April, 1977 Vol. Ten No. Two

FLUORIDE

OFFICIAL QUARTERLY JOURNAL

OF

INTERNATIONAL

SOCIETY for

FLUORIDE

RESEARCH

8th CONFERENCE
May 29 thru May 31
Oxford, England
See inside for details

Vol. Ten No. Two April, 1977 Pages 45-94

FLUORIDE

Quarterly Reports

Issued by

THE INTERNATIONAL SOCIETY FOR FLUORIDE RESEARCH

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Due to the sudden departure of Dr. H.A. Cook who was in charge of arrangements, the Eighth Conference of the International Society for Fluoride Research will be held May 29-31 in Oxford, England, at the Oxford Union instead of in London. For reservations contact immediately the Old Parsonage Hotel, 3 Bambury Road, Tel. 54843 (Single rooms L 4.75, Double L 9.00) or St. Giles Hotel, 56 St. Giles, Tel. 54620 (Single L 5.50, Double L 11.00) since rooms are difficult to secure. Reservations can also be made through Mrs. H.D. Larive, "Avalon", Badger Lane, Hinksey Hill, Oxford OX1 5BL England, Tel. Oxford 0865-730486. Please state price range and number of nights. An extensive program is scheduled with papers on the effect of fluorides on human health and on the botanical, veterinary and dental aspects of fluoride research. Please make reservations immediately.

FLUORIDE is published quarterly by THE INTERNATIONAL SOCIETY FOR FLUORIDE RESEARCH, INC.,

SUBSCRIPTION RATES - Price per annum in advance including postage $18.00; Single copies $5.00.

MANUSCRIPTS for publication should be submitted in English, double-spaced with generous margins. References should be arranged according to the order in which they are cited in the text, and written as follows: Author, title, journal, volume, pages and year. Each paper must contain a summary of not more than 12 lines.

Contributors will receive copies of the issue of FLUORIDE containing their paper, free of charge.

FLUORIDE is listed in
Current Contents Agricultural
Food and Veterinary Sciences

EDITORIAL

SKELETAL FLUOROSIS NEAR FLUORIDE-EMITTING FACTORIES

On page 89 of this issue the abstract of an article by Schmidt reveals x-ray evidence of skeletal fluorosis among persons residing close to a fluoride-emitting aluminum smelter.

That populations might be at risk due to fluoride emission from an industrial plant was first suggested in 1939 by Klotz, a German clinician. An infant residing near a phosphate fertilizer factory (1) had gastric disturbances simulating pyloric stenosis (vomiting, abdominal cramps) and muscular spasm, especially in the upper parts of the legs. The x-rays showed thickening of the periosteum of the long bones of the kind described in skeletal fluorosis. The rigidity of muscles had gradually extended over the entire body and the child expired at six months of age during a convulsion. Three additional children of a family in the same area who had mottled, stained, and fragile teeth, which Klotz diagnosed as dental fluorosis were similarly afflicted, although less severely.

In 1946, Murray and Wilson coined the term "neighborhood fluorosis" for a disease which they encountered in nine members of a farmer's household near a fluoride-emitting ironstone works in South Lincolnshire, England (2). The symptoms, which developed after 1 to 14 years of exposure in the polluted area, were persistent aches and pains, headaches, blurred vision, stiffness in muscles and joints, gastric upsets, cough, and a tendency to frequent upper respiratory infections. The x-rays of bones, however, were negative. The fluoride content of the urine ranged from 1.6 to 4.2 ppm. On the side facing the factory, the windows were etched, a typical sign of air pollution by hydrofluoric acid. Seven horses and eleven cows had died of fluoride poisoning. Grass samples within a few hundred yards of the burning ironstone mounds contained over 2000 ppm fluoride in dry matter, and the straw on the exterior of a stack about 0.5 mile away showed 490 ppm. Unfortunately, there was no follow-up on the subsequent fate of the nine cases.

The same disease was experienced in 1955 by a farm family of three residing close to an aluminum smelter in Troutdale, Oregon (3). During litigation of this case, muscular pains, general fatigue, and arthritis in conjunction with liver and kidney damage and with hypothyroidism were recorded. The court decision found a definite relationship between the disease and fluoride ingested from food grown in the contaminated area. Neither the British nor the Oregon patients displayed signs of skel-

etal changes which are characteristic of fluorosis.

The hematological findings of neighborhood fluorosis were investigated in 1969 on children 6 to 14 years old residing close to an aluminum factory in Czechoslovakia. Significantly lower hemoglobin but higher than normal red blood cell values were recorded, a condition encountered in certain lung diseases due to inhalation of toxic agents (4).

Waldbott (5) has elaborated on the initial stage of this disease. In residents of four widely separated areas he encountered a wide spectrum of manifestations which were identical with the syndrome of preskeletal fluorosis due to fluoridated water.

Typical skeletal changes in neighborhood fluorosis were first reported by Herbert et al. (6) in 1967 in a 46 year old worker who had been residing 10 km distant from an aluminum factory and who had not been exposed occupationally to fluoride.

Subsequently in a systematic survey of 2842 residents of the city of Dohna, DDR, Schmidt (7) established that 29 persons (24 men, 5 women) were afflicted with skeletal fluorosis. None of these persons were employed at the nearby hydrogen fluoride plant. They were residing 350 to 2100 m distant from the plant. Near the smelter, the air contained from 0.52 to 0.75 mg/m^3 (Maximum Allowable Concentration 0.03 mg/m^3), leaves of fruit trees 119 to 580 mg/kg (dry) and hay 88 to 91 ppm (7). By bringing his findings to the attention of the medical profession, Schmidt has made an important contribution to the role of fluoride as an air pollutant. Protective measures initiated in the contaminated area have already led to amelioration of the environmental damage.

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FLUORIDE EMISSIONS OF COAL-FIRED POWER PLANTS AND THEIR IMPACT UPON PLANT AND ANIMAL SPECIES

by

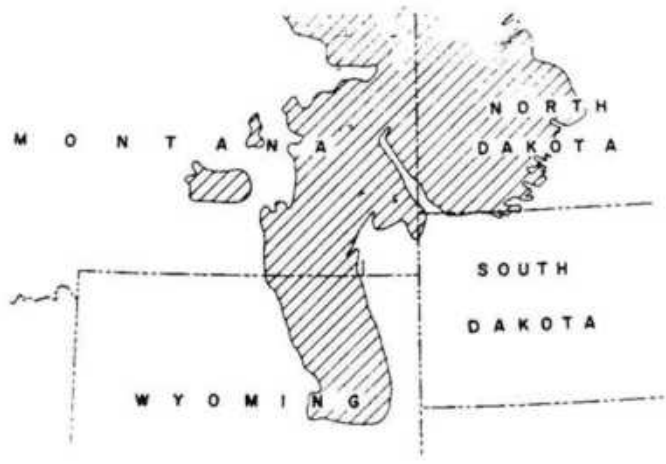
P.C. Tourangeau, C.C. Gordon and C.E. Carlson
Missoula, Montana

SUMMARY: The 40 lbs/day fluoride emissions from the Billings 180-megawatt coal-fired plant are substantial enough to accumulate at excessive levels to cause an impact upon the foliage of ponderosa pine and to increase the fluoride levels in the femurs of deer mice. The 40 lbs/day fluoride emissions from the Colstrip Units #1 and #2 will be released from 525 ft (161 meters) stacks and thus not fumigate and damage as seriously the ponderosa pine foliage of that area as at the Billings sites. Our past pre-operational baseline studies in the Colstrip area and the scavenging ability and partitioning of fluorides in the different portions of the needles of pondersosa pine indicate that the geographical extent of the trespass of Units #1 and #2 plume will not be difficult to evaluate in the future.

Introduction

During the past three years, we have conducted studies designed to determine the baseline, pre-operational conditions and to measure post-operational impacts resulting from coal-fired steam generation at 54 sites in a 5000 square mile portion of southeastern Montana.

Figure 1



INDEX MAP OF THE FORT UNION FORMATION IN MONTANA, WYOMING, NORTH DAKOTA, AND SOUTH DAKOTA

This study area lies within the Fort Union Basin (Fig. 1), which encompasses the Western portions of South and North Dakota, northeastern Wyoming, and the entire eastern third of Montana. Underlying the Basin is the Fort Union Formation, containing 1.3 trillion tons of coal, 40 percent of the coal reserves of the United States (1). Within the boundaries of Montana the strippable coal reserves are currently estimated to be over 31 billion tons. While the amount of fluoride contained within this vast quantity of coal is but an infinitesimal percent of the total coal tonnage which will be strip-mined to fuel coal-fired power plants located in the United States and other countries during the next 40-60 years, fluoride is, when released into the atmosphere, an extremely important pollutant. Very few past air pollution studies on coal-fired power plant emissions have included the impacts of the fluorides released during the burning of coal. This is unfortunate since fluoride accumulation and partitioning in plant tissues, especially species of pines, may assist researchers in locating other trace elements being emitted from coal-fired power plants.

To estimate the quantity of fluoride in the strippable coal reserves of Montana, we use here a conservative concentration of 40 ppm F^-, which is 10 ppm higher and 210 ppm lower than the concentrations of fluorides reported by state and federal agencies and the utility companies during the last 4 years of analyzing core samples of coal taken from southeastern Montana. At 40 ppm the 31 billion tons of strippable coal in eastern Montana contain 1,240,000 tons of fluoride. Of course only a small percentage of this fluoride should be released into the atmosphere if the utility companies install the "state of the art" abatement equipment which is available today. As we show from studies at Billings, Montana, however, it does not take many tons per year of atmospheric fluorides to cause an impact upon ponderosa pine Parkland-Savannah, which is the ecosystem of the 5000 square mile area around Colstrip, Montana, where our baseline biophysical studies were carried out prior to the start-up of two 350-megawatt coal-fired power plants during the last 11 months. These two plants, known as Colstrip Units 1 and 2, are owned and operated by the Montana Power Company and one West Coast utility company. They will, according to the companies' EIS (Environmental

Impact Statement) to Montana regulatory agencies, release into the atmosphere from two 525-foot stacks 7 tons of fluoride each year, or 40 lbs/day, when running at full capacity.

Billings, Montana, which is 100 air miles (160 km) west from Colstrip, is the site of a 180-megawatt coal-fired power plant operated by the Montana Power Company. This small power plant burns coal mined from the Rosebud seam at Colstrip, which is the same coal being fired in Colstrip Units 1 and 2 at Colstrip, and has been in operation since 1967. Analyses of its stack emissions have been carried out by Montana's air pollution regulatory agency (Department of Health and Environmental Sciences) during the last 3 years. The results of their analyses show that the plant emits 1.9 to 2.8 lbs of fluoride/hour for an average of 54.5 lbs/day and 9.9 tons/year when operating at full capacity, and that the bottom ash contains 60-80 ppm, the precipitator ash contains 170 ppm, and the stack particulate emissions contain 1,631 ppm. The particulate fluorides released from this 180-megawatt power plant stack are partitioned in greater concentrations in and on the smallest particulate which escapes the inadaquate (second rate) abatement equipment installed in this plant.

In Billings, 500 and 800 meters from this 180-megawatt coal-fired power plant on a bluff called Sacrifice Bluff which has an elevation equal to the top of the power plant stack, ponderosa pine sampling sites were established by us during the last 1 1/2 years. These two sites, as far as understory plant species are concerned, are typical of our permanent ponderosa pine sampling sites located throughout the 5000 square miles around Colstrip, Montana. These two Billings sites received what we consider the maximum possible impact of the power plant emissions when the winds are blowing from the stack toward our sites. In addition to its fluoride emissions the plant injects 28.9 tons of SO_2 and 16 tons of nitrogen oxide into the atmosphere each day, along with substantial quantities of particulate. Also in Billings Montana, two oil refineries release fluorides and sulfur compounds into the atmosphere surrounding our study sites. Thus, a portion of the concentration of fluorides and sulfur found in and on the foliage of plant species, as well as the damage to the ponderosa pines at our Billings, Montana, sampling sites, is being partially emitted by other stationary sources besides the 180-megawatt coal-fired power plant.

In this paper we will briefly present the results of baseline studies of fluoride in ponderosa pine, one of the most susceptible species known to fluoride pollution. We include the results of studies conducted at Billings and other areas polluted by fluoride. We will also summarize the results of studies on the fluoride concentrations in bone tissues of deer mice collected in pristine and fluoride-polluted areas.

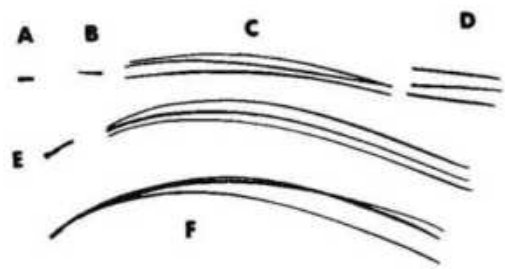
Methods and Materials

Each of our study sites are located on the most elevated terrain in its immediate vicinity (5 - 10 km). Five young and five old ponderosa pine trees were tagged at each site. Four branches were removed from each of two crown positions (upper half of tree and lower half of tree) on the side of the tree facing Colstrip. The following pine growth-health and chemical varibles were measured for each of the last four years of foliage growth (1972-75) to retain on the trees: (1) total sulfur and fluoride content, (2) total chlorophyll, (3) needle retention, (4) needle length, (5) fascicular cross-sectioned area, (6) moisture percentage, and (7) ten separate needle pathologies, which include fungal and insect damage, tip burn, basal necrosis, and scale beneath the fascicular sheaths of the needles.

Histological studies of needles manifesting the different pathologies were carried out independently by personnel of both the U.S. Forest Service and the University of Montana Environ. Studies Lab. Needles of a given age from each tree and crown position were prepared for chemical analysis. Fluoride analysis was carried out with the Orion fluoride specific ion electrode and sulfur analysis with the Leco induction furnace. Portions of selected ponderosa pine samples from study sites remote from, and close to, Colstrip, Montana, and our two Billings sites were sent to either the Ames, Iowa, ERDA National Laboratory or to the Battelle Pacific Northwest Laboratory at Richland, Washington, for trace element analysis by neutron activation.

Fluoride and sulfur analytical studies of partitioned ponderosa pine needles collected from five of our Colstrip sites located in close proximity (3 to 4.5 km) and at far distances (80 km) from Colstrip were carried out during the 1975 - 76 study period. Similar studies of partitioned needles were carried out at the two Billings sites on Sacrifice Bluff close to the 180-megawatt coal-fired power plant. Figure 2 depicts the different portions of the partitioned needles. The fascicular sheaths of the needles of pine species are composed of 2 to 5 layers of overlapping scale-like struc-

Figure 2 Separation of Various Needle Portions for Chemical Analysis



A= fascicular sheath separated from basal needle tissue; B=basal needle tissue which was previously covered by fascicular sheath; C=middle needle portion; D=needle tip portion--sometimes necrotic, sometimes healthy; E= fascicular sheath and needle base--analyzed separately to compare with analytical results of A & B above; F= whole needles analyzed for comparisons of A, B, C, D, and E.

tures (cataphylls) composed of thin-walled cells in early morphogenesis of the needle, which differentiate to thick-walled sclerenchymatous cells approximately 10 to 90 days after needle emergence with the outer cataphylls differentiating the earliest. During early development of the needle (1 to 2 weeks) in the spring, the fascicular sheath surrounds the entire needle and protects the thin-walled cells of the needle from external adverse conditions. As the apical external tissues of the needle differentiate into protective thick-walled cells (epidermis and hypodermis), they are pushed out beyond the protection of the fascicular sheaths by the production and growth of new cells, which are produced by the intercallary meristem located at the base of the needle where the cataphylls (scales) are attached to the needle and the stem. Once the growing needles emerge beyond the protection of the fascicular sheath, the sheath is very similar to a vase-like structure which can be a receptacle for all sorts of abiotic and biotic agents which are present in the environment of that needle. This vase-like fascicular sheath is what we believe to be the Achilles heel of the several pine species we have studied in various air polluted areas of the United States.

Results

Selected Growth-Health and Chemical Parameter Data

Foliage Data: Table 1 shows the results of the analysis of chemical and physical characteristics measured for foliage of four different years from all trees (10 trees/site), namely from both upper and lower crown positions at 16 sites located 5 to 83 km from Colstrip, Montana, before Colstrip Unit #1 went into production in September 1975.

Table 1

Colstrip Control Plots

Parameter	42-month Foliage	30-month Foliage	18-month Foliage	6-month Foliage
ppm Fluoride	1.53	1.44	1.46	1.14
ppm Sulfur	463	485	501	513
% Needle rentention	82.2	83.6	94.9	96.5
% Tip burn	5.6	3.5	1.9	0.06
% Basal necrosis & scale	8.3	5.5	1.0	0.3
% Total necrosis	8.0	7.5	4.5	1.0

All crown positions; all tree ages

On Table 2 are similar data from ponderosa pine foliage collected at our Sacrifice Bluff site located 800 meters from the 180-megawatt coal-fired power plant (Corrette) in Billings, Montana. As can be easily ascer-

tained by comparing the sulfur concentrations in the foliage from the Billings area with that from the 16 control sites from southeastern Montana, the sulfur levels are approximately twice as high in the Billings foliage regardless of the exposure time to the sulfur pollution in the Billings area. The fluoride levels in the Billings foliage, however, in general tend to increase with exposure time. After 32 months of exposure to the ambient air of Billings foliage contains approximately 18 times more fluoride than similar aged foliage at the pristine sites in the Colstrip area during our 1975 study period. Needle retention for the ponderosa pine trees in the Billings area

Table 2

Physical-Chemical-Pathological Parameters
Billings, Montana, 800 Meters from 180 MW Coal-Fired Power Plant
GB#1 Site, (5 trees)

Parameter	44-month Foliage	32-month Foliage	20-month Foliage	8-month Foliage
ppm Fluoride	22.3	26.7	21.5	10.5
ppm Sulfur	1037	1050	1062	950
% Needle retention	62.3	71.6	93	96.3
% Tip burn	47	28	7.2	0.9
% Basal necrosis & scale	48	44.2	18.3	8.0
% Total necrosis	13.0	11.0	5.0	4.0

All samples taken on side facing 180-megawatt utility plant.

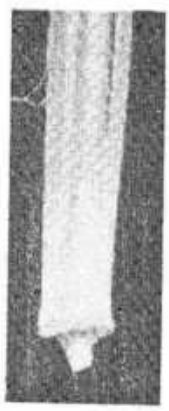
(see Table 1) for the 8- and 20-month-old foliage remains very similar to what we found for similar aged foliage at the pristine sites in the Colstrip area during our 1975 study period. However, after 32 and 44 months of exposure to the ambient air of Billings, the percent needle retention of the ponderosa pine foliage starts to become reduced over that found at our southeastern Montana Colstrip sites.

Of the needle pathology data depicted on Tables 1 and 2 for the Colstrip and Billings sites, one notes that the percent of needles exhibiting tip necrosis at the Billings site for foliage exposed 20 months to that atmosphere is higher than that found in foliage from our Colstrip sites exposed to that atmosphere for 42 months. After ponderosa pine foliage exposure to the Billings atmosphere at our site 800 meters from the Corrette plant for 44 months, the percentage of needles with tip burn is over 8 times greater than that found on 42-month-old ponderosa pine foliage from 16 of our Colstrip sites.

Prior to discussing the amount of basal necrosis and scale occurring in the foliage collected from the Billings and Colstrip sites, macrophotographs are present in Figure 3 which depict the appearence of each of these needle pathologies. Basal needle scale is a blister or raised eruption

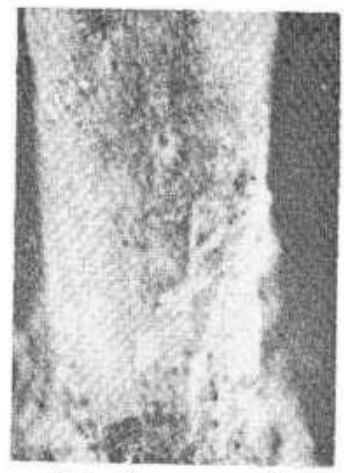
where the needle protrudes beyond the sheath. The blister is caused by localized hypertrophy of mesophyll cells which sometimes, because of excessive hypertrophy, causes a split in the epidermal and hypodermal cells. Basal necrosis is caused by the death and collapse of epidermal, hypodermal, mesophyll and, sometimes, the deeper endodermal and transfusion tissues. Basal needle tissue necrosis also occurs in between the needles (interfacial areas) where the dwarf shoot bud is located in the axis of the needle set.

Figure 3 Difference Between Basal Scale and Basal Necrosis



Normal

Basal scale



Basal necrosis

Necrotic swelling associated with basal scale.

Tables 1 and 2 show that the percentage of basal needle scale and necrosis on variously aged foliage from both the Colstrip and Billings sites increased with the exposure time to the atmosphere. After 20 months' exposure to the Sacrifice Bluff site, 800 meters from the Corette power plant, basal needle necrosis and scale is 18 times greater than similarly aged foliage from the Colstrip sites. After 30- to 32- month-exposure, the difference between the basal needle scale and necrosis of both areas is 8 times greater at the Billings site.

Fluoride scavenging: As previously mentioned, all foliage samples collected from the 16 Colstrip sites were taken from the side of the tree facing Colstrip. At the Billings sites, foliage was also collected from both the side facing the 180-megawatt Corrette coal-fired power plant and the side facing away from the plant. In Figure 4 are the average fluoride concentrations found on the foliage from both sides of the trees located 500 meters from the Corrette plant. One notes that for any given exposure period of the foliage, the fluoride concentrations on the side facing the fluoride-emitting source is 3 to 6 times greater. This phenomenon of fluoride scavenging by conifer foliage was first reported by Knabe (2)

in Germany, and we have utilized it in several areas of fluoride pollution in the United States. Sulfur analysis of the foliage from both sides of the trees collected at the Billings 500 meter site disclosed that there was little difference between the sulfur content of foliage on one side of the tree vs. the other. Figure 5 demonstrates the small differences in sulfur content between the two sides of the tree.

Figure 4
Atmospheric F Scavenging by Ponderosa Foliage on 2 Sides of Trees at Billings Site. 40 lbs Fluoride Emissions/Day. Collections 500 Meters from Source

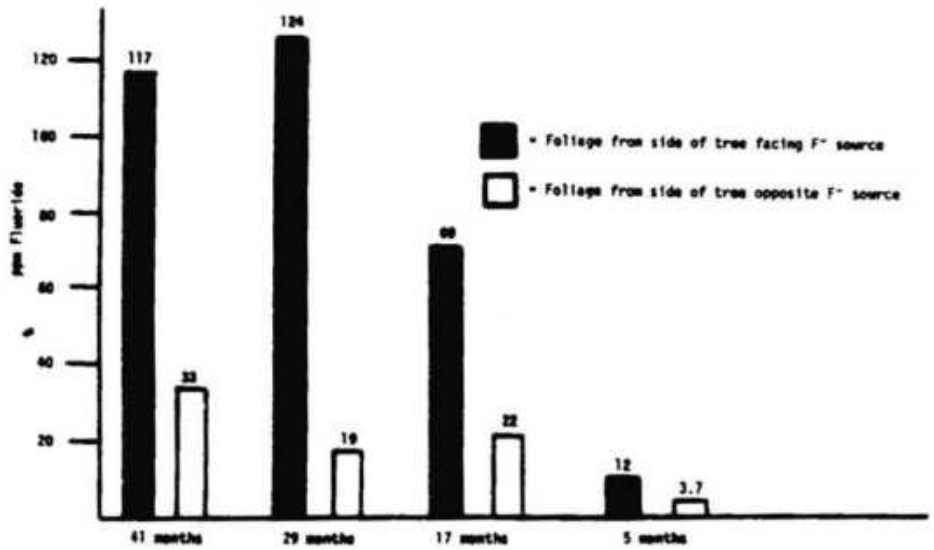
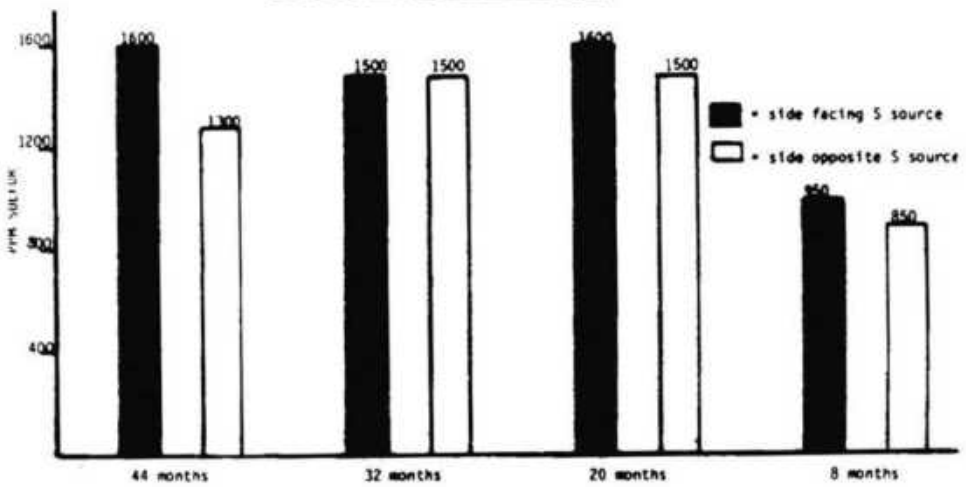
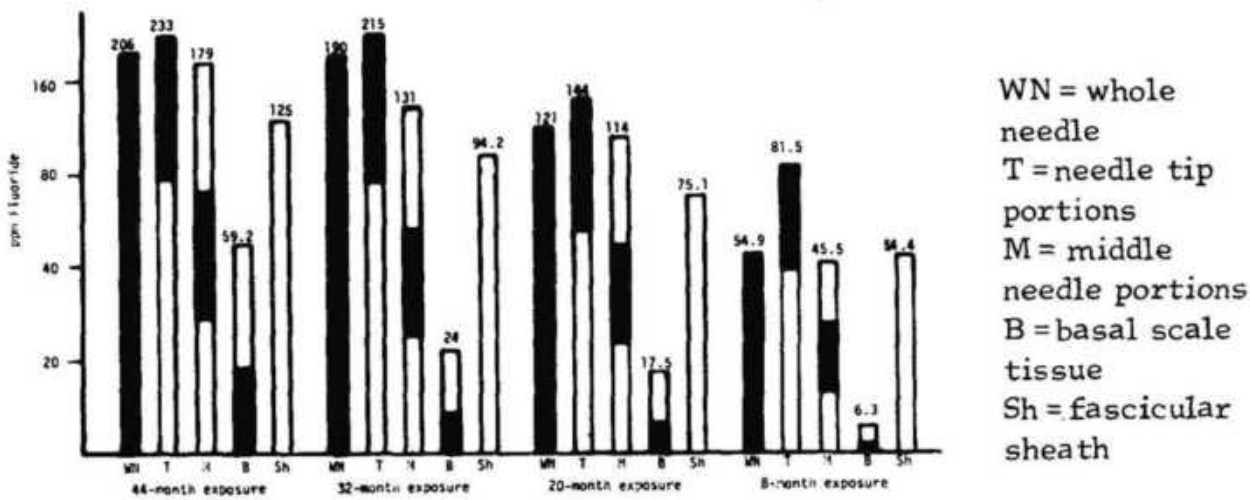


Figure 5 Average Sulfur Concentrations in Ponderosa Foliage Collected on 2 Sides of Trees Located 500 Meters from Atmospheric Sulfur Source (29 tons of Sulfur Emissions/Day)



Fluoride Partitioning in Needles: In Figures 6 and 7 are the average fluoride concentrations found in and on the partitioned foliage of ponderosa pine collected 500 and 800 meters from the 180-megawatt Corrette plant at Billings, Montana. The much higher fluoride levels found in the partitioned needles from the site 500 meters from the power plant are probably due to the fact that this foliage was heavily laden with flyash which was not the case at the 800 meter site. Where flyash contaminates the trees the fluoride levels in and on the middle portions of the 20- and 44-month-old partitioned needles are substantially higher than in the fas-

Figure 6 F Concentrations (ppm) in and on the Needle Tissues of Ponderosa Foliage Exposed to the Ambient Air of Billings for Varying Periods of Time Collection Site 500 Meters from 180 mW Coal - Fired Corrette Utility Plant



cicular sheaths. Figure 7 shows that the sheaths of the partitioned foliage from the 800 meter site at Billings contain substantially more fluorides than the middle portions. We have found this consistently in several fluoride-polluted areas where we have carried out similar studies For instance, Figures 8 through 11 depict the fluoride concentrations which were found partitioned in conifer foliage from other fluoride-polluted areas. Figure 8 is a bar graph which depicts the fluoride concentrations found in partitioned Scotch pine foliage collected during 1975, 10 and 20 miles (16 and 33 km) from a 1600-megawatt coal-fired power plant in the Mt. Storm area of West Virginia and Maryland. One notes from this bar graph that the fluoride levels in the fascicular sheaths are approximately 2.5 to 3.5 times higher than those in the whole needles, and approximately 2 times higher than at the tip portions of the needle.

Figure 7 F Concentrations in and on the Tissues of Ponderosa Foliage Collected 800 Meters from the 180 mW Coal-Fired Corrette Plant, Billings

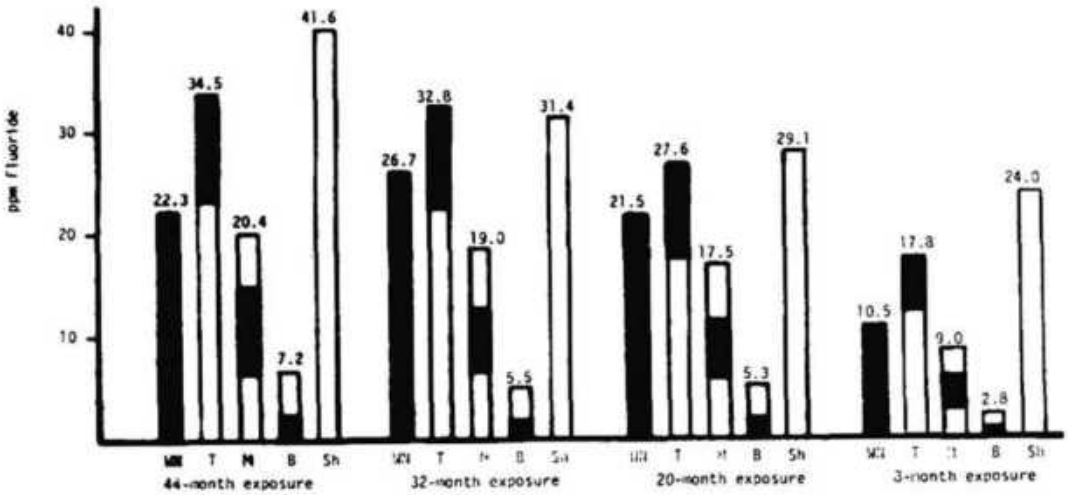
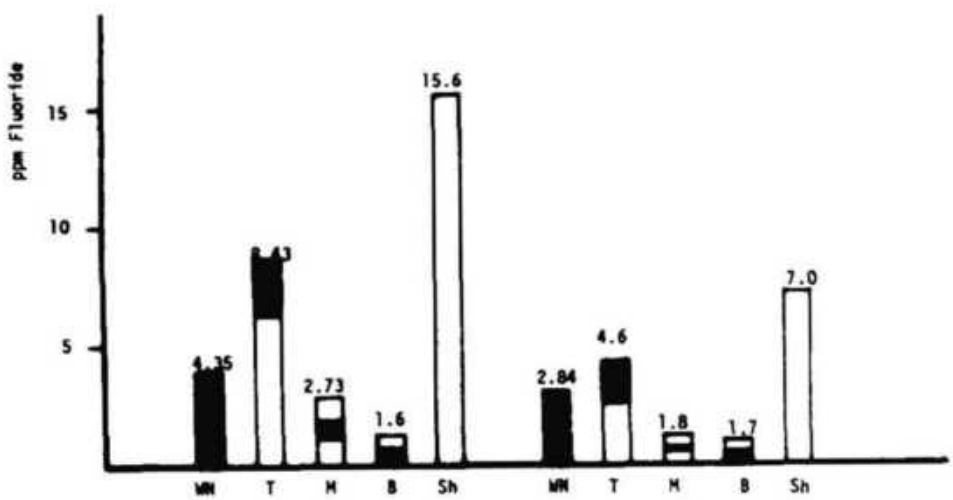


Figure 8 F Concentrations in and on the Needle Tissues of Scotch Pine Exposed to the Ambient Air of the Mount Storm Area of West Virginia and Maryland for 18- and 6-Month Periods



Figures 9 and 10 demonstrate that partitioning of fluoride occurs in other fluoride-polluted areas. Figure 9 depicts the fluoride concentrations

in partitioned ponderosa pine foliage collected 1 to 5.8 km from an aluminum plant located in The Dalles, Oregon area. This aluminum plant, according to EPA emission studies, is believed to release less fluoride per ton of aluminum produced than any other aluminum plant studied so far.

Figure 9 Average F Concentrations in and on the Tissues of Ponderosa Foliage Collection Exposed to the Ambient Air in The Dalles Martin Marietta Aluminum Plant - 400-800 lbs of F⁻ Emissions/Day

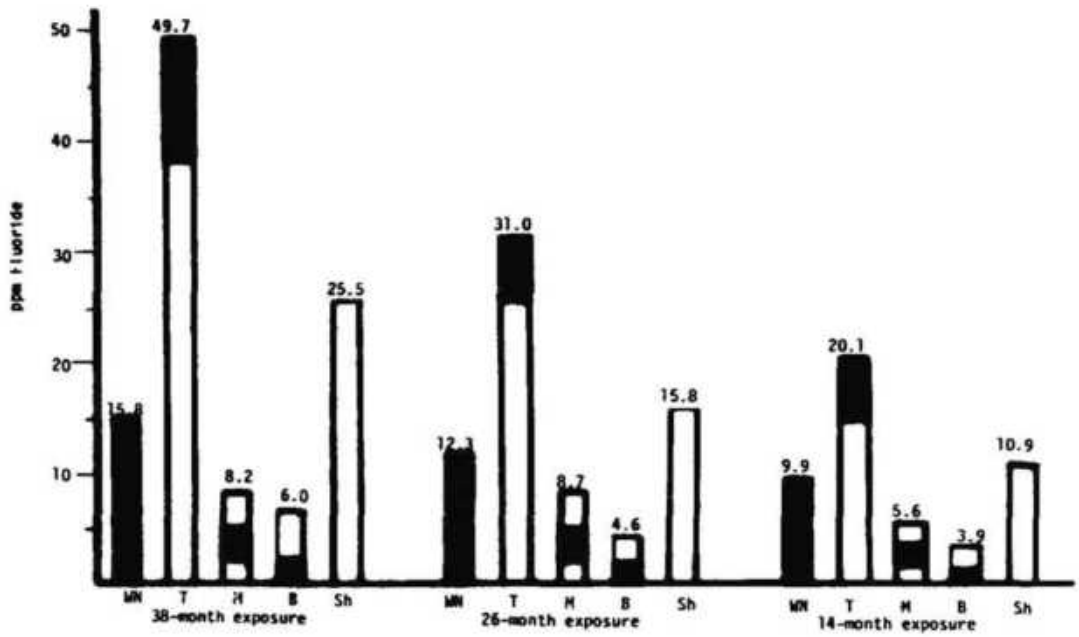


Figure 10 shows the fluoride levels in and on partitioned lodgepole pine (Pinus contorta) collected 1.5 km from an aluminum plant located in Columbia Falls, Montana. This plant emits in excess of 2500 lbs of fluoride each day into the atmosphere, probably the largest quantity of fluoride released from any single stationary source in the United States. One notes that while the fluoride concentrations on the various partitioned needles are elevated over concentrations previously discussed, the fluoride partitioning pattern remains very similar to other fluoride-polluted areas regardless of the amounts of fluoride emitted daily by the stationary source.

Figure 11, depicting fluoride partitioning in conifer needles, demonstrates the baseline levels in and on foliage collected in pristine areas

Figure 10 F Concentrations in and on the Needle Tissues of Lodgepole Pine Exposed to the Ambient Air of Columbia Falls for 15- and 3-Month Periods. Aluminum Plant Emits 2000 to 3000 lbs of Fluoride/Day

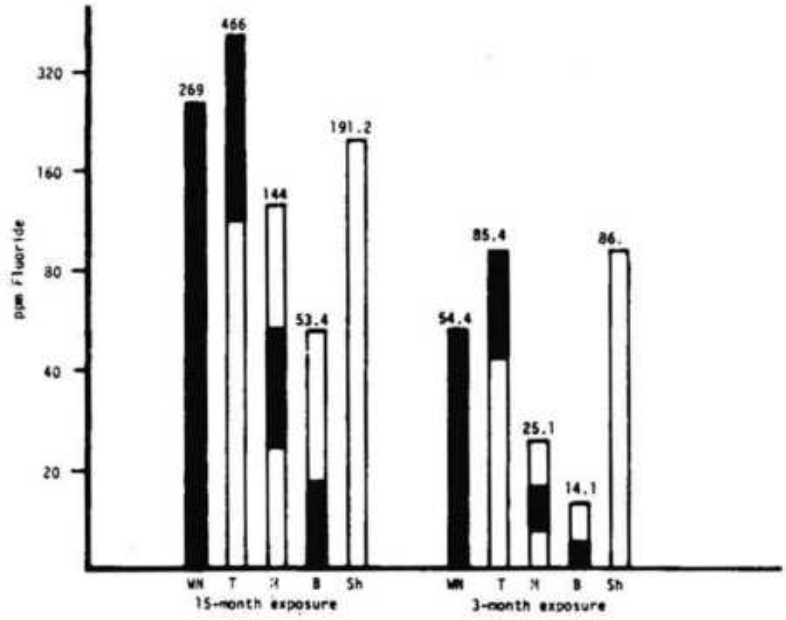
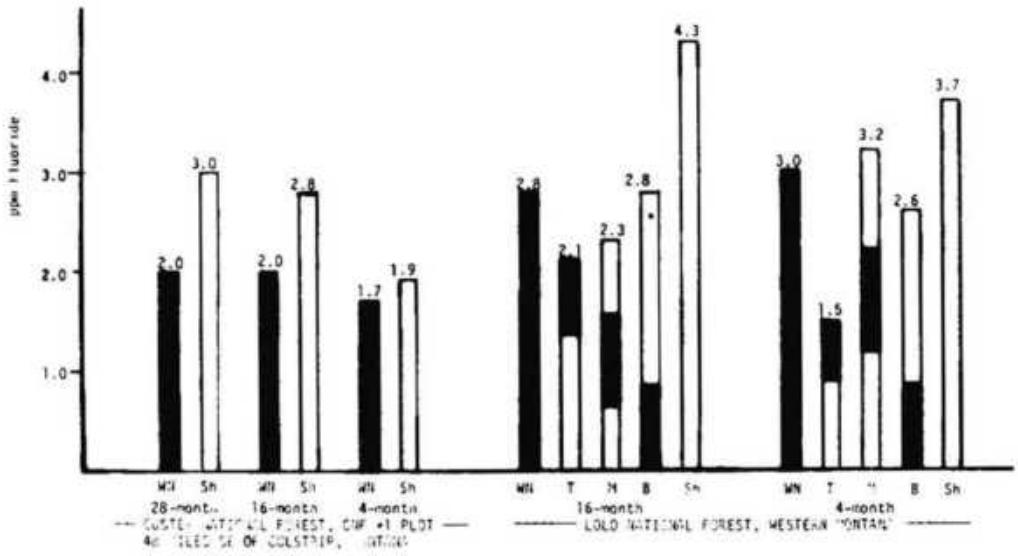


Figure 11 F Concentrations in and on Ponderosa Foliage Exposed to Ambient Air for Varying Periods of Time in Pristine Areas of Montana



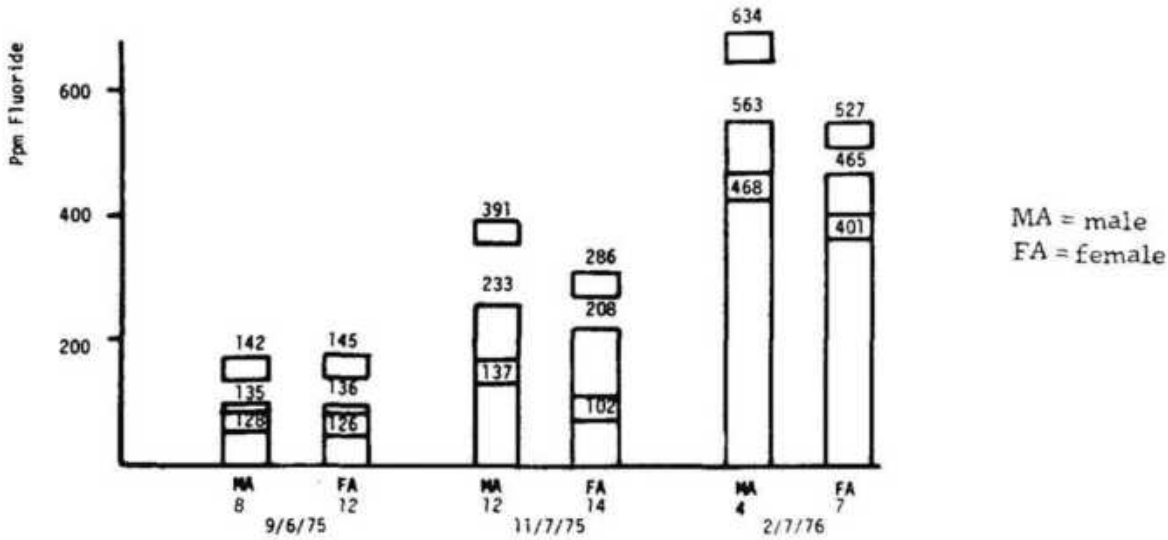
of eastern and western Montana. The highest fluoride concentration found in any partitioned portion of ponderosa pine needles from these two pristine areas is in the fascicular sheath; however, the fluoride concentrations of whole needles and the exposed sheaths are not significantly different from each other, even though the trend for higher fluorides in the fascicular sheaths seems evident.

Fluoride Concentrations in the Femur Bones of Deer Mice: Deer mice have been collected at 8 sites located 3 to 11 km from the town of Colstrip, Montana, during the last 1-1/2 years. We present here fluoride concentrations in the femurs of 151 deer mice collected at 3 of our 8 Colstrip sites and the fluoride levels in the femurs of 79 deer mice collected approximately 800 meters from the Corrette power plant in Billings, Montana.

The data reported pertain to only the femur bones of adult deer mice, following the suggestions of Lewis et al. (3) and Wright (4), who employ body weights of 13 grams for females and 14 grams for males to differentiate between adult and juvenile deer mice.

Colstrip Unit #1 commenced firing coal in mid-September, 1975. The data in figure 12 are the fluoride levels of deer mice collected during

Figure 12 F Concentration in Deer Mice at BNW #1 Site 4.51 Km SE of Colstrip



pre- and post-operational periods of Colstrip Unit #1 at a collecting site located 4.5 km southeast of Colstrip. The fluoride level of the femur bones of 20 animals collected prior to the operation of Unit #1 averaged 135 ppm (126-145 ppm). Five months later (February 7, 1976) the fluoride levels found in 11 deer mice at this same site averaged 501 ppm (401 - 634 ppm). At two other sites (Figures 13 and 14) located 3.3 km east and 3.0 km northwest of Colstrip the fluoride concentrations of the femurs also increased during the winter months of 1975-76 from an average concentration below 200 ppm to levels over 300 ppm (at BNW#3 site) and 490 ppm (at BNW#2 site).

This continuing increase during the first 5 to 6 months of operation might seem to indicate an increase of fluoride contamination at the Colstrip collecting sites, but it is too early in the study to arrive at this

Figure 13 F Concentration in Deer Mice at BNW #2 Site 3.3 Km East of Colstrip

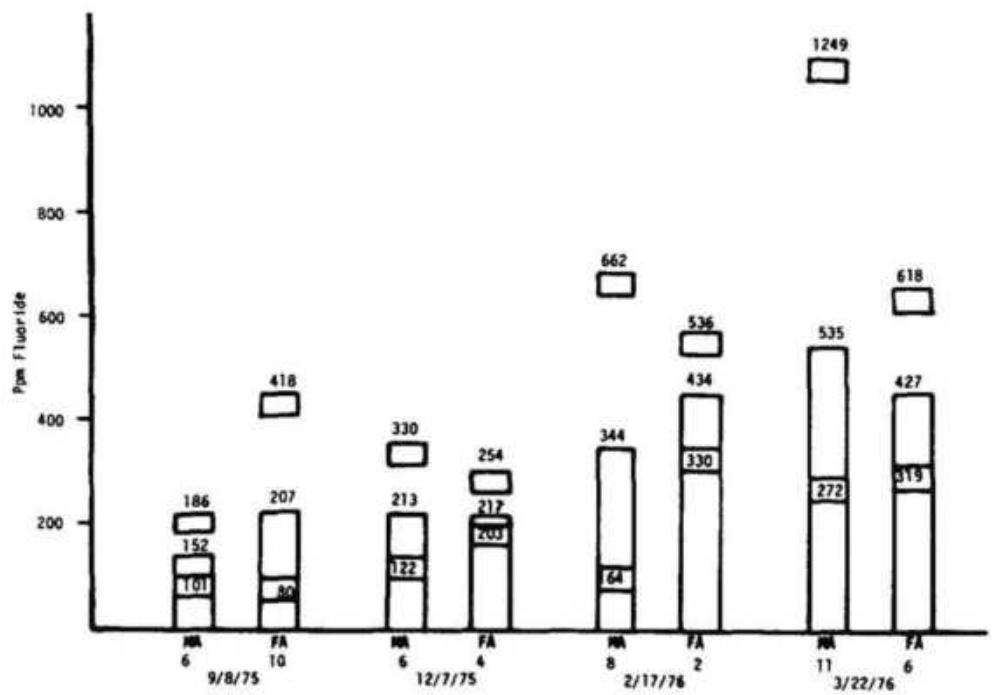
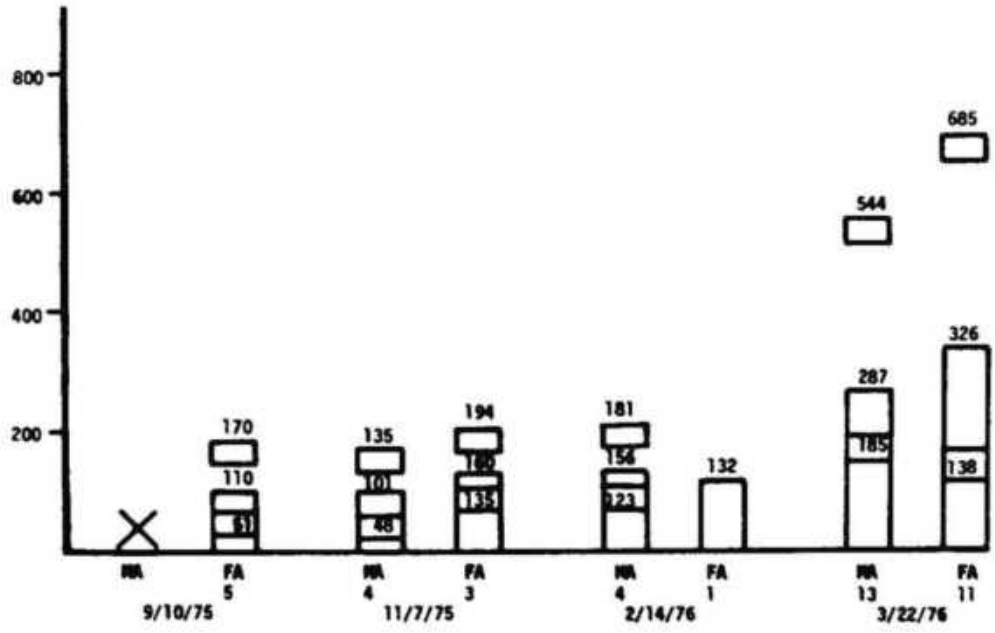


Figure 14 F Concentrations in Deer Mice at BNW #3 Site 3 Km NW of Colstrip



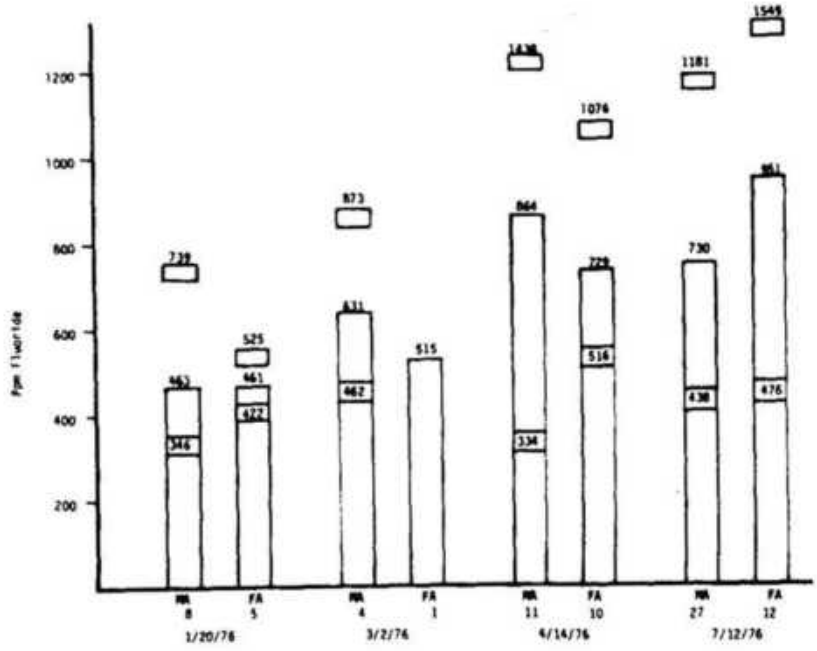
conclusion because of the fluoride concentrations found in the deer mice collected at our Billings site during this same period of time presented in figure 15. One notes that the trend for fluoride concentrations in the femur bones of the mice from the Billings site is lowest during the winter period and steadily increases during the spring and early summer period (Figures 12-14).

Current incompleted studies of the fluoride levels of the stomach contents as well as the identification of the flora and fauna (when possible) in the stomachs of the omniverous deer mice may help us understand the seasonal fluctuation of fluoride accumulation of these animals in the future.

Acknowledgments

This work was performed for the U.S. Energy Research and Development Administration (ERDA), and supported in part by the Interagency

Figure 15 F Concentrations in Deer Mice Collected 800 Meters from 180 MW Coal-Fired Power Plant



Energy Environment Research and Development Program of the U.S. Environmental Protection Agency (Contract #EPA-IAG-D5-E681) and the U.S Forest Service/Surface Environment and Mining (SEAM) (Cooperative Agreement #01-013).

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SEPARATION OF MALIC DEHYDROGENASE ISOENZYMES FROM SOYBEAN TISSUE IN RELATION TO FLUORIDE TREATMENT

by

M. Psenak[1], G.W. Miller[2], M.H. Yu[3], and C.J. Lovelace[4]

Logan, Utah

SUMMARY: Three isoenzymes of malic dehydrogenase were separated from soybean cotyledons: glyoxysomal (G-MDH), mitochondrial (M-MDH) and soluble (S_1-S_2-MDH). The effects of citrate, fluorocitrate, pyruvate, fluoropyruvate, inorganic fluoride and pyridin carboxylic acids on the malic dehydrogenase enzymes were determined. All three isoenzymes were less sensitive to fluorocitrate than to citrate. Pyruvate had little effect in contrast to fluoropyruvate which was an inhibitor. Both nicotinic acid and quinolinic acid are inhibitors of the isoenzymes. Succinate and malate oxidation by intact mitochondria is inhibited by potassium fluoride, but sodium or potassium fluoride at similar concentrations does not affect purified MDH-isoenzymes. This may indicate an effect of fluoride on mitochondrial membranes and not on the enzyme per se. In general, fluoride seems to have a dual effect on cell metabolism namely involvement of the structural integrity of the cell and its organelles, and a specific interaction with some enzymes, i.e., enolase, phosphoglucomutase.

It is now well established that an elevated concentration of fluoride, which occurs particularly in industrial areas, results in injury to plants. The general symptoms of fluoride injury, necrotic lesions and burning, appear first in the leaf tips and margins. Although the intensive quest for definitive answers about mechanisms by which fluoride damages plants has spanned two decades, no encompassing explanation is, as yet, available.

Fluoride effects on plants at different levels have been studied. By correlating intact leaf tissues with a decrease of chlorophyll content (1), changes in the rate of photosynthesis (2), decreased growth depending upon concentration (3), and both increased and decreased respiration (4) have been reported. Fluoride fumigation of soybean leaves has resulted in marked changes in free sugars, total organic and amino acids (5). These

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findings would appear to indicate that fluoride exhibits its effect at the level of the enzyme. When the fluoride effect on crude or partially purified enzymes was studied, it was found that some enzymes are able to tolerate the presence of elevated concentrations of fluoride (6) whereas others are quite sensitive to it (6, 7). Studies at the cellular and, particularly, the subcellular level (8) strongly indicate that fluoride exerts its effect through alterations of subcellular organization and upon the integrity of cellular and subcellular membranes (9).

The effect of fluoride on the oxidative capacity of intact, coupled mitochondria from etiolated soybean hypocotyls was examined by Miller and Miller (9). When succinate, malate and NADH were used as substrates, the respiration and ADP/O ratios were inhibited by potassium fluoride. The oxidation of succinate appeared to be more sensitive to fluoride than either malate or NADH. Similar results were reported by Lovelace and Miller for cauliflower mitochondria (10).

The current study was initiated to determine the effect of inorganic fluoride and some fluoro-organic compounds on individual malic acid dehydrogenase isoenzymes from etiolated soybean hypocotyls.

Materials and Methods

Plant Material: Soybean seeds (Glycine max, Merr.) were germinated and grown in vermiculite which was moistened with deionized water. After 8 to 9 days of germination in the dark at 25°C, cotyledons were used for preparation of a crude enzyme solution.

Purification and Separation of Malic Dehydrogenase Isoenzymes: All operations were carried out at 0 to 4°C unless otherwise specified.

Step 1. Crude enzyme extract. Forty g of soybean cotyledons were homogenized in a Waring blender with 0.05 M Tris-HCl buffer (pH 7.5) containing 5 mM EDTA and 2 mM 2-mercaptoethanol (120 ml) for two intervals of 15-20 seconds at low speed. The slurry was filtered through nylon cloth and the resultant filtrate was centrifuged at 300 x g for 5 min. Following this, the supernatant was recentrifuged at 17,000 x g for 15 min. to obtain the main supernatant fraction. The pellet obtained through these procedures was resuspended in the homogenization medium and ground in a Cold Ten Broek homogenizer for 10 intervals, each of 30 seconds' duration. After centrifugation, the resulting supernatant was pooled with the main supernatant fraction and used as a crude enzyme source for ammonium sulfate fractionation.

To obtain a crude mitochondria and glyoxysome fraction, the coty-

ledons were macerated gently with mortar and pestle using the same homogenization medium with the addition of 0.5 M sucrose. After the first centrifugation (300 x g, 5 min), the resulting supernatant was centrifuged at 1500 x g for 10 min. to spin down the nuclei and etioplasts. The supernatant obtained was recentrifuged at 17,000 x g for 25 min and the crude pellet fraction obtained was considered to contain a combined fraction of mitochondria and glyoxysomes contaminated with broken etioplast fractions. This pellet fraction was resuspended in 0.005 M sodium phosphate buffer, pH 7.2, and ground by means of a Ten Broek homogenizer for 10 intervals each of 30 seconds' duration. After centrifugation (17,000 x g, 15 min) and dialysis against 2 l of 0.005 M phosphate buffer overnight, the enzyme solution was again centrifuged (17,000 x g, 15 min) and used for separation of particulate malic dehydrogenase isoenzymes on a DEAE cellulose column.

Step 2. Ammonium sulfate fractionation. The crude enzyme extract (containing soluble and pellet supernatant) was brought to 30% saturation with ammonium sulfate and allowed to stand for 2 hrs. After centrifugation (15,000 x g, 25 min), the resulting supernatant was brought to 80% saturation with ammonium sulfate and allowed to stand for 3 hrs. The 30 to 80% fraction was collected by centrifugation (15,000 x g, 25 min). The precipitate was resuspended in 0.005 M sodium phosphate buffer, pH 7.2, and dialyzed overnight against 3 l of the same buffer. After centrifugation (15,000 x g, 25 min), the resulting supernatant was used for subsequent column separation.

Step 3. DEAE-cellulose chromatography. Four ml of enzyme solution from Step 2 were layered on a DEAE-cellulose column (1 x 25 cm) and equilibrated with 0.005 M sodium phosphate buffer, pH 7.2. After washing the column with 0.005 M phosphate buffer (100 to 120 ml), a linear phosphate gradient was applied to the column and 3 ml fractions were collected and assayed for malic dehydrogenase activity. The peak fractions were pooled and used for further analysis.

Enzyme Assay: All spectrophotometric assays were conducted at 25° C by means of a Hitachi-Perkin Elmer UV-VIS spectrophotometer. Malic dehydrogenase activity was determined in the direction of oxalacetate reduction. The standard assay medium contained: 0.3 µmole of oxalacetate, 0.3 µmole NADH, enzyme (10 to 12 µg of protein) and 0.05 M Tris-HCl buffer, pH 7.4, in a total volume of 3 ml. The reaction was started after 5 min. preincubation at 25° C with addition of NADH solution. The oxidation of NADH was measured at 340 nm; initial velocities were used to calculate the activities.

Manometric Polarographic Oxidase Studies: Oxygen consumption was measured using a Gilson Medical Electronics differential respirometer as outlined by Lovelace and Miller (10), using mitochondria from cauliflower Brassica oleracese L. It was also measured by polarographic techniques and mitochondria from soybeans as described by Miller and Miller (9).

Protein Determination: Protein content was measured by the methods of Lowry et al. (11) with crystalline bovine serum albumin used as standard.

Results and Discussion

The effect of KF on TCA oxidase systems in mitochondria from cauliflower is shown in Table 1. The mitochondria had low respiratory control (low coupling) and phosphrylation rates were poor. Succinate oxidase was markedly inhibited at 10^{-3} M and almost totally inhibited at 5×10^{-2} M. Both malate and NADH oxidases were insensitive at low concentrations and only inhibited 20% at this high concentration.

Using mitochondria isolated from etiolated hypocotyl soybean sections, we obtained good respiratory control with average P/O ratios (Table 1). Table 2 shows the effects of a range of fluoride concentrations on NADH, malate and succinate. Comparable concentrations of KCl had no effect on oxidation or phosphorylation. At the higher concentration of KF, all State 3 respiratory rates with the various substrates were inhibited and succinate, as before, was the most sensitive. State 4 respiration was not sensitive to KF up to 6.7×10^{-2} M. The sensitivity of succinate oxidase to KF may be related to its being a membrane-bound enzyme, whereas malate oxidase with less

Table 1

The Effect of Fluoride on TCA Oxidase Activity* from Cauliflower Mitochondria

KF concentration	NADH	Substrate Malate extract µl O_2/hr/ml	Succinate
0	90	80	86
1×10^{-3}	86	78	70
5×10^{-3}	87	72	66
1×10^{-2}	88	74	61
5×10^{-2}	71	57	19

*Figures represent average of 3 replications. Measurements using manometric techniques.

Table 2

The Effect of Fluoride on TCA Oxidase* Activity from Soybean Mitochondria

KF concentration	NADH	Malate O_2 nmoles/min/mg Protein	Succinate
0	543	278	264
6.7×10^{-3}	526	260	235
5.0×10^{-2}	475	218	147
6.7×10^{-2}	440	152	98
State 4	319	60	98

*Control contained KCl to compare to KF concentrations. Measurements using polarographic techniques.

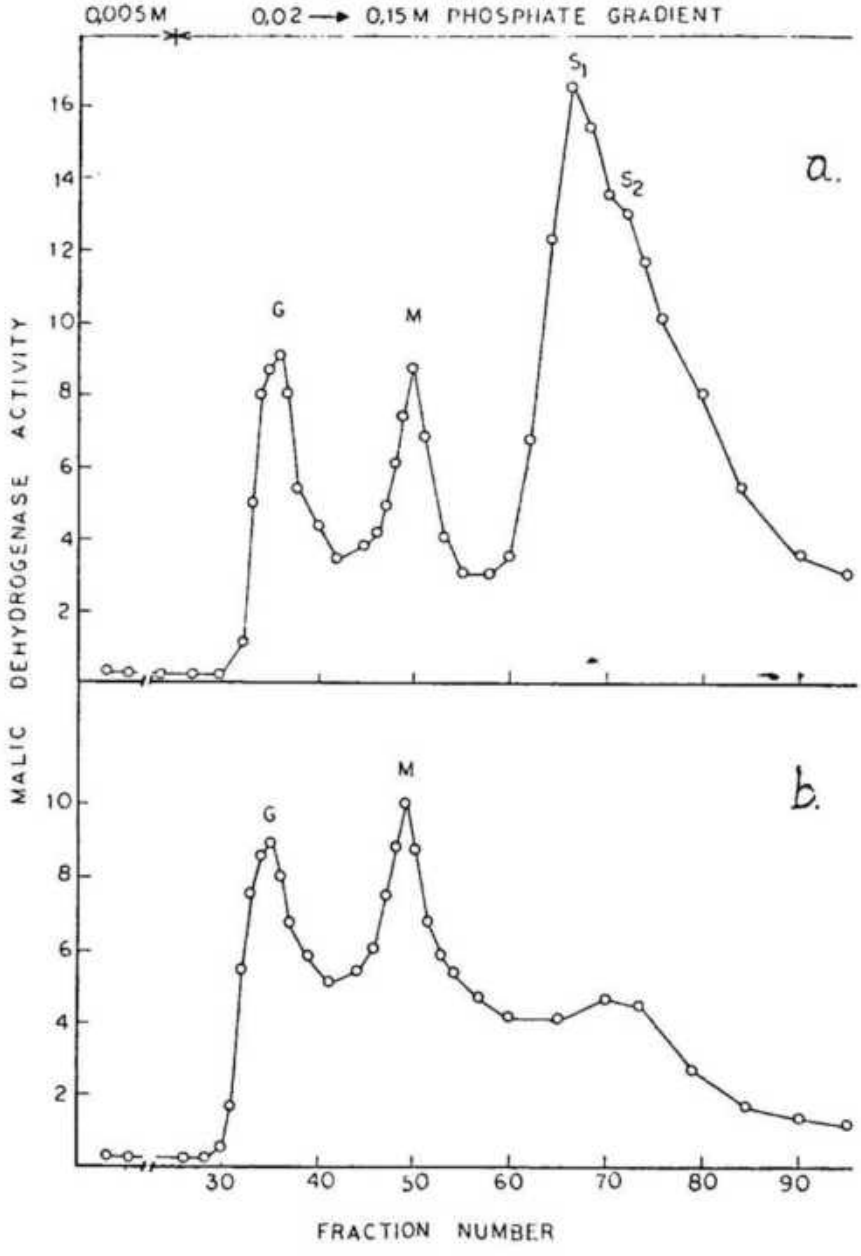
sensitivity to KF is found in the matrix.

It is now well established that multiple molecular forms of malic acid dehydrogenase (EC 1.1.1.37) exist in different plant tissues. In several tissues which have been examined, these isoenzymes have been shown to be associated with subcellular compartments: mitochondria, chloroplasts, peroxisomes and glyoxysomes (12, 13, 14, 15) and in the soluble fraction. When an enzyme extract containing soluble and particulate malate dehydrogenases from soybean cotyledons was separated on a DEAE-cellulose column (Fig. 1a), three distinctive peaks exhibiting malate dehydrogenase activity were eluted with a phosphate gradient. When a disintegrated crude mitochondrial and glyoxysome fraction was applied on the identical columns, only two malate dehydrogenase peaks were obtained.

The dominant metabolic event during germination of fatty seeds is the conversion of lipids to carbohydrates. Soybean seeds contain, on an average, 21% oil which is mainly located in cotyledon tissue (16). In the conversion of lipids to carbohydrates the glyoxylate cycle plays a central role (17, 18). The complete glyoxylate cycle is located in characteristic microbodies - glyoxysomes (19, 20) which are present only in the cotyledons or the endosperm of germinating fatty seeds (21, 22). Because of this, the first malate dehydrogenase peak is considered to be the glyoxysomal isoenzyme (G-MDH); the second is mitochondrial (M-MDH), and the third peak corresponds to soluble isoenzymes (S_1-, S_2-MDH (see Figure 1). The elution profile of MDH-isoenzymes extracted from soybean cotyledons is practically identical with those reported by Rocha and Ting (13, 23). In this preliminary report no further identification of individual MDH-isoenzymes was carried out. The peak fractions corresponding to individual MDH-isoenzymes (S_1-MDH was used) were pooled with corresponding fractions and after determination of protein content used for examination of their sensitivity to inorganic fluoride, some fluoro-organic acids and pyridine carboxylic acids.

To obtain approximately similar activities, the S_1-MDH fraction was diluted one to one with 0.005 M phosphate buffer, pH 7.2. The activities were measured only in the direction of oxalacetate reduction. When sodium and potassium fluoride (up to 6.6×10^{-2} M, final concentration in incubation mixture) were used, no significant inhibition was observed on the MDH-isoenzyme activity. The results obtained by testing the effect of citrate, fluorocitrate, pyruvate and fluoropyruvate are presented in Table 3. G-MDH appeared to be the most sensitive isoenzyme to citrate and fluorocitrate. The sensitivity of M-MDH and S_1-MDH to citrate is essentially identical to that reported by Mukerji and Ting (12) for these isoenzymes obtained from green stem tissue of Opuntia ficus indica, where the mitochondrial isoenzyme was more sensitive than the soluble isoenzyme.

Figure 1. Elution of Malic Dehydrogenase Isoenzymes from a DEAE-Cellulose Anion-Exchange Column



a. G-isoenzyme associated with the glyoxysomal fraction; M-isoenzyme associated with mitochondrial fraction and S_1 - (S_2-) -isoenzymes associated with soluble fraction. b. Elution profile of MDH-isoenzymes associated with pellet fraction (15,000 x g - 17,000 x g).

Table 3

Effect of Organic Acids and Their Fluoro-Analogs and Pyridine Carboxylic Acids on the Activity of Soybean Malic Dehydrogenase Isoenzymes

Additions	Final concentration (M)	Percentage of inhibition m-MDH	g-MDH	S_1-MDH
Citric acid	3.3×10^{-2}	30	39	24
	6.6×10^{-2}	40	53	27
F-citric acid	3.3×10^{-2}	20	34	25
	6.6×10^{-2}	26	44	27
Pyruvic	1.6×10^{-2}	10	2	7
F-Pyruvic acid	6.6×10^{-3}	21	16	12
	1.6×10^{-2}	30	21	27
Nicotinic acid	3.3×10^{-2}	35	43	37
	5.0×10^{-2}	51	54	--
Quinolinic acid	3.3×10^{-2}	64	70	53
	5.0×10^{-2}	70	—	64
KF or NaF	6.6×10^{-2}	0	0	0

Cinnamo et al. (24), using a mitochondrial enzyme obtained commercially from pig heart, reported this enzyme was specifically inhibited by citrate. The soluble malate dehydrogenase was found to be insensitive to citrate. They showed that the citrate inhibition was competitive with NADH and noncompetitive with oxalacetate. They also assumed that citrate binds at the same site as oxalacetate.

All three isoenzymes appeared to be less sensitive to fluorocitrate than to citrate (Table 3). This may be partially explained, by assumption, that the presence of fluoride in the citrate molecule decreases its reactivity with the enzyme molecule. As seen in Table 3, pyruvic acid did not have any significant effect on the activities of MDH-isoenzymes. These observations are consistent with those reported by Mukerji and Ting (12). Fluoropyruvic acid, however, appeared to be an inhibitor of MDH-isoenzymes. According to Avi-Dor and Mager (25) fluoropyruvic acid is a strong SH-inhibitor which, in our experiments, could react with the SH groups of enzymes. How-

ever, when the assay medium contained an equal amount of glutathione and F-pyruvate, the degree of inhibition was the same as without glutathione. In view of the fact that the purest F-pyruvate (26) is not a vigorous alkylating reagent and because the purity of F-pyruvate used in our experiments was not established, further experiments are necessary to clarify the interaction of fluoropyruvate with malic dehydrogenase isoenzymes.

The effect of various organic and amino acids on the activity of individual MDH-isoenzymes was examined by Mukerji and Ting (12) and by Cinnamo et al. (24). Pyridine carboxylic acids are formed in higher plants by condensation of C_4-dicarboxylic acid (aspartate) with glycerol or its biochemical equivalents (27, 28, 29). Quinolinic and nicotinic acids are precursors of the pyridine ring of pyridine nucleotides. Aspartate is formed from oxalacetate, which is at the same time (from the point of view of gluconeogenesis in fatty seeds) a precursor of C_3-intermediates in glycolysis. From this point of view, malic acid dehydrogenases are of primary importance in the formation of precursors of the pyridine ring of pyridine co-enzymes. Both nicotinic acid, and quinolinic acid appeared to be inhibitors of malate dehydrogenase isoenzymes (Table 3). This could indicate that the pyridine carboxylic acids are involved in the regulation of pyridine nucleotide biosynthesis. In this preliminary report, however, the kinetics of inhibition were not investigated.

As previously mentioned, the succinate and malate oxidation by intact mitochondria was found to be inhibited by potassium fluoride (9, 10), but sodium and potassium fluoride at almost the same concentrations did not have any effect on the purified MDH-isoenzymes. This indicates that the inhibition of the mitochondrial malate oxidation is a consequence of fluoride interaction with the mitochondrial membrane system. Taking into account the fluoride sensitivity of some purified enzymes (enolase, phosphoglucomutase), in general fluoride seems to have a dual effect on cellular metabolism namely, involvement of the structural integrity of the cell and its organelles, and a specific interaction with some enzymes.

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Discussion

Dr. Johnson: Was phosphate, Mg or Ca used in the enzyme assay?
Dr. Miller: No, tris buffer was used and the assay did not include Mg or Ca.
Dr. Waldbott: Could your data apply to animal cells as well as plant cells?
Dr. Miller: Yes, when one works with organelles, results among organisms may be similar. One would, of course, have to make a study with isoenzymes (malic dehydrogenase) from animal cells before definite conclusions could be made. A difference between plant and animal cells is that plant cells have vacuoles that may accumulate fluoride. Disruption of the vacuole would cause increase of fluoride around such organelles as the mitochondria. In animals, of course, most fluoride accumulates in the skeleton. Basic information is helpful regardless of the tissue with which one is working.

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SENSITIVITY OF THE HOUSE MARTIN, DELICHON URBICA, TO FLUORIDE EMISSIONS

by

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Bellingham, Washington

SUMMARY: The nesting patterns of the House Martins, Delichon urbica were found to be affected by fluoride emissions. In an area of high fluoride concentration the birds were rare while other species of birds were common. In areas of moderate fluoride pollution the population of House Martins were similar to control areas.

Introduction

Although much is known about the effects of fluoride on domestic animals only a few studies have considered the effects on wildlife (1, 2). Fluoride is known to have harmful effects on deer and to concentrate in the tissues of numerous species of rodents (3, 4, 5, 6, 7). Studies involving birds are even rarer. Feriancova-Masarova and Kalivodova (8) investigated the quantity of birds in the vicinity of an aluminum plant in Czechoslovakia. They found an inverse relationship between the concentration of atmospheric fluoride and the number of birds, especially insectivorous birds. This article describes a recent assessment of the nesting pattern and status of one of these birds, Delichon urbica, the House Martin in the vicinity of the same aluminum plant in Czechoslovakia. Delichon urbica is a migratory passerine bird which nests in Czechoslovakia from mid-April to September (9). It builds its nest of mud on the perpendicular masonry walls adjoining eaves of roofs, cornics and window overlappings. It feeds primarily on aphids and flies (10, 11).

Methods

Population census of the House Martin in central Slovakia was made by direct counting of the number of active nests on sides of buildings with suitable nesting conditions. Suitable nesting conditions were defined as buildings with masonry walls with eaves, cornices and window overhangs at

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least 90° angles to the walls, and sides of buildings with available flight area of at least two meters between adjacent buildings. The sides of buildings averaged 20 meters in length. Buildings with sides greater than 20 meters were divided into 20 meter segments. Where possible a minimum of 25 buildings with suitable nesting conditions were censused. Only buildings away from heavy vehicular traffic were censused. Censuses were carried out in July, 1976, during the nesting season in the early morning or late afternoon at the height of activity around nests at seven sites (immediate vicinity of plant, Horne Opatovce, Ladomerska-Vieska, Ziar, Lehotka, Dodina and Zvolen) at varying distances from the aluminum plant. The density of nesting birds was compared with available information on fluoride concentration in the air (12, 13).

Results

Delichon urbica was absent or extremely rare in the immediate vicinity of the fluoride source and zone of heaviest pollution (Table 1). Only one nest was found and no other attempts at nesting (partially completed nests) were observed. In areas of light to no fluoride concentration the House Martin was 33 times more common. In the intermediate zone the density of House Martins was comparable to control sites.

Table 1

Comparison of Nesting Density of the House Martin (Delichon urbica) with Fluoride Pollution

Site	Sample Size (No. of building sides)	No. of Nests	Mean Density (No. nests per side of building)
Zone I (> 100 µg/m) Alum. plant Horne Opatovce	76	1	.013 ± .110*
Zone II (60-100 µg/m) Ladomerska-Vieska Lehotka Ziar	188	81	.431 ± 1.000
Zone III (0-30 µg/m) Dolina Zvolen	147	64	.435 ± 1.370*

*significantly different $t_{(.01)(221)} = 2.671$

Discussion

The trend in declining House Martin populations has continued since 1963 in the vicinity of the aluminum plant to the point that they are almost completely absent. The House Martin species are migratory and do return in succeeding years to their original nesting areas (14). The absence of the House Martin in areas of potential suitable nesting habitat but with high fluoride emissions possibly reflects an avoidance of these areas. Other species of birds were observed in the zone of heaviest fluoride pollution. Although density estimates were not made the House Sparrow (Passer domesticus) the Pigeon (Columba palumbus), and a closely related insectivorous bird, the Swallow (Hirundo rustica) were quite common in this zone where the House Martin was rare. Further information is needed on the effect of fluoride emissions on nest site selection of the House Martin.

Acknowledgements

This study was supported by a research exchange grant of the National Academy of Science and the Czechoslovakia Academy of Science. Special thanks are given to the Research Institute of Forestry, Zvolen, for their assistance, and to Ann Drake, Bureau of Faculty Research, Western Washington State College for typing the manuscript.

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ALKALINE PHOSPHATASE ACTIVITY, FLUORIDE CITRIC ACID, CALCIUM, AND PHOSPHORUS CONTENT IN BONES OF COWS WITH OSTEOPOROSIS

by

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SUMMARY: Ribs, metacarpal, and metatarsal bones were obtained from cows subjected to excessive fluoride ingestion and examined for fluorine, citric acid, calcium, and phosphorus content, and alkaline phosphatase activity. The fluorine content increased approximately 10- to 20-fold over the controls, the increase being greater in the periosteal zones of the bones than in the endosteal zones. The alkaline phosphatase activity exhibited a 3.58 to 6.92 fold increase in the osteofluorotic bone, and the activity generally closely correlated with the fluorine concentrations of the bone. The citric acid concentration in bones showed a wide range variation; however, no significant changes were observed with the citric acid calculated on an average base between normal and osteofluorotic bones. Bone cal-

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cium was somewhat higher and phosphorus level lower in the bones from the cows with osteofluorosis.

Introduction

It has been established that ingestion of excessive fluorine by animals for a prolonged period of time causes abnormal bone metabolism. Shupe et al. (1, 2) reported that approximately 95% of total fluoride in the body was present in the bone, enamel, cementum, and dentine. Soft tissues had only trace amounts of fluoride, and no significant soft tissue changes could be correlated with fluoride ingestion. Fluoride deposition in bone and the accompanying decrease in bone citrate were also reported in heifers(3), rats, and in mice (5). Fluoride accumulation in the bone and abnormal bone metabolism were also accompanied by an increase in alkaline bone phosphatase activity (6).

As the cattle grazing on a pasture adjacent to an industrial phosphate operation evidenced severe fluoride toxicosis, attempts were made to investigate the cause of the injury. Soft tissues from these cattle were analyzed and certain biochemical changes were noted (7). This paper reports some results obtained from analyses made on the bone fluoride, citric acid, calcium and phosphorus content and on the alkaline phosphatase activity.

Materials and Methods

Bone samples (ribs, metacarpal, and metatarsal) were obtained from four normal cows and twelve cows with osteofluorosis as described previously (7). Samples were taken from the endosteal and periosteal zones of the metacarpal and metatarsal bones from osteofluorotic animals and analyzed separately.

Inorganic fluorine was determined by ashing the bone in the presence of calcium oxide, dissolving the ash with 0.1 N HCl and then following the method of Nielson (8). The procedures used in the extraction and the determination of alkaline bone phosphatase were the same as those described by Miller and Shupe (7). Enzyme activity was expressed as micrograms of P hydrolyzed per hour per milligram of bone. Phosphorus was determined by the method of Chen et al. (9). For calcium determination the bones were subjected to wet digestion and the digested bone samples determined by atomic absorption spectrophotometry. Citric acid was determined by the method of Taylor (10). Values for fluoride, citric acid, calcium and phosphorus are given in ug/g in fat free bones.

Results

A marked increase in fluorine concentration was shown in the osteofluorotic bones (Table 1). The increase was 15.4 fold in ribs, 9.7 fold in the endosteal portion of the metacarpus, 15.9 fold in the periosteal portion of the metacarpus, 10.1 fold in the endosteal portion of the metatarsus and 20.3 fold in the periosteal portion of the metatarsus. The alkaline phosphatase activity of the osteofluorotic bones was also increased several fold (Table 2). The increase in the alkaline phosphatase activity was higher in the periosteal portion of the metacarpal and metatarsal bones than in the endosteal portion of the same bones, similar to the anatomical location of high bone fluoride. Although the highest activity was in the periosteal zone of the metatarsal bone (11.7 fold), the difference was slight between the activity of rib (5.5 fold) and the periosteal zone of the metacarpal bone (5.2 fold increase). The degree of activity was related to the degree or severity of osteofluorotic lesions. There was a slight increase in the citric acid content in the osteofluorotic bones calculated on an average base of the group (Table 3). The increase was a 3.3% in ribs, 4.8 - 12.2% in the endosteal and periosteal portion of the metacarpal bones respectively and 13.8 and 9.7% in the metatarsal bones. However, on an individual basis, a definite decrease was found in the citric

Table 1

Fluorine Content of Bones (μg F/g dry fat-free bone)

		Metacarpal		Metatarsal	
Cows	Rib	Endosteal	Periosteal	Endosteal	Periosteal
Control (4)*	333.5 ± 121.8**	347.0 ± 182.2		281.75 ± 129.7	
Osteofluorotic (12)	5139.4 ± 1890.4	3370.3 ± 1687.5	5541.29 ± 1788.1	2851.3 ± 1199.1	5735.7 ± 2045.1
		Average 4455.79		Average 4293.3	

*( ) = numbers tested, ** = sample mean and standard deviation.

Table 2

Alkaline Bone Phosphatase Activity (μg P/hr/mg bone)

		Metacarpal		Metatarsal	
Cows	Rib	Endosteal	Periosteal	Endosteal	Periosteal
Control (3)	0.35 ± 0.13	0.31 ± 0.16		0.27 ± 0.13	
Osteofluorotic (12)	1.94 ± 1.37	0.61 ± 0.24	1.61 ± 0.80	0.52 ± 0.30	3.16 ± 1.9
		Average 1.11		Average 1.84	

Table 3
Citric Acid Content of Bones (μg/g dry fat-free bone)

Cows	Rib	Metacarpal Endosteal	Metacarpal Periosteal	Metatarsal Endosteal	Metatarsal Periosteal
Control (4)*	15476.3±2212.9**	11516.9±1281.0		10031.2±1521.8	
Osteo-fluorotic (12)	159997.5±3455.8	12092.2±3456.7	13106.6±4063.7	11635.0±3453.7	11548.7±2786.3
		Average 12599.4		Average 11591.8	

acid content of some osteofluorotic bones: 13.3 - 26.1% decrease in ribs, 11.3 - 49.5% decrease in the endosteal zone of the metacarpal bone, 26.4 - 55.3% in the periosteal zone of the metacarpal bone, 8 - 40.3% in the endosteal part of the metatarsal bone and 28.3 - 48.0% in the periosteal zone of the metatarsal bones.

In some bone samples there was no apparent difference in citric acid content between control and osteofluorotic bones, while in the rest of the samples there was a definite increase in the citric acid content of osteofluorotic bones. These changes, however, do not seem to be statistically significant due to the great variation within the group of osteofluorotic bones, as reflected by high standard deviation.

Our results indicate a slight increase of calcium content and decrease of phosphorus content in bones from animals with osteofluorosis (Table 4 and 5), and as a result the Ca:P ratio was wider in the osteofluorotic group than in the control group (Table 6).

Table 4
Calcium Content of Bones (μg/g dry fat-free bone)

Cows	Rib	Metacarpal Endosteal	Metacarpal Periosteal	Metatarsal Endosteal	Metatarsal Periosteal
Control (4)	237114.5±9083.2	230641.2±6580.6		246132.0±5715	
Osteo-fluorotic (12)	258744.8±17828.3	271679.5±10529.3	255467.7±14248.1	280214.4±134690.0	252820.8±16650.1
		Average 263573.6		Average 266517.6	

DISCUSSION

The results obtained from these studies showed that cows grazing on a pasture adjacent to an industrial operation accumulated high concentrations of fluoride in the bones. The fluoride bone accumulation was apparently accompanied by alteration of metabolism of the bone. It has been demon-

Table 5

Phosphorus Content of Bones (μg/g dry fat-free bone)

Animal	Rib	Metacarpal		Metatarsal	
		Endosteal	Periosteal	Endosteal	Periosteal
Control(4)	118666±5426.3	118662.1±4218.1		125075.7±4686.9	
Osteo-fluorotic(12)	111016.7±44091	114047.7±23100.6	121007.8±24026.0	117266.2±23578.5	110012.8±19471.9
		Average 117527.7		Average 113639.5	

Table 6

Ca:P Ratio in Normal and Osteofluorotic Bones

Animal	Bones		
	Rib	Metacarpal	Metatarsal
Normal	1.99	1.95	1.96
Osteofluorotic	2.33	2.24	2.34

strated that the periosteal zone of the bones had a higher fluoride content than the endosteal bone (Table 1). This finding confirmed the observations of several authors who emphasized the importance of the method of sampling (2, 11, 12). Similarly, the alkaline phosphatase activity was also significantly elevated in the osteofluorotic bones. Miller and Shupe (6) reported similar findings. It should be noted that the highest enzyme activity was found in extract from the periosteal zones of both metacarpal and metatarsal, where fluoride concentrations were also the highest (Table 2). Our data show a close correlation between bone fluoride content and alkaline phosphatase activity.

Our results obtained from bone citric analyses (Table 3) do not agree completely with those reported by other authors who worked with various species of animals (3, 4, 5) and human bones (12) and found a definite decrease in bone citrate in fluorotic bones. In our case the citric acid content in normal and osteofluorotic bones showed the same pattern i.e., the highest concentration was found in the ribs, lower in the metacarpal and metatarsal bones. Although in some bone samples there was a definite decrease in citrate content, in others there was no change or even increase as compared with control samples. The reason for the great variations of bone citrate within one group in the osteofluorotic animals is not known; however, this might be due to other factors, to date not investigated. It is interesting to note that a definite decrease of citrate content was found in the hearts and kidneys of osteofluorotic cattle (7).

Fluoride ingestion by animals apparently did not alter significantly the concentrations of either calcium or phosphate in bones (Table 4 and 5);

however, the Ca:P ratio was wider in the osteofluorotic group (Table 6) as a result of slight increase in calcium and some decrease in phosphorus content. Here again the variations within osteofluorotic groups were much higher than in the controls. There is also an uncertainty in the literature whether excess fluoride causes positive or negative Ca:P balance (13, 14).

Acknowledgements

The authors wish to thank Mrs. Sally Cotter, Mrs. Gertrude Hsieh, Mr. D. Wang and Mr. A.E. Olson for their assistance. Some financial support was provided by the National Air Pollution Control Administration, Consumer Protection and Environmental Health Service, Public Health Service, Grant AP 00276-06, and the Agricultural Experiment Station and the Pollution Center, Utah State University.

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LONG-TERM EFFECTS OF FLUORIDE ADMINISTRATION AN EXPERIMENTAL STUDY

I. RADIOLOGICAL ASPECT

by

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SUMMARY: In the current study the effect of fluoride on the skeleton of rabbits was investigated. Excessive amounts of fluoride given for prolonged periods produced adverse effects in the nature of osteoporosis.

Endemic skeletal fluorosis has been reported from various parts of the world. In India the disease was first described by Shortt et al. (1) in the Nellore district of Madras Presidency and subsequently by Singh et al. (2-4) in the Northern part of India, Punjab, which was recognized as an endemic belt. Our own experimental work to assess the action of fluoride on human health was started in 1968. We noted that the skeletal changes in the rabbits do not resemble those seen in the endemic belt. It appears that there are some factors yet unknown which play a part in determining the pattern of skeletal changes. The current research was carried out to explore this question.

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Method

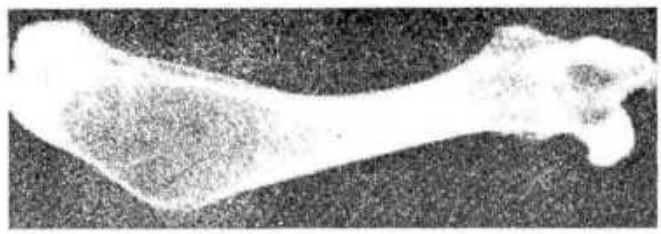
The animals were divided into four groups of 75 rabbits each, of either sex. Groups one and two were given sodium fluoride in distilled water in the dosage of 0.25 mg to 20 mg per kg of body weight orally and parenterally respectively. The salt was administered either in single or divided doses over a period of 2 to 3 years. These animals consumed municipal tap water. To the third group only water containing sodium fluoride in a concentration varying from 10 to 500 parts per million was administered for the same length of time. The fourth group constituted the control. The animals were kept on a well balanced high protein diet. The skeleton was procured either after their natural death or after they were sacrificed. Sodium fluoride was chosen because calcium fluoride and compounds of similar solubility are less toxic than sodium fluoride (5).

Results

The architectural pattern of the bones was found to be in a state of diminished density. The cortex of the long bones is seen as a distinct white homogeneous line, the thickness of which decreases both proximally and distally (Figure 1 and 2). The cancellous bone at the articular extremities is represented by a series of white lines, forming a honey comb-like pattern. Some of the trabeculae are prominent and may simulate the pressure and traction lamellae of the human skeleton. In some of the specimens, the honey comb-like spaces are much widened and are bordered by ill-defined trabeculae, giving the appearance of cystic changes in the cancellous tissue. In extreme forms, the metaphyseal segment of the bone is grossly expanded and irregular in outline. The cortex is reduced to a thin paper-like lamina. The trabecular pattern either disappears or becomes attenuated. The bone subsequently shows a pathological fracture resulting in mal-union of segments (Figures 3 - 6). The medullary cavity can be easily mapped out although its limits, unlike the cortex, is not sharply delineated. The articular extremities are smooth and regular in appearance. The vertebrae as seen in lateral radiographs are normal in configuration, bordered by sharply demarcated lines indicating the superior and inferior surfaces of bodies (Figure 7). The intervertebral spaces are in the form of clear translucent areas and are not encroached upon by any extraneous shadows of calcification or ossification of various ligaments, which are characteristic of human skeletal fluorosis.

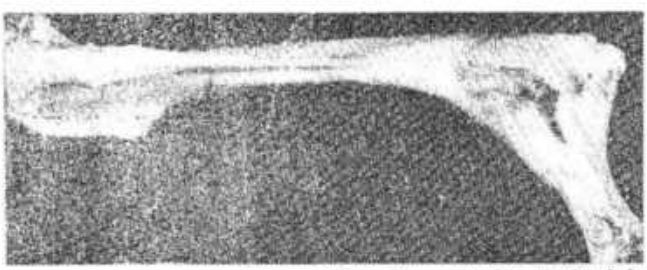
Thus, in general, the poor state of mineralization of the framework of the skeleton is manifested by the comparatively less density of the cortical and cancellous layers of bone. These changes partially substantiate the observations of Leone, Stevenson, Hilbish and Sosman (5), Shupe, Harris, Greenwood, Butcher and Nielsen (6) and Shupe and Alther (7) that the skeleton

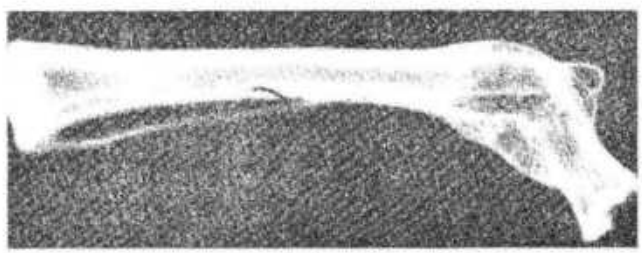
Figures 1 and 2 Specimen and X-Ray of Fluorosed Femur



Marked irregular expansion of distal segment of femur, more conspicuous dorsally. Irregular and pitted surface showing minute pinpoint apertures. Cortex of this expanded segment reduced to a thin linear line, especially on its inner aspect. Loss of trabeculation, causing a cystic cavity.

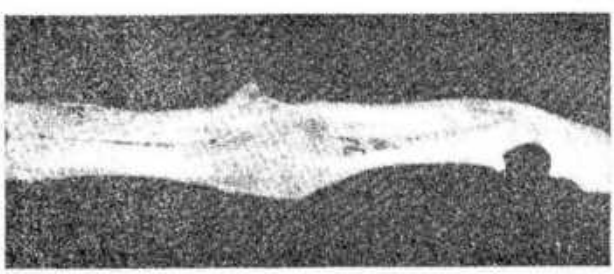
Figures 3 and 4 Fluorosed Tibia and Fibula

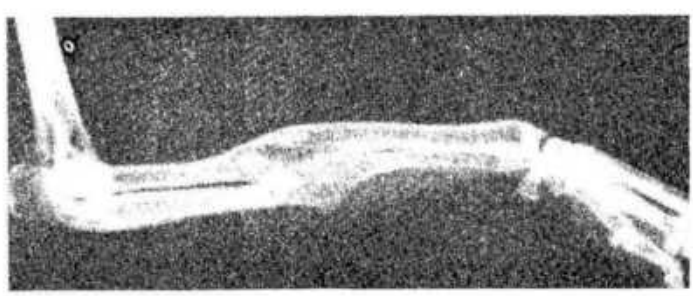




Poor mineralization at distal third. Healed fracture. Malunion of segments. External callus, formed irregularly. Note appearance of a medullary cavity.

Figures 5 and 6 Fluorosed Radius and Ulna

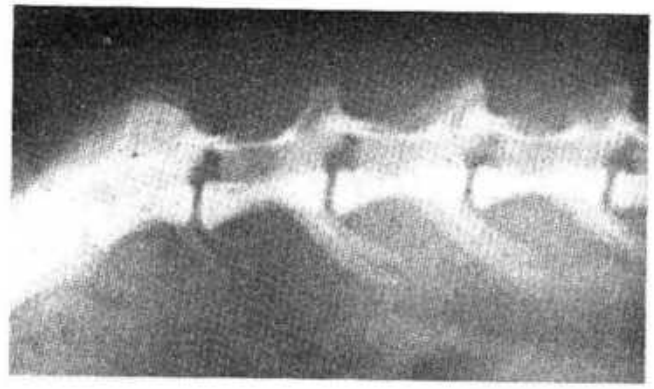




Fracture involves radius and ulna at its central thickest part of the shaft. The final process of healing and repair obscures the separate delineation of cortex of the two bones.

Figure 7 Advanced Osteoporosis of Vertebrae

Fluorosed vertebrae



Normal control

Linear crack on dorsal aspect of vertebral body. Wooly opacity encroaches vertebral canal and intervertebral foramen. Intervertebral space narrowed. Adjacent margins of vertebrae not sharp in outline.

may exhibit the appearance of porosis. However, these workers have also observed by roentgenograms sclerosis, malacia, hyperostosis, osteophytosis or any combination of these conditions in animals that have been given excessive amounts of fluoride over long periods of time. In the current series, two cases of compression paraplegia of hind limbs were seen due to fracture of the vertebrae which showed marked degrees of osteoporosis. This is an interesting and unusual finding not reported, to date, in experimental studies.

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TEMPORAL BONE IN FLUOROSIS

by

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SUMMARY: Twelve temporal bones of clinically proved cases of fluorosis have been examined. The bone presents features which are within the normal range of anatomical variations. Audiometric studies conducted in 25 male patients at ages 45 - 60 years, revealed mild sensori-neural deafness at higher frequencies.

Fluoride has received considerable attention by otologists since Shambaugh et al. first recommended its use in the treatment of otosclerosis (1). Also a report by Siddiqui (2) suggests that partial deafness might be a feature of skeletal fluorosis. It was, therefore, of interest to determine whether or not any unusual changes in the ear were discernible at autopsy.

This paper presents the gross anatomical findings in the fluorotic temporal bone as a whole, with special reference to the cavity of the middle ear on 12 cases of skeletal fluorosis. In the following our findings are described and illustrated in Figures 1 to 4.

The bone is thick and heavy, its transparency is significantly reduced. The sites of attachment of muscles, ligaments and fascia are prominent. The configuration of Macewen's triangle is conspicuous and its spine of Henle is prominent with rough and irregular margins. The foramina are normal in contour and reveal no narrowing or exostosis. The ear

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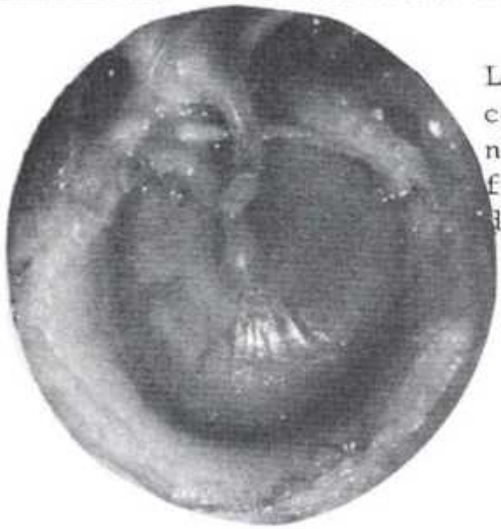
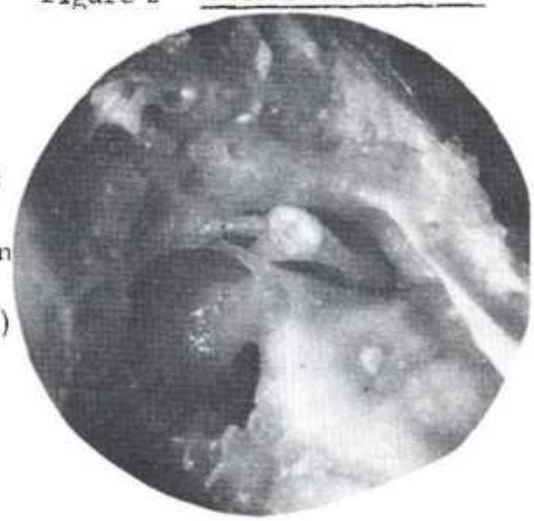
Figure 1

Medial Surface of Tympanic Membrane



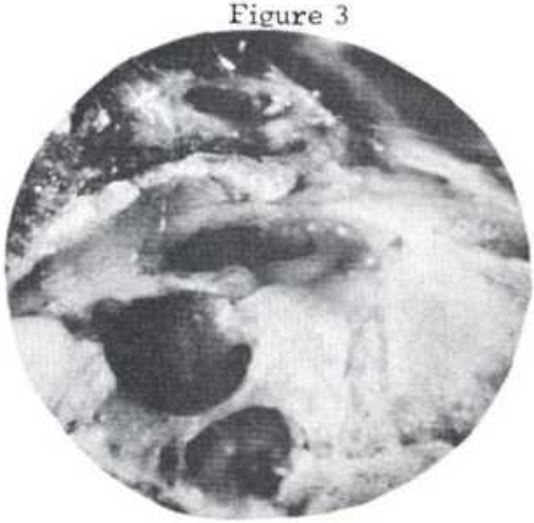
Long process of incus curved in outline. Lenticular process overrides the handle of malleus. Chorda tympani nerve in normal position. No ossification of peripheral fibrocartilaginous rim of the membrane which is easily dislodged from the tympanic sulcus (Fig. 1).

Figure 2 Facial Nerve Canal



A bulge hides lateral part of superior border of foot plate of stapes. Promontory connected to pyramid by fine spicule of bone. The stapedius muscle tendon attached to posterior aspect of neck of stapes. (Fig. 2)

Figure 3



The anterior crus of stapes prominent, showing a well marked bending. Fenestra cochleae divided by a spicule of bone into two areas (Fig. 3).

Figure 4

Bony canal of facial nerve deficient. Nerve in exposed position and covered by mucous membrane. Two spicules extending from promontory and pyramid toward each other (Fig. 4)

ossicles are freely mobile at their incudo-stapedial and incudo-malleolar joints. The foot plate of the stapes is also movable and can be easily dislodged from the fenestra vestibuli. There is no involvement of any ligaments binding these ossicles. The tendons of stapedius and tensor tympani show no ossification. The promontory is often completely or partially connected to the pyramid by bony spicules. The canal for the facial nerve shows some dehisence. The mastoid process is cellular in character. These features are all within the normal anatomical variations. The ear ossicles are normal in configuration and weight.

No conductive deafness occurred in advanced cases of fluorosis as demonstrated by the audiometric graphs. They show only mild sensorineural deafness at higher frequencies in aged patients explained by the presence of presbyacusis at this age.

A plausible explanation for the immunity enjoyed by the middle ear cavity might be that the osteosclerotic changes involve only the fairly prominent bone structures. No pathological changes are seen in the crico-arytenoid articulations. The greater parts of hyaline arytenoid cartilage do not show any calcification. The phonation and articulation is normal; the low tone is a normal phenomenon of advancing age affecting the intrinsic musculature of the larnyx.

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AUFTRETEN VON NACHBARSCHAFTSFLUOROSE UNTER DER BEVOLKERUNG EINER SACHSISCHEN KLEINSTADT

by

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(Abstracted from Deutsch. Gesundh.-Wesen, 31:1271-1274, 1976)

In the vicinity of an aluminum smelter near the city of Dohna, DDR, where trees, food and vegetables and domestic animals were considerably damaged by fluoride, the author examined x-ray films of 20 subjects, 16 men and 4 women. These individuals were residing within a radius of 50 to 450 m of the plant for an average of 28 years. The only one of the group who had been in factory employment was working in a different plant in the same area, which produces hydrogen fluoride. Two persons were residing within 2 km of the second factory. All had been consuming food, vegetables and meat grown near the aluminum smelter. In four persons, x-ray findings were normal, 11 showed minor periosteal depositions and slight thickening of the bone structure and 5 exhibited evidence of typical skeletal fluorosis. The air near the aluminum factory contained 0.5 mg/m^3 fluoride; leaves of fruit trees close to the factory contained 440 ppm in dry substance; cabbage, 150 ppm and hay, 88 to 91 ppm. Measures to counter the fluoride emission have somewhat improved the situation.

THE EFFECT OF NaF AND SnF_2 MOUTHRINSES ON BACTERIAL COLONIZATION OF TOOTH ENAMEL: TEM AND SEM STUDIES

by

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(Abstracted from Caries Res., 10:415-426, 1976)

Whereas the caries preventive effect of fluoride is usually attributed to the reduction in solubility of the tooth enamel there might also

be a direct effect of fluoride on the microorganisms in dental plaque. Several authors have reported that concentrations of fluoride between 2 and 100 ppm inhibit the glycolytic activity of microorganisms and that higher concentrations of fluoride have been found to be bactericidal. Other research has shown that fluoride suppresses bacterial colonization on enamel after treatment with stannous fluoride. Fluoride may also decrease the surface energy of enamel which in turn may influence the adsorption of protein. A significant bacterial reduction in saliva followed the use of SnF_2 (1200 ppm) but did not occur after mouthrinsing with NaF in comparable concentration.

In the current investigation the authors used electron microscopy to explain the variables affecting the suppression of plaque development by the two mouthrinses, SnF_2 and NaF (100 ppm) in vivo.

In a volunteer, cylinders of surface enamel were sectioned out of the smooth surfaces of extracted molars by means of a cylindrical diamond drill. Two to eight specimens were embedded with sticky wax to the upper jaw in a Hawley appliance. The appliance was removed for meals. In 13 different periods a total of 40 enamel cylinders were worn for two to seven days. All specimens were derived from the one individual who wore the appliance.

In the solution of aqueous stannous chloride (0.049%) the tin was equimolar to the tin found in stannous fluoride (100 ppm F^-). The aqueous solution of NaF also contained 100 ppm F^-. The mouth was rinsed for 1 minute with 10 ml of the respective solution in the evening before retiring in the once a day regime and, after rising and before retiring in the twice a day regime. Two weeks were allowed between the two experimental periods in order to permit equilibration of a possibly altered flora. The enamel cylinders were removed from the appliance and prepared for electron microscopy.

NaF: On all specimens worn for two days and subjected to NaF (100 ppm) mouthrinses, plaque formation was apparent macroscopically which was more pronounced in the specimen rinsed only once a day. Transmission microscopy showed that the bacteria were adsorbed to the enamel surface with a pellicular deposit often present between the enamel and the plaque. Specimens which had been worn for two days and had been rinsed twice daily with NaF appeared, on scanning electron microscopy, to have a separation between the plaque and enamel. Variation in the microbial colonization was due to alteration of adhesion of bacteria to enamel and of bacteria to bacteria.

SnF_2: Mouthrinsing with SnF_2 for two days showed almost total absence of bacteria, but when the solution was aged there was some bacterial colonization. Control rinsing with $SnCl_2$ showed less reduction in bacterial growth than with SnF_2.

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INDUSTRIAL SKELETAL FLUOROSIS AND PROBLEMS OF TREATMENT OSTEOFLUOROSIS WITH FLUORINE

by

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(Abstracted from Zeitschr. für Altersforsch., 29:277-283, 1975)

Twelve workers at the hydrogen fluoride plant in Dohna, Saxony who had been under observation for over 22 years and had been exposed to fluoride for 15 to 20 years were autopsied and skeletal studies performed. In seven, complete skeletons were obtained which exhibited the following features:

The majority showed pronounced osteosclerosis and hypersclerosis involving mostly the endosteal and periosteal surfaces which lead to an increase in bone mass, both in the compacta and the spongeous bone. The new bone formation was more pronounced at the endosteal than at the periosteal surface. Whereas on cross section the thickness of the bone was not essentially increased, the bone marrow was distinctly smaller than in normal bone.

Numerous indications in the microscopic picture suggest a periodic accretion of new bone which accounted for a mosaic-like appearance. Fluorotic bone showed no change in the number of osteocytes as compared with normal bone. The blue-stain of the bone substance was indicative of an increased metabolic activity of the osteophytes. The author found no fibrosis of the bone marrow, no osteoporosis, no evidence of osteomalacia.

The second important characteristic sign of fluorosis was multiple periosteal appositions on bone. The osteophytes thus formed consisted of mature bone substance which because of its porosity could be clearly dis-

tinguished from original compact bone. No evidence of inflammation was found. The periosteal bone originated without mediation of cartilage. Furthermore, ossification of tendons and muscle attachments appeared as pointed protrusions. The first changes of fluorosis occured in the spinal column, the ribs, and pelvic bones. No tumors were observed in the study, nor was there clinical evidence of anemia.

The author's observations suggest the following considerations concerning treatment with sodium fluoride:

1. Industrial fluorosis can be considered a model for long-term application of fluoride. Since atrophy of bones in old age and osteoporosis are characterized principally by endosteal distribution of bones and since fluoride stimulates mainly endosteal apposition, it appears likely that the destruction of bone can be ameliorated by fluoride.

2. Fluoride treatment must be initiated at a time when there is sufficient activity of osteoblast, i.e. during mid-life, not in advanced age.

3. Other essential factors are likely to influence fluoride therapy, especially nutritional habits. With simultaneous supplementation of calcium and phosphorus, marked apposition of bone occurs. The author considers prophylaxis of osteoporosis with sodium fluoride desirable.

J. F.

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EFFECT OF DIETARY FAT ON FLUORIDE ABSORPTION AND TISSUE FLUORIDE RETENTION IN RATS

by

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(Abstracted from the J. Nutr., 106:575-579, 1976)

The authors investigated the effect of graded levels of fat intake on the toxicity of dietary fluoride. Rats were administered an iso-energetic diet with graded levels of fat (O, 10%, 30%, 50%) containing 400 ppm fluoride. All animals received the control diet for one week before the start of the ex-

periment. Samples of plasma, liver and bone collected at the end of the study were assayed for their fluoride content. Urinary and fecal fluoride excretion were also studied in order to determine the effect of dietary fat on fluoride absorption.

A depression in growth occurred in all fluoride groups but was most dramatic in the 50% fat group. This decrease in weight lasted for two weeks, but in the 50% fat group remained significantly depressed. The fluoride concentration in the tissue correlated with the fat intake. All plasma fluoride concentrations were higher in the evening. The increased fluoride concentration in plasma and tissue were attributed to greater absorption of fluoride, since urinary fluoride rose and fecal fluoride fell in direct relation to the elevation in fat consumption. The authors believe that fluoride absorption was enhanced due to the delaying effect of fat and fluoride upon gastric emptying, which provided more time for fluoride to be absorbed through the stomach.

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EFFECT OF FLUORINE AIR POLLUTION ON FLUORIDE CONTENT OF DECIDUOUS TOOTH ENAMEL

by

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(Abstracted from Proc. Finn. Dent. Soc., 72:27-29, 1976)

In 14 children residing in the vicinity of a Finnish superphosphate factory located in the city of Oulu elevation of the fluoride content of the outermost surface of tooth enamel was statistically significant compared with that of 13 children residing in the most distant "fluoride-free" part of Oulu. The monthly fallout of fluoride in the contaminated air in 1970 ranged from 17 to 97 g/hectare. Drinking water in Oulu contained 0.1 ppm fluoride. The children ranged in age from 8 to 11. The fluoride analyses were made on the enamel layers of the vestibular surface of deciduous teeth.

The authors believe that inhalation of air contaminated by fluoride has a local effect on teeth but no systemic sequelae and that elevated fluoride levels in enamel constitute significant evidence of the presence of

fluoride in the atmosphere. Fluoride deposition in the enamel, the authors conclude, is not increased in a polluted area during the fetal or preeruptive period.

FLUORIDE EXCRETION IN HUMANS OF DIFFERENT AGE GROUPS

by

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(Abstracted from Wiener Klin. Wochensch., 88:209-12, 1976)

The authors studied 24-hour fluoride excretion in the urine of three groups each consisting of 5 male subjects in the following ages: Group A, 4 to 6 years; Group B, 25 to 45 years; Group C, 60 to 70 years. During the four weeks prior to the study, the individuals had no known fluoride intake.

Two kinds of studies were made. The three groups received 6 mg sodium fluoride within 24 hours and the hourly fluoride excretion was charted. In the second study each individual received 6 mg of sodium fluoride daily during a period of ten days and the daily 24-hour specimens were studied for their fluoride content.

In the hourly study, the peak of excretion occurred within 4 1/2 hours in groups A and B but in group C (the older individuals, aged 60-70) the maximum was reached at 5 hours. This finding suggests a delay in absorption or excretion in the older individuals probably due to altered fluoride metabolism in this group. The results of the second study which determined the daily urinary output for 15 days provides further support for this conclusion. When the fluoride was given daily in doses of 6 mg the peak was reached on the second day in groups A and B but in all five persons of group C the peak was not reached before day 11.

After discontinuance of the fluoride on the 11th day a prompt decrease in the fluoride level occurred in all three groups. Again in group C a slight delay was observed compared with the excretion in the two other groups. The authors noted that there were marked variations in the excretion of the urine between individuals in the three groups during the 11 day period. At each of the higher ages a decrease in the regulatory mechanism of the fluoride metabolism occurs.

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