCOOH. (5).

structure of ribosomal proteins. This was demonstrated by electrophoretic separation of acid-soluble ribosomal proteins from fluoride-treated corn seedling roots (Figure 5). The decreases in RNA to protein ratios in free and bound ribosomes (Table 6) therefore could be due to the reduction of ribosomal RNA (Table 4) and possible accumulation of structurally altered ribosomal protein components (Figure 5).

Fig. 5

Electrophoretic Patterns of Acid Soluble Ribosomal Proteins in Polyacrylamide Gel



The proteins migrated from right (cathode) to left (anode). The upper pattern represents control proteins and the lower one the sample from fluoride ($4 \times 10^{-3}$ M) - treated material (5).

During seed germination, corn plants depend on the endosperm and scutellar tissues for the raw materials for germination and growth. Three major types of hydrolytic enzymes such as amlyases, proteinases, and esterases (lipase and phosphatase) are responsible for hydrolysis of carbohydrates, protein, fats and phosphates, respectively (8). Plant seeds contain 50 to 88 percent of the total organic phosphate in the form of phytin (9). According to Ergle and Guinn (10) the increasing orthophosphate contents coupled with the diminishing amounts of phytin in germinating seeds parallel the increasing RNA and DNA contents during the growth of embryonic plants. Therefore, the effect of fluoride on phytase enzyme during corn germination and subsequent growth was investigated.

The author (11) observed that the highest total phytase activity is located at the 1700 x g fraction of endosperm-scutellum tissue homogenate of corn seedlings. The Michaelis constant (Km) of this enzyme is $0.91 \times 10^{-4}$ moles/liter. In addition, the action of various salts on phytase activity was tested. The enzyme activity was inhibited most by sodium fluoride (Table 7). To determine the minimum range of fluoride concentrations which inhibits phytase activity, the author assayed the enzyme activity in the presence of various fluoride concentrations (Table 8). One-tenth to ten mM sodium fluoride inhibits the enzyme activi-

TABLE 7

Effect of Various Salts on Phytase Activity

| Substance added | Activity (phytate P released) | % of control | Substance added | Activity (phytate P released) | % of control |
|---|---|---|---|---|---|
| | γ/hr./ml. enzyme | | | γ/hr./ml. enzyme | |
| Control | 103.0 | 100 | Sodium nitrate | 105.1 | 102 |
| Sodium fluoride | 48.4 | 47 | Calcium chloride | 124.6 | 121 |
| Sodium sulfate | 104.0 | 101 | Potassium cyanide | 98.8 | 96 |
| Magnesium sulfate | 101.9 | 99 | Sodium azide | 112.2 | 109 |

*Refer to reference 11 for assay conditions

TABLE 8

Effects of Various Concentrations of Sodium Fluoride on in vitro Phytase Activity

| Fluoride concentrations | Phytase activity (P released) | |
|---|---|---|
| mmoles | γ/hr./ml. enzyme | % of control |
| 0 ..... | 98 | 100 |
| 0.05 ..... | 93 | 95 |
| 0.1 ..... | 85 | 87 |
| 0.5 ..... | 81 | 82 |
| 1 ..... | 75 | 77 |
| 5 ..... | 52 | 53 |
| 10 ..... | 45 | 46 |

*Refer to reference 11 for assay conditions.

ty in vitro by 87 to 46% of control level. The action of such a low amount of fluoride suggests possible direct inhibition of this enzyme activity by fluoride in vivo. Fluoride also prevents the release of phytin phosphorus of endosperm-scutellar tissues during corn seed germination and growth (Table 9). This fact again implies the inhibitory action of fluoride on phytase enzyme activity in vivo. The breakdown of organic phosphate decreases as the fluoride concentration in the germination medium increases. The release of orthophosphate from phytin is blocked by fluoride most significantly at the 24- to 48-hour germination period. The kinetics of phytin phosphorus during seed germination (Table 9) is therefore related to the general pattern of growth inhibition caused by fluoride (Figure 1).

In 1966, Chang (1) demonstrated that fluoride accelerates the aging process in corn roots as well as inhibits growth of roots. This was accompanied by microscopic observation of the levels of vacuolated cortical cells of root tissue

TABLE 9

Effect of Various Concentrations of Fluoride on Phytase Contents of Endosperm-Scutellar Tissues During Corn Seed Germination and Growth (11)

| Germination media | Phytate P released Germination and growth period | | | | |
|---|---|---|---|---|---|
| | 0 h | 24 h | 48 h | 72 h | 96 h |
| | γ/g.* | γ/g. | γ/g. | γ/g. | γ/g. |
| Water | 0 | 135 | 492 | 1,089 | 1,886 |
| NaF | | | | | |
| 0.1 mM | 0 | 25 | 217 | 915 | 1,820 |
| 1 mM | 0 | 18 | 30 | 725 | 1,719 |
| 10 mM | 0 | 10 | 24 | 152 | 715 |

*Initial dry weight of seeds.

as an index of tissue aging. The number of such cells between 500 μ and 750 μ distant from root tips is related to fluoride treatment (Table 10).

TABLE 10

Effect of Fluoride on Cell Vacuolation in Corn Seedling Roots

| Experiment number | Number of cortical cells counted | Number of vacuolated cells counted | Vacuolated cells (%) |
|---|---|---|---|
| E-0 | 1612 | 14 | 0.9 |
| E-1 | 1408 | 110 | 7.8 |
| E-2 | 1638 | 186 | 11.3 |
| E-3 | 1392 | 208 | 14.0 |
| E-4 | 1320 | 266 | 20.2 |

For explanation of E-0 to E-4, see Table 1. Number of cortical cells was counted between 500 μ and 750 μ distant from the root tip of longitudinal root section, and the number of vacuolated cells in this same area by 400x microscopic magnification. Each value was the average of three determinations.

A decrease in RNA and protein content, and a decline in the capacity to synthesize these metabolic constituents during the aging process was shown by Srivastava (12). He (13) also suggested that protein synthesis is directly related to polysome content. Therefore, we investigated the amount and particle distribution of ribosomes and of the alterations associated with the ribosomal compo-

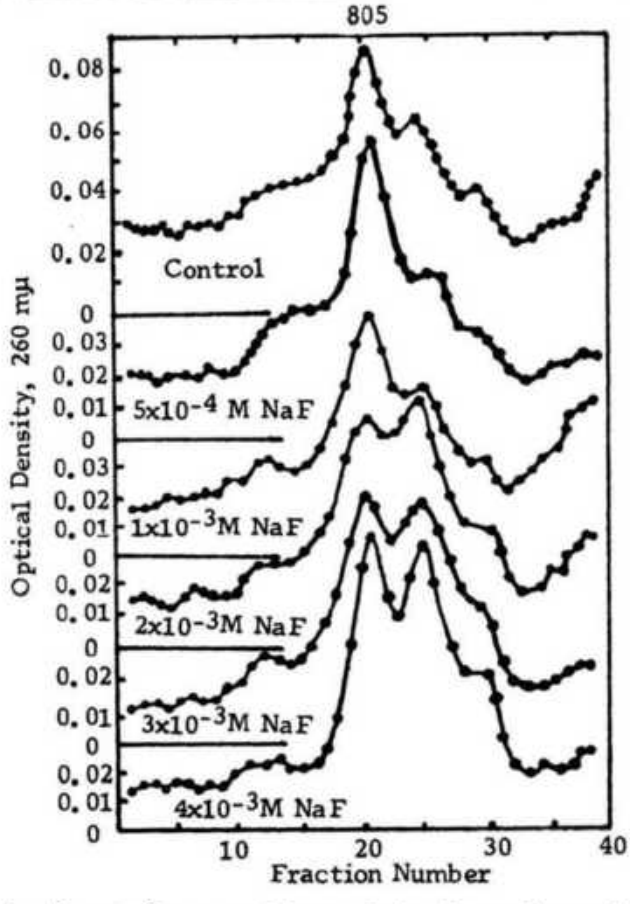
nents in fluoride-treated corn roots.

This study revealed (14) that fluoride causes a reduction in the total RNA and protein content of ribosomes in corn roots (Table 11). In addition, the analyses by sucrose density gradient revealed that fluoride induces a reduction of ribosome population in which a decrease in polysome level is accompanied by a combined increase in number and concentration of monosomes and subunits (Figure 6 and Table 12).

To find the factor which is responsible for the dissociation of polysomes, the influence of fluoride on ribonuclease activity in corn roots was studied (14). The assays of subcellular distribution of ribonuclease activity in fluoride-treated corn roots revealed that all subcellular components except plastid fraction show

Fig. 6

Sucrose Gradient Sedimentation Profiles of Ribosomes Isolated From Control Corn Roots and From Varying Concentrations of NaF




$0^1$, $0^2$, $0^3$, $0^4$, $0^5$ and $0^6$ are positions of abscissae for sedimentation profiles of samples a, b, c, d, e, and f, respectively. Each experiment is the result from 1 ml of ribosome sample having an absorbance of 3 optical density units at 260 mμ. Direction of sedimentation is from right to left (14).

TABLE 11

Amounts of Ribosomes From Control and Fluoride-Treated Corn Roots

| Treatment | Ribosomes* Fresh weight of roots (mg/g) | % of control |
|---|---|---|
| Control | 1.98 | 100 |
| $5 \times 10^{-4}$ M NaF | 1.51 | 76 |
| $1 \times 10^{-3}$ M NaF | 1.37 | 69 |
| $2 \times 10^{-3}$ M NaF | 1.29 | 65 |
| $4 \times 10^{-3}$ M NaF | 1.34 | 68 |

* Ribosomal contents were determined by the sum of RNA and protein contents analyzed (14).

TABLE 12

Relative Amount of Different Ribosomal Components Isolated from Control and Fluoride-Treated Corn Roots

| Treatment | Percentage absorbance of total components (V) at 260 mμ | | | | | |
|---|---|---|---|---|---|---|
| $10^{-3}$ x M Naf | I* | II | III | IV | V | VI |
| 0.0 | 44.4 | 26.5 | 15.7 | 13.4 | 100 | 55.6 |
| 0.5 | 39.1 | 34.0 | 15.4 | 11.5 | 100 | 60.9 |
| 1.0 | 37.9 | 30.3 | 19.1 | 12.7 | 100 | 62.1 |
| 2.0 | 34.1 | 21.9 | 31.7 | 12.3 | 100 | 65.9 |
| 3.0 | 30.6 | 26.4 | 27.2 | 15.8 | 100 | 69.4 |
| 4.0 | 26.3 | 31.3 | 25.3 | 17.1 | 100 | 73.7 |

To obtain these values, the areas under the peaks shown in Fig. 1 were integrated.

* Particles heavier than monomer (fractions 1-17), (II) monosomes (fractions 18-22), (III) subunit A (fractions 23-26), (IV) subjunit B (fractions 27-32), (V) sum of four components, total components (I + II + III + IV), (VI) sum of three components (II + III + IV) (14).

progressive increases in activity with increasing fluoride concentrations. However, fluoride increases mainly the specific enzyme activity associated with ribosomal protein (Table 13). The enhanced activity is most likely due to enzyme activation caused by fluoride. Hanson (15) indicated that this enzyme activity in vivo is normally controlled by the ratio of potassium, calcium, and magnesium ions in corn

roots. A complex formation of fluoride with magnesium ions, also, was shown by Miller (16).

The author (14) found that the fluoride-increased activity of ribosomal ribonuclease, as expected, degrades ribosomal RNA components. This was demonstrated by the fact that ribosome fractions analyzed by sucrose density gradient contain a mixture of heterogeneous RNA components (Figure 7). A possible destruction of messenger RNA, therefore, may be one of the factors which are linked with the disintegration of polysomes in corn roots treated with fluoride. This finding from higher plants contrasts with the dissociation of polysomes and preservation of messenger RNA observed in reticulocytes of animal cells treated with fluoride by Marks (17). Since Chang (2) has demonstrated that fluoride induces accumulation of ATP in corn roots, the decrease in ribosomal content may be secondary to the restricted supply of high energy phosphate compounds.

TABLE 13

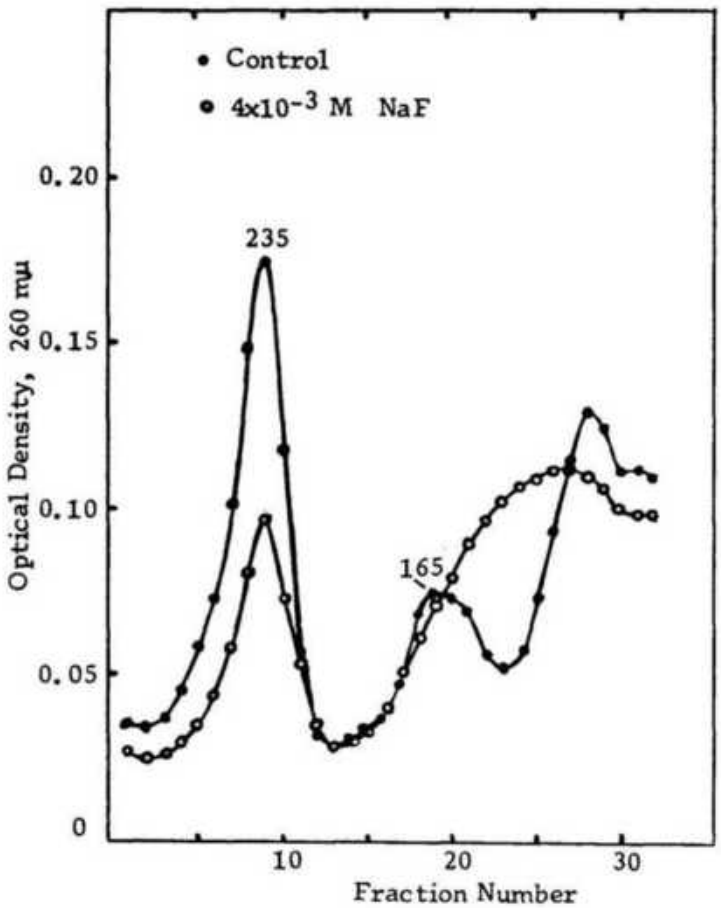
Cytoplasmic Subcellular Distribution of Ribonuclease Activity in Control and Fluoride-Treated Corn Roots

| Fraction* | | Control | NaF concentrations (M) $1 \times 10^{-3}$ | $2 \times 10^{-3}$ | $4 \times 10^{-3}$ |
|---|---|---|---|---|---|
| F-1 | | | | | |
| | A | 16.0 | 29.3 | 20.0 | 14.7 |
| | B | 50.0 | 63.0 | 63.5 | 53.8 |
| | C | 100.0 | 126.0 | 127.0 | 108.0 |
| F-2 | | | | | |
| | A | 8.8 | 17.6 | 13.6 | 14.0 |
| | B | 22.2 | 43.8 | 46.3 | 51.3 |
| | C | 100.0 | 197.0 | 209.0 | 231.0 |
| F-3 | | | | | |
| | A | 12.0 | 28.3 | 29.3 | 31.6 |
| | B | 27.4 | 83.5 | 107.3 | 128.5 |
| | C | 100.0 | 305.0 | 392.0 | 469.0 |
| F-4 | | | | | |
| | A | 205.3 | 479.1 | 422.4 | 370.9 |
| | B | 134.3 | 311.1 | 372.5 | 380.8 |
| | C | 100.0 | 232.0 | 277.0 | 284.0 |

*Fractions: F-1, plastid; F-2, mitochondria; F-3, microsome; F-4, soluble (arbitrary designation). Units: A, micrograms RNA hydrolyzed per minute; B, micrograms RNA hydrolyzed per minute per milligram protein; C, percent specific enzyme activity (14).

Fig. 7

Size Distribution of Ribosomal RNA From Control Corn Roots and Those Treated With $4 \times 10^{-3}$ M Sodium Fluoride




Ribosomes were isolated and RNA was extracted with cold phenol and sodium lauryl sulfate procedure. Sucrose density gradients (28 ml) were prepared from 5-20% sucrose in 0.05 M sodium phosphate buffer (pH 6.8) containing 1 mM $MgCl_2$. Samples having bsorbances of 5 optical density units at 260 mu were overlayered. After centrifugation for 15 hours at 25,000 rpm, 28 drops were collected for a fraction. Fractions were measured at 260 mu after dilution. (•) Control, (o) $4 \times 10^{-3}$ M NaF. Refer to reference (14) for additional procedures in detail.

The dissociation of polysomes occurring in aged tissue such as fluoride-treated corn roots is consistent with findings in higher plants aged by other causal factors. Srivastava and Arglebe (18) showed that polysomes and ribosomes are lost in senescing barley leaves. The author (19) also has recently discovered ozone's preferential disintegration of chloroplast polysomes and ribosomes in pinto bean leaves and related it to the ozone-accelerated aging process in higher plants (20). However, the mechanism associated with this finding differs from that linked with the polysome dissociation in corn roots treated with fluoride (14). Ozone directly disintegrates chloroplast ribosomes in pinto bean leaves by the interaction with sulfhydryl groups of chloroplast ribosomal protein (21).

This review is limited to a consideration of biochemical and biophysical data on growth and aging in sodium fluoride-treated corn seedlings from the author's five recent publications (1966-1970).

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# THE MUTAGENIC ACTIVITY OF INORGANIC FLUORIDE COMPOUNDS

by

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(Abstracted from the Gig. Sanit., 37:9-12, 1972)

In order to evaluate the mutagenic action of inorganic fluoride compounds the authors studied the occurrence of chromosomal aberrations in bone marrow and the mytotic activity of epitheleal cells of the cornea in white female rats. The animals were exposed six hours daily for five months (except on Sundays) to cryolite ($Na_3AlF_6$) in concentrations of 3, 1 and 0.5 mg/m$^3$ (as calculated for fluoride ion) and to a mixture of 0.5 mg/m$^3$ of cryolite and 0.35 mg/m$^3$ of hydrogen fluoride. Such levels are encountered in the air in the electrolytic manufacture of aluminum.

## Method

For cytogenetic analysis, four animals were injected with colchicine an hour before being killed and their corneal epithelium was compared with that of uncolchicinized animals to determine mytotic activity. Bone marrow preparations were stained with azure-eosin. All types of chromosomal and chromatid aberrations were studied in the metaphases. For analysis only undamaged metaphasal plates with the typical number of chromosomes for a given variety (42) were selected. Instances of hyperploidia were also taken into account. Gaps were not included in the number of aberrations. An average of eighty metaphasal plates were analyzed for each rat. Total corneal preparations were stained with hematoxilin according to Bemer's method. Mytotic activity was determined by the number of mytoses per 15,000 cells in each cornea. The experimental results were processed by dispersion analysis.

## Results

The combination of cryolite with hydrogen fluoride had no effect on the mytotic activity of the corneal epithelium. However at the end of the recovery period a significant increase in mytotic activity in the corneal epithelium was noted in all experimental animals which was attributed to seasonal variations in the rate of cell division. The phase coefficient which is equivalent to the ratio between the portion of cells in the prophase and metaphase and the portion of cells in the anaphase and telephase, which was practically the same in all variants of the experiments, averaged 1.4. Therefore the authors concluded that there was no cytostatic effect on the cells of the cor-

From the Sverdlovsk Institute of Labor, Hygiene and Occupational Diseases

neal epithelium even though fluorides are known to retard cell division in tissue cultures significantly.

TABLE 1

Mytotic Activity in the Corneal Epithelial Cells in 0/00

| Period of Experiments | Mytotic Activity ($\bar{x} + S_{\bar{x}}$) | | | | |
|---|---|---|---|---|---|
| | control | cryolite in mg/m$^3$ 0.5 | 1.0 | 3.0 | cryolite 0.5 mg/m$^3$ +HF 0.35 mg/m$^3$ |
| At end of 5 months | 5.4 ± 0.5 | 5.5 ± 0.3 | 5.8 ± 0.4 | 6.0 ± 0.9 | 5.9 ± 0.4 |
| 1 month later recuperation | 6.0 ± 0.5 | 6.0 ± 0.3 | 6.3 ± 0.3 | 6.3 ± 0.2 | 6.9 ± 0.7 |

With respect to bone marrow, the authors found an increase in the percentage of aberrant cells only after inhalation of the highest concentration of cryolite (3mg/m$^3$) and following inhalation of a mixture of cryolite and hydrogen fluoride. The fact that almost 3 1/2 times as many aberrations occurred in animals poisoned with a mixture of cryolite (0.5 mg/m$^3$) and hydrogen fluoride (0.35 mg/m$^3$) as in those poisoned solely by cryolite (0.5 mg/m$^3$) indicates that the cytogenetic effect must be ascribed primarily to fluoride rather than to aluminum or sodium: Both are constituents of cryolite. Furthermore their concentrations in the experiment were identical whereas the fluoride concentration in the first case was 1.7 times as high as in the second. Moreover, fluoride which enters the respiratory organs in the form of hydrogen fluoride is fully absorbed by them. On the other hand, fluoride is assimilated to a much lesser degree and with much greater difficulty when it enters the organism in the form of cryolite (M. S. Sadilova and E. G. Plotko).

TABLE 2

Number of Cells (in %) With Chromosomal Aberrations in the Bone Marrow of Rats Subjected to Poisoning With Fluoride Compounds

| Index | Experimental Variant | | | | |
|---|---|---|---|---|---|
| | control | cryolite in mg/m$^3$ 0.5 | 1.0 | 3.0 | cryolite (0.5 mg/m$^3$) + HF (0.35 mg/m$^3$ |
| Number of cells with chromosomal aberrations, $x - S_x$ | 1.40 ± 0.88 | 1.50 ± 0.56 | 2.40 ± 0.59 | 6.50 ± 0.66 | 5.10 ± 0.55 |
| P values | --- | 0.05 | 0.05 | 0.001 | 0.01 |

Most of the aberrations caused by the fluoride compounds were of the chromatid type and more than 70% of the damage was represented by individual fragments.

The authors concluded that fluoride stimulates the formation of mutagenic metabolites in the organism of rats. They further pointed out that the comparatively weak mutagenic activity of inorganic substances which was observed in these experiments does not detract from their potential genetic danger to humans. With long exposure even weak mutagens can cause considerable damage to the mechanism of inheritance because genetic changes are practically irreversible.

During their experiments the authors also observed development of retardation processes in the central nervous system, supression of the activity of a number of ferments, morphologic changes in the intermal organs and tissues. Cryolite in the concentration of 0.5 mg/$m^3$, however, revealed no such toxic effects.

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## EFFECT OF REDUCED FLUORIDE INTAKE BY MICE ON HAEMATOCRIT VALUES

by

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(Abstracted from Nature New Biology, 240:218- 219, 1972)

The fact that fluoride is ubiquitous in natural foods and water has hampered attempts to demonstrate its dietary essentiality. It is difficult to prepare a diet devoid of fluoride. Commercial rodent food contains between 40 and 60 ppm fluoride.

The authors used a diet low in fluoride consisting mainly of milk and cereals (48% whole wheat flour and 21.6% skim milk which contained 0.1-0.3 ppm fluoride). This diet interfered markedly with haemopoiesis, a condition which was prevented by the addition of fluoride.

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The study was concerned with the influence of fluoride intake on two forms of stress on the haemopoietic system in mice, namely the period of pregnancy and that of rapid growth before weaning.

## Method

Twenty weanling female albino mice received dionized water and 16 animals water containing 50 ppm of fluoride as sodium fluoride. All mice were mated at 10 weeks of age and blood from the tail was taken into pre-heparinized micro-haematocrit tubes two days before and 5, 10, 15 and 19 days after mating. The red cell-plasma volumes were measured after centrifugation.

Ten "low fluoride" and 7 "high fluoride" mice were conceived from the mating. The body weight of the two groups before mating was not affected nor was the litter size influenced by the maternal intake of fluoride. Both groups appeared to be in normal health. In the low fluoride group the haematocrit values of adult mice before mating was 49.9 $\pm$ 1.2%. These values declined and after 19 days of pregnancy the mean values were significantly lower in the low fluoride group than in the high fluoride group ($P < 0.005$).

Similar studies were made of the pups born of the mothers in both groups. Blood was collected from the tails of the newborn pups and from pups aged 5, 10, 20 and 60 days. For the latter determinations, the pups were weaned at 21 days of age and given the same food and water as their mothers. Each fluoride group at each age constituted 20 pups, except at 60 days when 8 pups in each group were used. Only 2 pups were taken from any one litter at a given age.

The maternal fluoride intake did not affect the weight of the pups at birth or the increase in weight with age. The fluoride level in bones in the two groups differed markedly: In the low fluoride group the fluoride concentration averaged 0.007% of the ash at birth and 0.009% at 60 days of age. The corresponding values in the high fluoride group were 0.08% and 0.26% (800 and 260 ppm).

At birth and at 20 days of age the haematocrit values remained approximately constant in pups from mothers whose fluoride intake was high. By 60 days of age the haematocrit had increased to 51.5 $\pm$ 0.85%, a value which is similar to that recorded by dams before mating. In the low fluoride group, the haematocrit declined from birth (38.5 $\pm$ 1.43%) to 10 days of age (25.1 $\pm$ 0.08%). Following weaning these values increased to 53.9% $\pm$ 0.77% by 60 days of age. However the haematocrit values in the low fluoride pups were significantly less than those in the high fluoride pups at 5 days but not significantly different at birth and at 60 days of age.

## Discussion

The haematocrit values of both the non-pregnant adult female mice and the newborn pups were influenced by the low fluoride intake. Under the

stress of pregnancy and rapid growth in the newborn period, the low fluoride mice developed a severe anemia. The authors suggested that fluoride may play an unidentified role in haemopoiesis which is only manifest during stress on the haemopoietic system. On the other hand, fluoride may be required for intestinal absorption or utilization of another factor involved in haemopoiesis. In the latter case, fluoride might affect the excretion of iron or other trace elements obtained from the milk. A low concentration of iron in the milk of "low fluoride" mothers and reduced intestinal absorption of iron in all the pups could produce anemia.

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## EFFECTS OF DIALYSATE CALCIUM AND FLUORIDE ON BONE DISEASE DURING REGULAR HEMODIALYSIS

by

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(Abstracted from J. Lab. Clin. Med., 79:204-214, 1972)

In a previous study, the authors had noted an increase in calcification of soft tissues and in hypercalcemia following long-term hemodialysis in kidney patients. This condition developed almost exclusively when the individuals were treated with a low calcium dialysate, as a rule less than 5.7 mg per 100 ml. Also the authors had found elevated serum immunoreactive parathyroid hormone (iPTH) in patients with bone disease which correlated with the low serum-dialysate calcium concentration and with high serum phosphates. Furthermore they had recognized that fluoride in the dialysate at a concentration of 50 $\mu$M (1 ppm) induced multiple fractures in spite of the presence of calcium in high concentrations (7.2 mg per 100 ml). In individuals with clinically apparent bone disease, the concentrations of ser-

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um fluoride were generally higher than in patients without calcifications. In the current study, the authors investigated the effect of various concentrations of calcium and fluoride in the dialysate on bone resorption and bone mineralization.

Fourteen patients aged 19 to 56 were selected who had had no previous prolonged periods of immobilization, no steroid, thyroid, androgen or high-dose vitamin D therapy for at least 1 year prior to the study and had been free of any other skeletal disorder. Seven patients received dialysis with low calcium concentrations (5 to 5.7 mg per 100 ml) and 7 with high concentrations (6 to 7.4 mg per 100 ml). Two in each group were exposed to dialysate high in fluoride. For histologic examination, bone biopsy specimens from the iliac crest were taken at the beginning and at the end of 2 to 9 months' treatment.

At the beginning of the treatment, an increase in bone resorption was observed in all but one patient. At the termination of the treatment, bone resorption had increased in 5 of the 7 exposed to low calcium dialysate and had decreased significantly in all exposed to high calcium dialysate. In all but one individual of each group, bone formation was below normal and did not appear to change between the first and second biopsies.

With respect to fluoride, all four patients exposed to high fluoride dialysate showed excessive osteoid formation with little change during the three to nine months' interval. In five additional patients in whom single biopsies were carried out, who had been maintained by dialysis for 13 months or longer, osteoid formation was 9 times greater in those exposed to high fluoride dialysis (50 μM) than in those exposed to lower concentrations (5 μM).

This study established that high calcium concentrations in the dialysate reduces significantly resorption of bone substance whereas a low concentration of calcium in the dialysate enhances bone resorption. On the other hand, high fluoride concentrations in the dialysate increase the amount of osteoid tissue but, by itself, fluoride does not appear to enhance calcification of bone.

The authors conclude that the use of fluoride-free dialysate decreases the risk of severe osteomalacia. Neither of the two measures, either addition of calcium or the elimination of fluoride from the dialysate is likely to affect bone formation.

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Correction:

Volume 6, page 81: In Table 3 in the article by E. Auermann "Fluoride Uptake in Humans", the values should be mg/l not μg/l.

# EFFECT OF SODIUM FLUORIDE AND SODIUM PYRUVATE ON PALATAL DEVELOPMENT IN VITRO

by

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(Abstracted from the Anat. Rec., 171:39-52, 1971)

Little is known of the biochemical reactions involved in the normal development of the palate. The author studied one of the metabolic functions likely to be involved - namely the carbohydrate catabolism and energy metabolism - by the administration of compounds which are known to affect this pathway. This article is concerned with the addition of sodium fluoride to the medium on which palatal explants are grown. In addition the effects of sodium pyruvate on explants treated with fluoride and non-treated palates is described.

## Methods

Female Sprague-Dawley rats were used for all experiments. The females were mated and on the 16th day post-mating (when fusion of the palate in this species normally occurs), the pregnant female was killed by cervical dislocation and the embryos were dissected. The palatal explants were cultured for 72 hours and at the end of the incubation period the palates were examined at low magnification to determine the extent of fusion and then sectioned and stained for histological examination.

The defined medium used for culture was Leibovits Medium L-15 (Leibovits, '63). The experimental culture media was prepared by adding, a) sodium fluoride, b) sodium pyruvate and, c) a combination of fluoride and pyruvate to the medium. The final concentrations of sodium fluoride used were 2.0-, 2.5-, 3.0-, 3.5-, 4.0 and $4.5 \times 10^{-3}$ M. Higher concentrations caused extensive tissue necrosis.

## Results

Controls: Growth of palatal explants on semi-defined medium resulted in complete fusions of 84% of the palates, partial fusion of 11% and non-fusion of 5%.

Sodium Fluoride: The extent of fusion was directly related to the concentration of fluoride added, ranging from complete fusion of all palates at the lowest level of fluoride ($2 \times 10^{-3}$ M) to an incidence of 90% non-fusion at the highest level ($4.5 \times 10^{-3}$ M).

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TABLE 1

Effect of Sodium Fluoride On Palatal Fusion In Vitro

| Concentration of Na F (M) | No. palates cultured | Fused | Partially fused | Not fused |
|---|---|---|---|---|
| | | % | % | % |
| 0 | 64 | 54 ( 84) | 7 (11) | 3 ( 5) |
| $2.0 \times 10^{-3}$ | 10 | 10 (100) | --- | --- |
| $2.5 \times 10^{-3}$ | 14 | 12 ( 86) | 2 (14) | --- |
| $3.0 \times 10^{-3}$ | 40 | 7 ( 18) | 26 (64) | 7 (18) |
| $3.5 \times 10^{-3}$ | 5 | --- | 18 (51) | 17 (49) |
| $4.0 \times 10^{-3}$ | 15 | --- | 2 (13) | 13 (87) |
| $4.5 \times 10^{-3}$ | 10 | --- | 1 (10) | 9 (90) |

Sodium Pyruvate: Sodium pyruvate at a concentration of $5 \times 10^{-2}$ M produced explants noticeably larger than controls and fusion occurred from 12 to 24 hours earlier. Pyruvate levels of $10^{-1}$ M delayed time of fusion and lowered the incidence of fusion in all cases.

Sodium Fluoride-Sodium Pyruvate: Very low pyruvate levels ($10^{-3}$ - $10^{-2}$ M) had no effect on fusion. However, $5 \times 10^{-2}$ M pyruvate reversed the effect of $3.0 - 3.5 \times 10^{-3}$ M fluoride and permitted a large percentage of fusions to occur. If maximal fluoride concentrations and intermediate or high pyruvate levels were present simultaneously in the medium, some explants were capable of complete fusion and the incidence of partial fusions was greater.

Palate Transfers: Palates transferred from a medium containing $3.5 \times 10^{-3}$ M fluoride to a fluoride-free medium demonstrated a limited ability to recover and eventually fused when no excess pyruvate was present in the recovery medium. However, addition of $5 \times 10^{-2}$ M pyruvate to the second medium greatly enhanced the incidence and extent of fusion.

The effects of a higher initial fluoride concentration ($4.0 \times 10^{-3}$ M) were not as readily reversed by transfer to a medium with no excess pyruvate. An excess pyruvate level in the second medium ($5 \times 10^{-2}$ M) again proved most effective in that instances of complete fusion were found and total nonfusion did not occur.

The experiments demonstrate an effect of fluoride on embryonic palatal explants which is characterized by "retardation of shelf growth toward the midline, precluding normal fusion". The effect can be reversed by pyruvate. The mechanism of action of fluoride and pyruvate under these conditions was not determined.

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