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The 13th Conference of the International Society for Fluoride Research is scheduled to convene in New Delhi, India, November 14-17, 1983. The program committee is soliciting abstracts (up to 300 words) of papers to be presented at the conference dealing with the action of fluoride on (1) Ecology and Environment (2) Geology and Geochemistry and (3) Health. Abstracts should be submitted prior to March 15, 1983. Authors will be notified of acceptance of papers by June 1, 1983.

Kindly send abstracts to Dr. A.K. Susheela, Organizing Secretary, 13th I.S.F.R. Conference, Department of Anatomy, All India Institute of Medical Sciences, New Delhi 110029, India.

Further information concerning the conference will appear in FLUORIDE January 1983.

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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.

FLUORIDE is listed in
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IN MEMORIUM

George L. Waldbott, M.D.
Jan. 14, 1898 - July 17, 1982



The officers, and members of the Editorial and Advisory Boards of the International Society for Fluoride Research wish to express their deep and heartfelt sorrow at the sudden demise of the society's founder and the editor of its official journal FLUORIDE since its inception in 1968, George L. Waldbott, M.D., 84, following open heart surgery. A new aortic valve was successfully implanted, he was regaining his strength when postoperative complications developed.

Dr. Waldbott was residing in Leonard, Michigan. He was a practicing physician in the State of Michigan since November 23, 1923. A specialist in allergic diseases, he was a graduate of The University of Heidelberg, Germany, Medical School in 1921 and then interned at Henry Ford Hospital, Detroit, 1923 to 1924. He was a member of the American Medical Association, Michigan

State and Wayne County Medical Societies, a diplomate of the American Board of Internal Medicine since July 1, 1937; a diplomate of the specialty of allergy since April 19, 1941; co-founder and former president of the Michigan Allergy Society (1936); Fellow of the American College of Physicians; Fellow of the American College of Chest Physicians; Fellow of the Academy of Allergy; Fellow of the American College of Allergists; honorary member of the French and Spanish Allergy Societies.

He was founder and chief of allergy clinics in four Detroit hospitals: Grace, Harper and Children's Hospitals of Michigan, and the North End Clinic (now Sinai Hospital); Emeritus Physician in Allergy at Harper Hospital and Honorary Physician at Hutzel Hospital, Detroit; former President of the Michigan Branch of the American College of Chest Physicians; former Chairman of the Air Pollution Committee of the American College of Chest Physicians, and of the American Academy of Allergy.

Dr. Waldbott was a pioneer in the specialty of allergy. His extensive clinical research has appeared in more than 200 publications, many in American Medical Association journals. Early in his career, his original research on human anaphylaxis, published in a series of articles, has been responsible for saving numerous lives.

He was first to report many new observations in his specialty. For example:

He was first to investigate the effect of tonsillectomy in allergic respiratory disease.

He was first to report allergy (asthma) due to local anesthesia.

He was first to call attention to the relationship of the thymus gland and lymphoid tissue to allergy.

He was first to describe allergic pneumonitis.

In 1927, he carried out the first pollen survey in Michigan and, in 1937, the first comprehensive annual fungus survey ever published.

He determined that rust and smut are major causes in respiratory allergy.

He was first to describe a case of allergic pneumonitis in a pigeon breeder (1943).

He presented one of the first clinical studies on antihistamines before the general session of the American Medical Association at its hundredth anniversary celebration.

He evaluated the effect of diets in chronic asthma and countered the abuse of dieting in chronic asthma.

He presented the first fatality of human anaphylaxis due to penicillin.

He introduced bronchoscopic lavage as an emergency treatment in status asthmaticus, which has saved numerous lives.

He presented the first case of urticaria due to pollen.

He carried out an experimental study on drug tolerance in allergic diseases.

He made the first clinical observations on the effect of smoking (other than cancer) on the respiratory tract.

His book on "Contact Dermatitis" (1953) in which he presented an original method of determining the source of the lesion by observing the pattern - termed a classic for many years to come - has been invaluable to physicians as well as to patients themselves in diagnosing the source of their ailment. He was contributing author of several other books pertaining to allergy.

His report in 1954 of the first fatality of human anaphylaxis from penicillin received editorial commendation in the Journal of the American Medical Association.

His experience with intolerance to drugs in his patients led him to the study of the effect of fluoride and of other environmental pollutants

on the human body. His book "Health Effects of Environmental Pollutants", second edition, March 1978 - one of the first on the subject - is being used as a textbook in universities here and abroad.

For the past 25 years, since 1955, he has been carrying out basic clinical research on how fluoride affects the human organism. His data have been presented in more than 80 reports in some of the most important medical journals in the U.S.A. and abroad. These publications include two monographs, one entitled "Fluoride in Clinical Medicine", the other "Acute Fluoride Intoxication", an article entitled "Fluoride in Food", and another article "The Physiologic and Hygienic Aspects of the Absorption of Inorganic Fluorides, Comments on the Symposium", the last-mentioned of which appeared in an American Medical Association publication. A chapter on the "Health Impact of Fluoride in Air and Water -- International Clinical Data" in the Health Handbook edited by G.K. Chacko appeared in 1979 by the North-Holland Publishing Company, Amsterdam.

His most recent book "Fluoridation: The Great Dilemma", 1978, in collaboration with Professors A.W. Burgstahler and H.L. McKinney is the most encompassing presentation available on this subject.

His studies on fluoride include the administration of test doses of fluoride to allergic and non-allergic individuals and to those suspected of being intolerant to fluoridated water. During the course of those studies he had urinary analyses made for fluoride on more than 300 individuals. He determined levels in blood of various biochemical agents, especially calcium and phosphorus, before and after test doses with fluoride. He had analyses done for fluoride in food, eye cataracts, bones and other organs. He compared the fluoride content of normal-appearing aortas with that of calcified aortas, of normal skin with that of diseased skin, of normal lung tissue with that of diseased lung tissue.

He studied cases of fluorosis in Tampa, Florida (air pollution from fertilizer factories); Lubbock, Texas (natural fluoride water - 4.4 ppm); Saginaw, Michigan (fluoridated water); Palermo, Italy (natural fluoride water - 3 to 6 ppm); Port Maitland, Ontario, and Walcott, Iowa (air pollution due to a fertilizer factory); Wabash, Indiana (pollution from secondary aluminum smelters); Moehlin, Switzerland (pollution from an aluminum factory); Barcelona, Spain (fluoride-contaminated wine); Bolzano, Italy (aluminum and magnesium manufacturing); Kitimat, British Columbia (aluminum factory); Clarington, Ohio (near an aluminum plant). In Hannover, Germany he observed fluorosed cattle; in Stockholm, Sweden, fluorosed calves and horses; in Brussels, Belgium, fluorosed sheep. Just prior to his death he was engaged in the study of the health effects of environmental pollutants in Urbana, Ohio and in Hemlock, Michigan.

As founder of the International Society for Fluoride Research, a multi-disciplinary organization, the purpose of which is to investigate the biological effects of fluoride, and editor of FLUORIDE, its official publication, he has made an invaluable contribution toward understanding how fluoride in water, air, food and pharmaceuticals affects humans, vegetation and animals, both wild and domestic.

Among awards, he received first prize for his exhibit on Occupational Allergy at the Congress of the European Academy of Allergy, The Hague,

Holland (May 11, 1958); another first prize from the journal "Cutis" in collaboration with Dr. V.A. Cecilioni, in March 1972 (page 331), for his manuscript on Chizzola Maculae, the description of a skin lesion which is a diagnostic tool in chronic fluoride poisoning. He was presented with a distinguished "Award of Merit" by the Board of Regents of the American College of Allergists, March 30, 1977 "in recognition of professional achievements, contributions to the medical literature, teaching on allergy and immunology and for more than 25 years service to patients and the profession of medicine, particularly in his field (of allergy)."

A comprehensive article in the Southern Medical Journal, March 1980, which includes case histories on the preskeletal phase of fluoride intoxication; presentation to his colleagues in October 1980 of a poster exhibit on "The Role of Fluoride in Clinical Medicine" - a condensation of his vast research on fluoride - at The Michigan Chapter of the College of Physicians at Sugar Loaf Mountain in Northern Michigan, and in the following January in Atlanta at the 1981 Winter Session of the American Medical Association - represent fitting highlights in his long and distinguished career in medicine in service to mankind. He will be missed not only in this country but in countries throughout the world.

OVERFLUORIDATION

Formerly acute fluoride intoxication usually resulted from suicide or homicide attempts, or from ingestion of fluoride compounds mistaken for a non-toxic agent such as baking powder(1). In recent years, however, several cases of acute fluoride intoxication have been associated with prophylaxis for tooth decay. In general, accidental mass poisoning from fluoride taken by mistake seems to have decreased, whereas accidents due to fluoridation of public water supplies have become more prevalent.

In at least 9 such accidents, the health of humans was affected (Table 1), and in 6 others the spill contributed to environmental pollution (Table 2). Ten incidents involved fluoridated municipal water supplies (1 ppm), whereas five occurred in rural schools where hydrofluosilic acid was being added to school drinking water (5 ppm).

It is difficult to evaluate the extent of adverse health effects caused by such accidents because temporary illness may not be severe enough to warrant hospitalization. Therefore no case records are available. Indeed, in some instances, neither the patients nor the physicians were sufficiently versed concerning the symptoms of fluoride poisoning even to suspect it. Besides, lay people and scientists have been constantly reassured that no harm can result from fluoridation. Furthermore, the responsible authorities - desirous to maintain a good image for fluoridation - tend to minimize the damage. For instance, in Harbor Springs the illness was attributed, at first, to a high iron content of the water. It was four years before the true nature of the episode was publicized through the press (2).

The most serious spill recorded to date occurred on November 11, 1979 in Annapolis, Maryland, where one death and one near fatality were officially acknowledged among 8 patients undergoing hemodialysis. A sample of "softened" water used for dialysis November 13th contained 50 ppm (3). Officially the symptoms in the 8 dialysis patients were nausea, hypotension, substernal pain or pressure, diarrhea, itching, vomiting, malaise, dyspnea, flushing, localized numbness, diaphoresis, and headache (3).

Regarding this accident, the following facts are noteworthy:

1. Poisoning was not solely the result of drinking water but was also due to food and commercial beverage preparations, especially soft drinks, which had been contaminated by the overfluoridated water. In 13 of 103 individuals, the disease started 10 to 14 days after the spill undoubtedly due to consumption of food and beverages (4) processed with water which contained up to 30 ppm fluoride (3).
2. Existing diseases, such as diabetes and a tendency to kidney stones, became aggravated during the spill (4).
3. In 25 subjects, who vomited, the illness lasted from a few hours to 2 days. In three, the disease lingered up to day 18 (4).
4. Of four pregnant women, who feared possible damage to the fetus,

Table 1

FLUORIDE "SPILLS" REPORTED IN THE LITERATURE

Name of Town Reference	Date	Cause	F ⁻ Content (ppm)	Duration of spill	Number Affected
<u>Szolnok, Hungary</u> Orvosí Hetilap 108:306-7, 1967	3/29 1965	Soda water plant failed to flush pipes after F ⁻ equipment plant had been closed for repair.	<650:orangeade 900:soda water		15-20 in restaurant 55 children 5 adults in kindergarten
*N.E. Bradford Elem. School, Rome, PA. Dept. Health Ment. Hyg. Field Investigation Report 35-36 (3-19-80) State of MD.	6/6/72	BIF feeder by-pass blocked	48 to 67ppm in water 230 in coffee		150 Students at school picnic vomited after drinking orange juice made with water.
*Stanly, N.C. M.M.W.R., 23: 190, 1974	3/16 1974	F ⁻ feeder pump malfunctioned	270:orange juice 125:water		201 students 12 adults
<u>Harbor Springs, MI.</u> PHS-CDC- Atlanta, 12-14-78. J.Amer. Water Works Assoc. 72: 238-243, 1980.	11/22 1977	Tree fell on electric wire causing failure of F ⁻ feeder control.	<2400 mg/l: water	<25 min.	4
*Los Lunas, N.M. Pediatrics, 65: 897-900, 1980	11/17 1978	Malfunction of electric relay switch.	375(Bldg. A) 9.35(Bldg.B)		34
<u>Island Falls, ME.</u> Report by Water Dist. Mgr.C. Given, 9/21/81.	May 1979	Extra F ⁻ flowed into water system during change of meter head.			Gastrointestinal symptoms
<u>Annapolis, MD.</u> M.M.W.R. 29: 134-36, 1980	11/13 1979	Worker neglected to close valve	36:city water 50:water of dialysis unit	17 1/2 hrs.	<6000 (?) 22 attending farmers' market on school grounds: Nausea, vomiting, headache, cramps, dizziness, diarrhea
*Vermont Elem. School. Amer. J. Public Health 72:1168-1169, 1982	8/30/80	Continuous operation of school fluoridator	1041 mg/l: water		
*Jonesboro Elem. School, Dept. of Human Services State of Maine, Augusta 10/22/81	10/6 1981	Defective valve	25.3:water fountain 84:coffee pot 236:left over cup of coffee		57 transported to hospital, 38 including 3 teachers given emetics, 2 hospitalized temporarily

* School fluoridation

Table 2
Fluoride "Spills" Causing Environmental Pollution

Town & Ref.	Date	Cause of Accident	Results
<u>Lebanon, Pa.</u> Lebanon Daily News 8/22/75	8/20 1975	Ruptured storage tank holding 6000 gal. of H_2SiF_6	About 1500 gal. leaked into holding ponds and Swatara Creek, killing fish.
<u>Seattle, Wash.</u> Seattle Times 5/23/76	May 1976	Break in plastic fitting of rubber-lined steel tank.	5000 gals. spilled in- to Cedar River water- shed.
<u>Syracuse, N.Y.</u> Auburn Citizen 3/29/77	March 1977	Ruptured underground F^- tank.	1200 gals. accidentally pumped into Skaneateles Creek. Hundreds of fish dead on banks of creek.
<u>Marin Co. Calif.</u> San Rafael Inde- pendent Journal 11/25/77	10/27- 11/2 1977	F^- feeder valve malfunctioned.	Five communities re- ceived up to 5.4 ppm excess F^- for several days. Public not in- formed.
<u>Pendleton, Ore.</u> East Oregonian 7/20/81	6/2-8 1981	Burst pipe leading from F^- storage tank.	4000 gals. of F^- leaked into ground.
<u>Village of Potsdam, N.Y.</u> Courier-Freeman 8/18/81	8/10 1981	"Diffuser", a plastic pipe that controls F^- flow into the water system, broke off. Entire contents of a drum of F^- entered the water supply.	Residents in a state of "water emergency" for 9 hrs.

two had only minor symptoms, two others were completely symptom-free (4). In other kinds of mass poisoning as, for instance, the Minamata Bay mercury poisoning in Japan (5), pregnant women seemed to be unusually tolerant to toxic agents probably due in part to transfer of the poison across the placental barrier to the fetus. Such transfer of fluoride has been documented repeatedly (6).

5. In the fatal and near-fatal hemodialysis cases, the critical stage was not immediate but occurred several hours after the patients had experienced temporary improvement.

In Annapolis, the accident was due to failure of a waterworks employee to close a control valve which meters 22% hydrofluosilic acid from a 4000 gallon storage tank to a 50 gallon fluoride feeder. "One thousand gallons of the acid overflowed into drains leading to sand-filter-backwash and sludge-decant tanks from which decanted liquid was recycled as raw water." (3).

In Harbor Springs a tree, which accidentally fell across city power lines, shut off the electricity which controlled the feeding of the acid into the water (7,8). According to water department records, as much as 86 kg of 25% hydrofluosilicic acid may have been pumped into the system (7). The maximum fluoride level in water may have reached as high as 2400 mg/l in some residences (7).

In the other 11 instances, the accidents were due to equipment failure. It appears likely that the highly corrosive fluosilicic acid itself might be the chief culprit as it is liable to damage the equipment. It is a fact that the accidents have become more prevalent during the past few years following prolonged use of the equipment.

Although waterworks and health officials carefully monitor the fluoride content of water supplies, it will be difficult if not impossible to prevent similar accidents in the future.

These "accidents" reveal that fluoridation is by no means "completely safe" as the public is being continuously assured. No other water treatment procedure is as potentially hazardous as addition of fluoride to drinking water.

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G.L.W.

FATE OF FLUORIDE FOLLOWING ITS ADMINISTRATION
INTO A BIOLOGICAL SYSTEM - AN IN VIVO STUDY

by

A.K. Susheela, Y.D. Sharma, Mohan Jha, M. Singh,
B. Jagannath, and S.K. Jain
New Delhi, India

SUMMARY: Although it is established that excessive ingestion of fluoride leads to its deposition in tissues, it is not fully understood how much of it is deposited, how much is excreted and what quantity of fluoride is in circulation. Whether the rate of uptake and retention of fluoride is the same or different with regard to different tissues is not known.

Some of these questions have been answered by conducting a survey on fluoride content in tissues of various organs of rabbits namely, calcified tissues, noncalcified tissues, serum and urine after ingestion of 10 mg NaF daily for varying periods of time.

Material and Methods

Fluoride Estimation (Serum): Rabbits, which were administered 10 mg NaF daily, were bled at an interval of 1,2,3,6,8, and 10 months after fluoride ingestion by marginal vein puncture of the pinna/ocular vein. Serum fluoride was determined by the method of Hall et al. (1) using fluoride ion specific electrode in a PHM 84 Research pH meter (Radiometer). The fluoride content of serum, expressed as ppm, is reported in Table 1.

Fluoride Estimation (Urine): Rabbits 2 months of age were administered (intragastrically) 10 mg NaF/kg body weight for varying periods of time. Urine samples were collected at different intervals starting from day one and fluoride content was estimated. The pH of the urine was adjusted between 2 and 3 with 30% perchloric acid and each sample was diluted to 5 ml with 0.1 M acetate buffer (pH 5.2). The fluoride ion concentration was determined by fluoride ion specific electrode as described by Hall et al. (1). The results are reported in Table 2.

Fluoride Estimation (Noncalcified Tissues): Samples of skeletal muscle, liver and kidney obtained from rabbits exposed daily to 10 mg NaF/kg body weight, sacrificed at 6 months, were ashed and fluoride content was determined by the method of Singer and Armstrong (2). Results are reported in Table 3.

From the Fluorosis Research Laboratory, Dept. of Anatomy, All India Institute of Medical Sciences, New Delhi, India. Presented at the 12th I.S.F.R. Conference, May 16-18, 1982, St. Petersburg Beach, Florida.

Table 1

Serum F^- Level in Rabbit Following F^- Administration
(10 mg/kg body weight)

	ppm F^- Mean \pm S.D.
Normal (3)	0.07 \pm 0.02
NaF Treated:	
1 month (5)*	0.29 \pm 0.04
2 months (5)*	0.35 \pm 0.08
3 months (5)*	0.40 \pm 0.02
6 months (3)**	0.36 \pm 0.08
8 months (3)**	0.45 \pm 0.06
10 months (3)**	0.50 \pm 0.01

Number in parenthesis indicate the number of experiments. S.D. - Standard Deviation; * P value $< .05$; ** P value < 0.005 .

Table 2

Urinary F^- Following Daily Administration of
10 mg NaF/kg Body Weight

	Mean \pm S.D.
Normal	1.46 \pm 0.62 (7)
1 day treated	1.50 \pm 0.00 (2)
5 days treated	2.83 \pm 0.66 (3)
10 days treated	2.94 \pm 2.20 (5)
12 days treated	2.02 \pm 2.90 (5)
15 days treated	4.39 \pm 3.22 (5)
16 days treated	5.18 \pm 2.88 (5)
30 days treated	5.66 \pm 4.06 (3)
45 days treated	4.12 \pm 1.15 (5)
46 days treated	3.63 \pm 3.32 (5)
10-11 months treated	2.59 \pm 1.52 (15)
20-25 months treated	1.64 \pm 1.22 (12)

Number of experiments indicated in parenthesis. Results expressed as ppm F^- . S.D. = Standard deviation.

Table 3

F⁻ in Non-Calcified Tissues Following Daily Administration of 10 mg NaF/kg Body Weight

Tissues	Normal Mean±S.D.	NaF Treated Mean±S.D.
Skeletal muscle	0.22±0.05	0.41±0.05
Liver	0.17±0.05	0.41±0.12
Kidney	0.16±0.04	0.50±0.10

ppm F⁻ on wet tissue weight.

Rabbits administered NaF for 6 mos.

Four experiments in each group.

S.D. = Standard deviation

All values significant at P < .005.

Fluoride Estimation (Erythrocyte Membrane and Hemolysate): Blood samples were drawn from normal rabbits, 3 and 6 months of age, which had been administered 10 mg NaF/kg body weight daily through intragastric route. Erythrocytes were separated by centrifugation at 300 x g for 10 min. at 4°C. using a SORVALL General Purpose Refrigerated Centrifuge (Model No. III). All subsequent centrifugations were carried out according to the modified method of Suketa et al. (3). Fluoride content of erythrocyte fluid (hemolysate) and that of the membrane was determined by the method of Hall et al. (1) using the fluoride ion specific electrode in a PHM 84 Research pH meter (Radiometer). The results, expressed in ppm fluoride, are reported in Table 4.

Table 4

F⁻ Content of Rabbit Erythrocyte Membrane and Hemolysate

	Erythrocyte Membrane*	Hemolysate**
Normal	0.032±0.006	0.006±0.004
<u>NaF Treated</u>		
3 months	0.042±0.009***	0.081±0.013****
6 months	0.043±0.005***	0.082±0.013****

* ppm F⁻ in membrane suspension containing 10 mg protein/ml

** ppm F⁻/gm% hemoglobin of hemolysate

*** P value <0.05; **** P value <0.005.

Fluoride Estimation (Calcified Tissues): Samples of cortical bone from diaphyseal region of femur and cancellous bone from the iliac crest region of the pelvic girdle of rabbits were dissected out. Marrow free cortical and cancellous bone were defatted in ether acetone mixture (1:1 v/v) and dried in acetone. Fluoride in dry defatted bone ash was determined using a PHM 84 Research pH meter (Radiometer, Copenhagen) with a fluoride specific electrode (2). Results are reported in Table 5.

Table 5

F⁻ in Calcified (Osseous) Tissues Following Daily Administration of 10 mg NaF/kg Body Weight

	Cortical Bone Mean±S.D.	Cancellous Bone Mean±S.D.
Normal	805±43.7	895±57.7
6 months	3873±468.2	5870± (2)
8 months	3817±407.2	7467±550.8
10 months	3700±264.6	7550±477.0

ppm F⁻ in dry defatted bone ash.

3 experiments were carried out, in all but one case. The one with 2 is indicated (2)

P value for bone fluoride is <.005.

S.D. = Standard deviation

Conclusion

The fluoride content of normal rabbit tissues and those administered NaF are reported in Tables 1-5.

The circulating level of fluoride is enhanced following fluoride ingestion. The increase in fluoride content is proportionate to the duration of fluoride administration, at least up to 10 months.

The data obtained on urinary fluoride content reveal that, due to fluoride ingestion, the amount of excreted fluoride increases up to 30 days. Thereafter, for unknown reasons, fluoride excretion gradually diminishes towards normal limits.

Among calcified tissues, cortical and cancellous bone differed significantly in their fluoride content. Cancellous bone, on NaF administration, revealed greater affinity for fluoride uptake, possibly due to its greater surface area exposed to circulation.

The data on fluoride content of noncalcified tissues, have revealed that less fluoride is incorporated into noncalcified tissues compared to calcified tissues. However, in noncalcified tissues it is evident that different organ tissues vary in their affinity for fluoride and in their fluoride content. On NaF administration, all soft tissues investigated, including the erythrocyte membrane and hemolysate, have shown enhanced fluoride content.

Excessive fluoride deposition in calcified and noncalcified tissues leads to certain specific manifestations. This aspect has been explored with special reference to collagenous and noncollagenous constituents.

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CERTAIN FACETS OF F^- ACTION ON COLLAGEN PROTEIN IN OSSEOUS AND NONOSSEOUS TISSUES

by

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SUMMARY: Collagen, a fibrous protein, constitutes the major bulk of the organic matrix of bone and tendon. In order to probe into the defective mineralization process known to occur as a result of fluoride toxicity and fluorosis, the collagenous constituents have been investigated with reference to 1) Amino acid composition, 2) Collagen content, 3) Collagen biosynthesis, 4) Collagen crosslink precursors and 5) Collagen bound collagenolytic activity.

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Due to the wide range of variation in the methodology employed for investigations, these five aspects are dealt with in separate sections, namely, Part I to V.

Part I - Amino Acid Composition of Bone and Tendon: Although different types of collagen have been identified in different tissues, tendon and bone are known to have the same type of collagen (Type I), but they differ in their amino acid composition (1,2). In collagen, some amino acids are introduced as a consequence of certain post-translational changes such as hydroxylation of proline and lysine giving rise to hydroxyproline and hydroxylysine. Both of these amino acids are important to make collagen biologically stable (3). Hydroxyproline participates in the stabilization of the triple helical structure of tropocollagen molecules. Hydroxylysine provides the base for introducing carbohydrate moieties into collagen. It is the carbohydrate moieties, that participate in the calcification process.

Material and Methods

Rabbits in two groups were pair fed and maintained under identical laboratory conditions. One group was given daily 10 mg NaF/kg body weight through the intragastric route. The second group, given no NaF, served as control. The animals were sacrificed after 8 months. Both cortical bone and tendon were dissected out and cleaned from extraneous material.

Preparation of Acid Soluble Collagen of Tendon: Tendon was cut into small pieces and ground at very low temperature. In tendon, the soluble collagen was preferred as adequate quantity was obtained from the tissue. The tissue was initially extracted with 0.05 M Tris-HCl buffer (pH 7.6) containing 1 M NaCl for 48 hours at 4°C. The residue was extracted with 0.5 M acetic acid for 48 hours at low temperature. The supernatant thus obtained containing acid soluble collagen was further purified by the method of Kang, et al. (4).

Preparation of Insoluble Collagen of Bone: In the bone tissue, the insoluble collagen was extracted as the mature collagen was considered for analysis. Bone was cut into small pieces, ground and demineralized with 0.35 M EDTA at very low temperature. The demineralized bone was extracted with Tris-HCl buffer containing NaCl and subsequently with 0.5 M acetic acid as described above and the insoluble collagen was prepared as described by Fujii and Tanzer (5).

Amino Acid Analysis: The acid soluble collagen fraction from tendon and insoluble collagen fraction from bone were hydrolyzed under nitrogen with 6 N HCl at 115°C. for 20 hours in sealed ampules. The hydrolysate, thus obtained, was dried in vacuo to remove the acid. Amino acid analysis was carried out with the Technicon Amino Acid Autoanalyzer.

Results and Conclusions

Results obtained on tendon and bone collagen are reported in Tables 1 and 2. In normal samples of tendon and bone, glycine showed the high-

Table 1

Effect of Excessive Ingestion of F⁻ on Amino Acid
Composition of Rabbit Tendon Collagen

Amino Acid	Normal	Experimental
Glycine	320	319
Proline	118	150
Alanine	105	107
Hydroxyproline	90	64
Glutamic acid	73	73
Arginine	46	47
Aspartic acid	45	43
Serine	33	32.5
Lysine	28	23
Leucine	26	25
Valine	21	21
Threonine	17	17
Isoleucine	13	12
Phenylalanine	13	13
Methionine	5	5
Hydroxylysine	6	4
Histidine	5	4.8
Tyrosine	3.9	4
Ammonia	35	37
Proline/Hydroxyproline Ratio	1.31	2.34

Values in both tables are expressed as residues per 1000 residues and are the mean of 3 experiments.

Table 2

Effect of Excessive Ingestion of F⁻ on Amino Acid
Composition of Rabbit Cortical Bone Collagen

Amino Acid	Normal Bone F ⁻	Treated Bone
Glycine	318	319
Proline	113	137
Alanine	104	103.5
Hydroxyproline	97.7	84
Glutamic acid	72	72
Arginine	48	47.8
Aspartic acid	47	47
Serine	34	33
Lysine	26	20
Leucine	24	24
Valine	21	20
Threonine	18	18.5
Isoleucine	10	10.6
Phenylalanine	12	12
Methionine	6	7
Hydroxylysine	5	4
Histidine	5.5	5
Tyrosine	4	4
Ammonia	34	32
Proline/Hydroxyproline Ratio	1.15	1.63

est concentration which is almost one third of the total amino acids. Proline and hydroxyproline also showed higher concentrations. The ratio of proline/hydroxyproline in normal tendon and bone collagen is recorded as 1.31 and 1.15 respectively. This ratio is an index of the rate of hydroxylation of proline residues.

In samples obtained from rabbits which had ingested fluoride, the hydroxyproline residues were decreased whereas proline residues were increased. This resulted in increased proline/hydroxyproline ratio in fluoride-treated samples. It was also observed that lysine residues were reduced in experimental samples. The concentration of other amino acids did not change following fluoride ingestion.

The present investigation indicates that fluoride ingestion leads to the reduction in hydroxyproline content and, in consequence, proline residues are increased. The reduction in hydroxylation could be due to the depletion in ascorbic acid content (6) a cofactor for prolyl hydroxylase (7).

The deficiency in hydroxyproline is likely to affect the stability of the collagen. The deficiency in lysine residues would ultimately decrease collagen crosslinks, increasing the solubility of the protein.

From the present study, therefore, it is concluded that fluoride interferes with the normal hydroxylation steps of protein producing inadequately hydroxylated collagen. The collagen would also be inadequately crosslinked due to reduced lysine content.

Part II - Collagen Content: Having observed reduction in the hydroxyproline and lysine content of cortical bone and tendon, it was of interest to investigate the status of hydroxyproline content in cancellous bone and other noncalcified tissues following fluoride ingestion. The collagen content was assessed in terms of hydroxyproline content in osseous and nonosseous tissues.

Material and Methods

Normal healthy rabbits and rabbits administered daily 10 mg NaF/kg body weight for varying periods of time were sacrificed. Both cancellous and cortical bone from the iliac crest and diaphyseal region of femur respectively were taken and bone from marrow cleaned. Bone samples were defatted and dried using a mixture of ether and acetone (1:1) and acetone. Dry fat free bone samples were analyzed for hydroxyproline content according to the method of Kivirikko et al. (8). The results obtained for hydroxyproline content of cancellous and cortical bone are shown in Table 3.

The hydroxyproline content was also determined in osseous and nonosseous tissues after hydrolyzing with 6 N HCl at 110°C. for 20 hours and measuring the content in hydroxylate according to the method of Kivirikko (8). In this series of experiments, the results are expressed as μg hydroxyproline/mg wet tissue (Table 4).

Table 3
 Hydroxyproline Content of Cortical and Cancellous Bone
 Before and After F⁻ Ingestion

	Cortical Bone Mean±S.D.	Cancellous Bone Mean±S.D.
Normal Bone (5)	2.15±0.61	3.32±0.30
<u>After F⁻ Ingestion</u>		
3 months (5)	1.45±0.61	2.76±0.24
6 months (5)	1.42±0.61	2.70±0.24
8 months (5)	1.81±0.01	2.96±0.26
10 months (5)	1.63±0.06	1.87±0.87
12 months (5)	1.78±0.02	1.96±0.05

The results are expressed as mg% on fat free dry weight

P value <.05

Table 4
 Effect of 10 mg NaF/kg Body Weight on Hydroxyproline Content
 of Osseous and Nonosseous Tissues After 6 Months Exposure

Tissue	Normal	NaF Treated
Bone*	34.44±1.08	27.92±3.17
Tendon*	66.23±2.81	60.08±1.78
Trachea*	35.90±1.45	31.06±2.73
Skin*	59.08±2.78	52.35±2.44
Lung*	11.08±0.90	9.69±0.80
Kidney**	4.78±0.70	4.11±0.56
Heart*	4.15±0.75	3.87±0.50

Values are expressed as µg hydroxyproline/mg wet tissue.

The number of experiments carried out are 5 in each group.

* P value <0.01 (students' t test applied)

** P value <0.05 (students' t test applied)

Results and Conclusions

Hydroxyproline content of cancellous bone is greater than that in cortical bone. The hydroxyproline content both in cancellous and cortical bone is reduced significantly due to fluoride ingestion.

The investigations on osseous and nonosseous tissues have revealed that tendon has the highest hydroxyproline content compared to the other nonosseous tissues investigated. After NaF ingestion for a period of 6 months, all the tissues investigated revealed a reduction in hydroxyproline content.

These observations suggest that in fluoride toxicity the hydroxyproline content is reduced both in osseous and nonosseous tissues. This possibly may reflect on the collagen content of the tissues.

Part III - Studies on ¹⁴C Proline Uptake(9); The primary structure of collagen protein is known to vary in different tissues from Type I to IV and proline is an essential amino acid component of collagen. Therefore the uptake of ¹⁴C labelled proline was assessed as an index of collagen synthesis in a wide range of tissues from rabbits after fluoride ingestion.

Material and Methods

Normal healthy young rabbits were treated intragastrically with 50mg NaF/kg body weight daily for periods ranging from 22 to 83 days. The rabbits intoxicated with NaF were injected subcutaneously with carbon labelled proline (1 μ Ci/100 gm body weight; Sp: activity 125 mCi/mmol, Radiochemical Centre, Amersham). The animals were sacrificed after 2 hours and tissues such as bone, tendon, pinna, trachea, skin, muscle, lung and kidney cortex were dissected out and homogenized in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.005 M CaCl₂.

A known volume of tissue homogenate was subjected to collagenase digestion at 37°C. (collagenase 140 units/mg Worthington Biochemicals) for 6 hours. The hydrolyzed collagen was separated from residual protein by centrifugation at 5000 x g for 10 min. according to the method of Chia Lin Hu et al. (10). The supernatant containing the hydrolyzed collagen was separated. The residual protein was dissolved in 0.5 N NaOH (11,12). Known aliquots of supernatant (i.e. hydrolyzed collagen) and the residual fractions were treated with toluene (Sample solubilizer) for 2 hours at 60°C. A known volume of sample scintillation cocktail containing PPO (5 mg) and POPOP (0.5 mg) in toluene (1 liter), was added and the rate of uptake of ¹⁴C proline was counted using a Packard Tricarb Liquid Scintillation Spectrometer. Counts per minute (cpm) obtained at 40% efficiency were corrected for 100% efficiency. The protein content was measured by Lowry's method (13). The rate of uptake of ¹⁴C proline is expressed as dpm/mg protein. The background count was subtracted from all the test samples reported under results.

Results and Conclusions

The details of the animals used for experimentation are reported in Table 5. Considerable variation in the rate of uptake of ¹⁴C proline was observed in different tissues (Bar 1-4). The highest rate of ¹⁴C proline uptake was observed in hydrolyzed collagen fractions of tissues of normal bone and tendon which are known to be mainly constituted of collagen Type I. Bone and tendon in fluorosed animals showed the maximal re-

Table 5

Details of Rabbits Used for Experimentation

Rabbit	Age When Sacrificed (in months)	Duration of NaF Treatment (in days)	Body Weight in gm	
			Initial	Final
N ₁	2			800
N ₂	1			600
N ₃	2			1000
F ₁	2.5	22	700	500
F ₂	3	24	800	500
F ₃	4	80	1000	800
F ₄	5	83	950	800

All animals used were male rabbits. N₁ - N₃ = Normal rabbits
F₁ - F₄ = Rabbits administered NaF.

duction in ¹⁴C proline uptake. A significant reduction in the rate of ¹⁴C proline uptake was also found in all other tissues studied which contain collagen Types II, III and IV.

Irrespective of whether a tissue contains collagen Type I, II, III or IV, the incorporation of ¹⁴C proline is severely impaired both in osseous and nonosseous tissues following fluoride ingestion. This finding has been further corroborated by analyzing the rate of uptake with reference to various collagen fractions namely, 1) Hydrolyzed collagen fraction obtained by collagenase digestion and separated at 9000 g (14), 2) Native collagen fibril obtained by thermal reconstitution (15-17), 3) Total and soluble fraction obtained following extraction, 4) Total noncollagenous protein fraction and 5) Alkaline soluble collagen fraction.

The result obtained for native collagen fibril is a specific index for ¹⁴C proline uptake and rate of collagen biosynthesis. The animals which have been subjected to fluoride intoxication for varying periods of time have shown significant reduction in the rate of uptake of ¹⁴C proline in all 5 fractions studied. However, the rate of uptake of ¹⁴C proline of native collagen fibril of both tendon (Table 4) and bone (Table 7) has been reduced significantly indicating reduced collagen biosynthesis in fluoride intoxication.

It can be argued that the changes observed in the rate of ¹⁴C proline uptake may not necessarily be due to the unique effect of fluoride, as a high degree of intoxication could be attributed to 50 mg dose. The extent of intoxication is also being revealed by the reduction in body weight by 150 to 300 gm over a period of 22 to 83 days.

Figure 1

EFFECT OF FLUORIDE TOXICITY ON THE INCORPORATION OF ^{14}C PROLINE IN HYDROLYZED COLLAGEN, RESIDUAL & TOTAL PROTEIN OF RABBITS

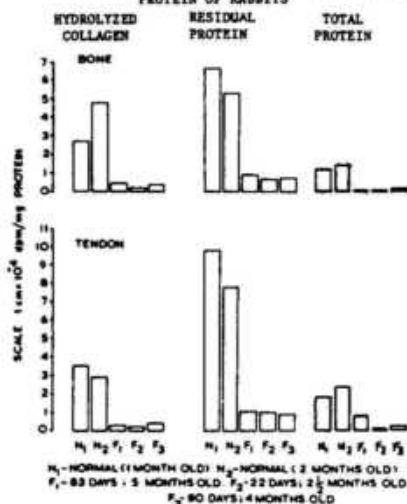


Figure 2

EFFECT OF FLUORIDE TOXICITY ON THE INCORPORATION OF ^{14}C PROLINE IN HYDROLYZED COLLAGEN, RESIDUAL & TOTAL PROTEIN OF RABBITS

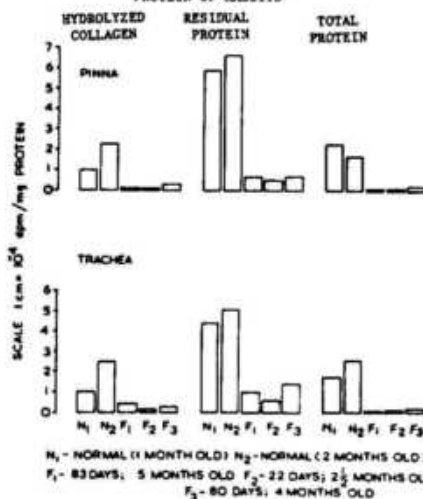


Figure 3

EFFECT OF FLUORIDE TOXICITY ON THE INCORPORATION OF ^{14}C PROLINE IN HYDROLYZED COLLAGEN, RESIDUAL & TOTAL PROTEIN OF RABBITS

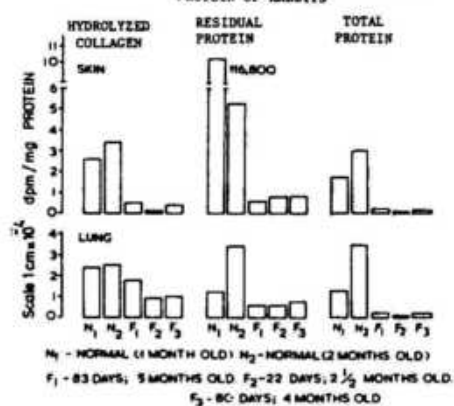


Figure 4

EFFECT OF FLUORIDE TOXICITY ON THE INCORPORATION OF ^{14}C PROLINE IN HYDROLYZED COLLAGEN, RESIDUAL & TOTAL PROTEIN OF RABBITS

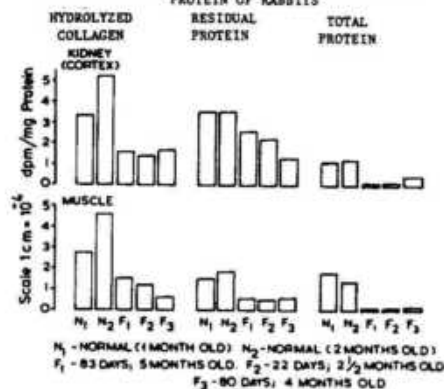


Table 6

Effect of NaF on the Incorporation of ^{14}C Proline in Collagenous and Noncollagenous Protein of Tendon in Rabbits

	Control			NaF Treated			
	N1	N2	N3	F1	F2	F3	F4
Collagenase digested fraction	9.5	4.9	5.6	1.4	1.7	1.7	1.8
Native collagen fibril	2.8	2.7	2.4	0.4	0.3	0.4	0.2
Acid soluble collagen*	3.4	3.3	3.0	1.1	1.6	1.8	1.5
Noncollagenous protein *	2.2	2.1	2.2	1.3	1.4	1.4	1.3
Alkali soluble collagen	6.5	9.6	4.7	5.7	3.2	4.3	4.6
Results expressed as 10^{-4} x dpm/mg protein.							

* Total count as 10^{-4} x dpm

The noncollagen protein fraction of both tendon and bone have also revealed a reduction in ^{14}C proline uptake, indicating that the high degree of intoxication has also affected the noncollagenous proteins. This

Table 7

Effect of NaF on the Incorporation of ^{14}C Proline in Collagenous and Noncollagenous Protein of Bone in Rabbits

	Control			NaF Treated			
	N1	N2	N3	F1	F2	F3	F4
Collagen digested fraction	4.5	7.2	7.9	0.6	2.0	0.9	1.8
Native collagen fibril	2.6	2.2	2.7	0.3	0.2	0.4	0.2
Acid soluble collagen*	2.9	3.0	2.7	0.8	1.7	1.1	1.5
Noncollagenous protein*	3.3	2.7	3.3	0.7	1.6	1.9	1.9
Alkali soluble collagen	3.8	4.4	3.1	1.9	1.3	2.5	2.3
Results expressed as 10^{-4} x dpm/mg protein.							

* Total count as 10^{-4} x dpm.

aspect has been further explored by carrying out yet another set of experiments on ¹⁴C proline uptake on rabbits by administering a low dose of NaF namely 10 mg/kg body weight.

Table 8 reveals the details on 10 mg dose of NaF and experimented upon for the 5 different fractions of collagen.

Table 8

Effect of NaF on the Incorporation of ¹⁴C Proline in Collagenous and Noncollagenous Protein of Bone and Tendon in Rabbit*
10 mg NaF Administered Daily for 175 Days

Bone	Control	NaF Treated
Collagenase digested fraction	6.2	2.5
Native collagen fibril	1.6	0.61
Acid soluble collagen **	2.1	1.0
Noncollagenous protein**	0.9	2.9
Alkali soluble collagen	1.7	0.7

Tendon

Collagenase digested fraction	4.8	2.5
Native collagen fibril	1.4	0.55
Acid soluble collagen**	2.3	1.4
Noncollagenous protein **	1.6	2.7
Alkali soluble collagen	2.0	4.4

Results expressed as 10^{-4} x dpm/mg protein. * Control animal: 1 month old, 600 gm body weight. NaF treated animal: Initial body weight 800 gm, final body weight 1050 gm, 1 animal in each group. ** Total count as 10^{-4} x dpm.

It is evident that, in the NaF treated animal, the first 3 fractions namely collagenase digested, native collagen fibril and acid soluble collagen of both bone and tendon have shown a reduced rate of ¹⁴C proline uptake compared to the control.

The nature of reduction in ¹⁴C proline uptake in the animal administered a low dose of NaF has been in the same pattern as those animals on

a high dose of NaF except for the noncollagen protein fraction which has shown an increased rate of ^{14}C uptake both in bone and tendon. This observation further confirms our finding that, in a high degree of intoxication, other proteins are likely to be affected. However, in low dose, the collagen protein is more specifically involved. Also in low dose of NaF estimation, the body weight of the animal has increased by 250 gm over a period of 175 days and, even under such circumstances, the collagen protein biosynthesis is considerably affected.

Part IV - Collagen Crosslink Precursors(18) In part I, II and III of this article, the data suggests that excessive ingestion of fluoride leads to the reduction in hydroxyproline, lysine and total collagen contents. Efforts have been made to study the nature of the collagen laid down by investigating the saturated peptide-bound aldehydes which are known to be the crosslink precursors. The present communication, therefore, reports the status of the saturated peptide-bound aldehyde content in salt soluble collagen following excessive fluoride ingestion.

Material and Methods

Rabbits, 1.3 to 1.5 kg body weight, were maintained in two groups under identical laboratory conditions. One group was given, every 24 hours, 50 mg NaF/kg body weight through the intragastric route. The other group served as controls. On day 80, 154 and 176 experimental animals and age-matched controls, were sacrificed. The neutral salt soluble collagen was extracted and purified as described by Kang et al. (4). The collagen samples were then dissolved in 0.1 M glycine buffer (pH 4.0) and denatured at 60°C for 20 minutes. The saturated aldehyde associated with salt soluble collagen was measured spectrophotometrically at 312 nm according to the method of Paz et al. (19) using N-methylbenzothiazolonehydrozone (MBTH) reagent.

Results and Conclusions

The results are presented in Table 9. Although salt soluble collagen is a minor fraction of total tissue collagen, this fraction was preferred for the present study as it contains mostly topocollagen molecules. The data obtained on bone, tendon, trachea and skin of the control animals revealed that the saturated aldehyde content increased with the duration of the experimental phase, possibly indicating increased crosslink precursors with advancing age. Age related changes in collagen crosslink are known to occur (20).

It is also evident that fluoride ingestion led to significant reduction in saturated aldehyde content (21) of all tissues investigated. However, the variation observed in the extent of reduction is possibly due to different tissues having different types of collagen. The reduction in saturated aldehyde content could be due to the impairment in its formation. It is known that the two major factors namely, the copper content and copper dependant lysyl oxidase, which are responsible for the formation of aldehyde, are also affected in fluoride toxicity (21,22).

Table 9

Effect of NaF on Saturated Aldehyde Content of Salt Soluble Collagen of Rabbit Tissues (Means \pm S.D., n=5)

	80 days	154 days	176 days
Bone			
Control	0.42 \pm 0.03	0.63 \pm 0.01	0.78 \pm 0.02
Experimental	0.24 \pm 0.03	0.34 \pm 0.03	0.21 \pm 0.03
Tendon			
Control	0.42 \pm 0.02	0.54 \pm 0.04	0.72 \pm 0.03
Experimental	0.12 \pm 0.03	0.12 \pm 0.03	0.15 \pm 0.03
Trachea			
Control	0.30 \pm 0.05	0.42 \pm 0.04	0.51 \pm 0.03
Experimental	0.22 \pm 0.03	0.34 \pm 0.01	0.12 \pm 0.03
Skin			
Control	0.27 \pm 0.03	0.60 \pm 0.02	0.57 \pm 0.08
Experimental	0.12 \pm 0.03	0.20 \pm 0.02	0.40 \pm 0.04

Values are expressed as μ M of acetaldehyde/100 mg of collagen. P < 0.001 (student's t test) for all comparisons with respective control tissues.

It is therefore suggested that due to excessive ingestion of fluoride, the tropocollagen molecules with a reduced number of aldehydes are likely to produce inadequately crosslinked collagen fibers. The lysine residues, which were also reduced due to fluoride ingestion, result in the formation of a lower number of covalent crosslinks in collagen.

Part V - Collagen Catabolism: The reports currently available in the literature are inadequate to elucidate fluoride action on collagen degradation. This aspect has been explored to some extent and data reported.

Material and Methods

Rabbits in two batches were pair fed and maintained under identical laboratory conditions. One batch of animals received 10 mg NaF/kg body weight daily through the intragastric route. Rabbits were sacrificed after 12 months and tissues such as bone, tendon, trachea, lung, kidney and heart were dissected out. The methodology employed for hydroxyproline estimation and the data obtained have been reported in Part II, Table 4.

The collagen bound collagenase activity in each of the tissues listed above, was determined. The tissues were homogenized in ice cold 0.01 M CaCl₂ containing 0.25% (v/v) Triton x 100. It was centrifuged at 6000 g for 20 min. at 4°C. A known amount of 6000 g pellet was hydrolyzed in 6

N HCl to determine its hydroxyproline content. The remaining part was further processed to determine the collagen bound collagenase activity(23). The results obtained on collagen bound collagenase activity are expressed as μg hydroxyproline released/mg hydroxyproline in 6000 g pellet/ hour at 37°C .

It is evident from the data that HP, a measure of collagen content, decreased in all tissues investigated. From the table on collagen bound collagenolytic activity it is noted that, as a consequence of fluoride ingestion, the HP released was enhanced in all the tissues investigated. This indicates that the collagen laid down/synthesized during fluoride ingestion is underhydroxylated and inadequately crosslinked and is rapidly catabolized.

Table 10
Effect of NaF on Collagen Bound Collagenase
Activity in Rabbit Tissues

Tissue	Normal Mean \pm S.D.	Experimental Mean \pm S.D.
Bone	2.50 \pm 0.70	6.50 \pm 0.90
Trachea	1.28 \pm 0.34	3.30 \pm 1.12
Tendon	2.42 \pm 1.14	6.73 \pm 2.07
Lung	0.64 \pm 0.38	1.30 \pm 0.51
Kidney	2.29 \pm 0.36	3.68 \pm 0.99
Heart	0.79 \pm 0.09	1.10 \pm 0.23

Mean of 5 number of experiments, P value < 0.01

The results obtained in Parts I through V, strongly suggest that, due to excessive ingestion of fluoride, the collagen laid down both in osseous and nonosseous tissues is abnormal.

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F⁻ INGESTION AND ITS INFLUENCE ON GLYCOSAMINOGLYCANS IN CANCELLOUS
AND CORTICAL BONE - A STRUCTURAL AND BIOCHEMICAL STUDY

by

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SUMMARY: The noncollagenous constituents, mainly the glycosaminoglycans, are also important as collagenous constituents in understanding fluoride action because the noncollagenous constituents provide the milieu/environment for calcification of collagen fibers. The noncollagenous constituents have been explored in rabbit cortical and cancellous bone after ingestion of 10 mg of sodium fluoride daily for varying time intervals. The major pathological lesion, namely osteoid formation in cancellous bone, its cellular and biochemical characteristics have been explored and results reported.

The sulphated glycosaminoglycans, which appeared in excessive amounts due to fluoride ingestion in cancellous bone, were extracted, purified and quantitated to assess the extent of accumulation and to identify the various isomeric forms. The sulphated glycosaminoglycans known to exist in normal cancellous bone contain chondroitin sulphate A, C and hyaluronic acid. In fluorosed cancellous bone, besides chondroitin sulphate A, C and hyaluronic acid, the presence of dermatan sulphate (chondroitin sulphate B) was detected and confirmed. The occurrence and accumulation of dermatan sulphate in the cartilagenous loci (osteoid) is possibly the major factor for the osteoid to remain unmineralized providing the embryonic nature of bone in fluorosis and fluoride toxicity.

The status of glycosaminoglycans in cancellous and cortical bone was investigated separately as these two types of bone may differ biochemically (1) and the response of cancellous and cortical bone to fluoride toxicity is unlikely to be the same. Moreover our objective was to investigate the reason(s) that the clinical manifestations of fluorosis is confined to specific bony sites of the body namely, vertebral column, pelvic girdle, joints, etc.

Part I of the report deals with the structural aspects and Part II with the biochemical aspects.

Part I - Structural Aspects

Material and Methods

Rabbits aged 2 months were fed daily 10 mg NaF/kg body weight through the intragastric route up to 10 months after which they were sacrificed.

From the Fluorosis Research Laboratory, Dept. of Anatomy, All India Institute of Medical Sciences, New Delhi, India. Presented at the 12th I.S.F.R. Conference, May 16-18, 1982, St. Petersburg Beach, Florida.

The iliac crest region of the pelvic girdle and thoracic vertebra were dissected out and fixed in 10% neutral formalin containing 1% cetyl pyridinium chloride (CPC) to preserve the glycosaminoglycans. The fixed tissue was decalcified in a mixture of 10% formic acid and 20% sodium citrate mixed in a ratio of 1:1. The decalcified tissue was blocked in paraffin and 5 μ thick sections were prepared. These sections were deparaffined and stained with Alcian blue (acidified with acetic acid to pH 2.6) and freshly prepared aqueous solution of Ruthenium red to localize glycosaminoglycans (GAG) and proteoglycans (protein complex of GAG) respectively.

Sections were also incubated in a solution containing 3000-4000 IU of hyaluronidase/ml of 0.15 M NaCl (pH 6) for 10-60 min. at 37°C. prior to Alcian blue staining to digest those glycosaminoglycans which are susceptible to enzymic digestion. Control sections were incubated for the same length of time under the same conditions in 0.15 M NaCl prior to Alcian blue staining. Bone sections obtained from age matched control animals, which did not receive NaF and cortical bone (diaphysis of long bones) samples were likewise treated in a similar way.

Results

Bone samples from the iliac crest region of the pelvic girdle and the body and median spine of the vertebra obtained from animals treated with fluoride for 8 and 10 months and various regions of the trabecular bone revealed osteoid formation. Morphologically, the cells confined to the osteoid region resemble chondrocytes. The intercellular matrix of the osteoid revealed Alcian blue and Ruthenium red positive material indicating the presence of glycosaminoglycans and proteoglycans. Isolated patches of Alcian blue and Ruthenium red positive areas were also observed in the trabeculae which revealed the presence of cells which were not well differentiated into chondrocytes. Osteoids at various stages of formation were thus seen in the bone sections. Even after hyaluronidase digestion, Alcian blue positive intercellular material was observed in the osteoids indicating the presence of hyaluronidase resistant glycosaminoglycans in the matrix (Figs. 1-3).

In cortical bone no such osteoid formation was seen. However, the matrix did reveal highly basophilic reaction, when Ruthenium red stain was used. The striking structural variations observed in the cortical bone due to excessive ingestion of fluoride are 1) increase in cortical thickness and 2) enhancement of the diameter of the osteon (Table 1).

Conclusions

Morphologically, the cells of the osteoid resemble chondrocytes. The chondrocytes also reveal stacking arrangement and it appears that the osteoid resemble fibrocartilage. The appearance of chondrocytes in the trabeculae may possibly be due to activated differentiation of totipotent mesenchymal cells into chondrocytes under the influence of fluoride. It is also possible that the previously existing osteocytes and osteoblasts become dedifferentiated into totipotent cells which further undergo re-

Figure 1Figure 2Figure 3

differentiation into chondrocytes.

The intercellular matrix of the osteoid has revealed high content of glycosaminoglycans and proteoglycans. The hyaluronidase resistant Alcian blue positive material is possibly due to the presence of dermatan sulphate.

Table 1

Morphometric Data on Cortical Bone of Normal and NaF Treated Rabbits

Parameters	Normal (5)	F ⁻ Treated (5)	P value
Cortical thickness in mm	1.39±0.08	2.50±0.12	P <0.05
Diameter of osteon in μ	3.80±0.80	5.80±0.40	P <0.05

Numbers in parenthesis indicate the number of experiments carried out. Exposure to F⁻ = 8-10 months.

Part II - Biochemical Aspects

The structural observations made in cancellous and cortical bone lead to the biochemical analysis of GAG and its characterization.

Chemical Analysis of GAG

Material and Methods

Rabbits aged 2 months were fed daily 10 mg NaF/kg body weight through the intragastric route up to 10 months. The rabbits were killed at intervals of 6, 8, and 10 months after fluoride ingestion. The cortical diaphyseal bone from the femur and cancellous bone from the iliac crest region were dissected out and cleaned from marrow. Fat free bone powder was prepared using an ether-acetone mixture (1:1) and acetone. One gram of bone powder both from cortical and cancellous bone was suspended in 40 ml of digestion mixture containing 0.005 M cystein hydrochloride and 0.2 M EDTA for simultaneous demineralization. An aqueous solution of papain (0.1 ml/40 ml of digestion mixture) was also added. The enzyme papain contained 1.7 mg of protein (10-15 units/mg protein). The GAG released after demineralization and proteolytic enzyme digestion was precipitated with cetyl pyridinium chloride (CPC) as described by Hjerquist and Vejens (2). Analysis of the various constituents of GAG namely, uronic acid, hexosamine (Galactosamine and Glucosamine) and sulphate was carried out (3-6).

Tissues obtained from normal rabbits aged 8-10 months maintained under the same laboratory conditions but without sodium fluoride were subjected to the same treatment as controls.

Characterization of GAG

Material and Methods

Rabbits weighing 600 to 800 gms were fed 10 mg NaF/kg body weight daily through intragastric route up to 8 months. Fluoride-treated rabbits along with age-matched controls were killed and the iliac crest region of the pelvic girdle was dissected out. Marrow free iliac crest bone was defatted in ether-acetone mixture (1:1) and dried in acetone for further analysis. One gm of bone powder was suspended in 40 ml of digestion mixture containing 0.005 M cystein hydrochloride and 0.2 M EDTA for simultaneous demineralization. An aqueous solution of papain (0.1 ml/40 ml of digestion mixture) was also added. The enzyme papain contained 1.7 mg of protein (10-15 units/mg protein). The GAG released after demineralization and proteolytic enzyme digestion were precipitated with CPC as described by Hjerquist and Vejens (2). The relative amount of isomeric chondroitin sulphate was determined by the method of Saito, et al. (7). Uronic acid was determined by the method of Bitter and Muir (3). Gel filtration of GAG was carried out using sephadex G-150 in columns of 2 x 40 cm. Samples (1.0 μ mole as uronic acid) were dissolved in 1 ml of 0.2 M NaCl and applied to the column. Elution was carried out with 0.2 M NaCl at a rate of 7 ml/hr at room temperature. Two ml fractions were collected and analyzed for uronic acid. The void volume of the column was 30 ml and column volume was 115 ml.

Electrophoresis of GAG was carried out on 6 cm long strips of cellulose acetate at a constant current of about 1 mA per cm. The buffer system used was pyridine-acetic acid-water in the ratio of 1:9:115 v/v at

Table 2
Chemical Analysis of CPC Precipitable GAG from Cancellous Bone
of Normal and NaF Treated Rabbits

	Total Hexosa- mines Mean±S.D.	Galactosa- mines Mean±S.D.	Glucosa- mines Mean±S.D.	Uronic Acid Mean±S.D.	Sulphate Mean±S.D.	Molar Ratios** Uronic Acid:hexos- amine	Sulphate Hexosamine	Ratios Galactos- amines: Glucos- amines
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Control (3)	21.2±1.1	56.3±2.5	43.7±2.5	23.8±0.5	17.5±0.5	1.04	1.53	1.29
6 months(2)	23.7	65.0	35.0	25.4	28.5*	0.99	2.24	1.86
8 months(3)	23.7±1.7	67.3±4.2	32.7±4.2	24.9±1.6	28.5*±1.3	0.97	2.24	2.06
10 months(3)	23.1±1.0	65.0±4.4	35.0±4.4	23.9±1.2	28.8*±0.8	0.96	2.32	1.86

* Significant at $P < 0.0005$. ** Molar ratios are based on hexosamine = 1.0. Data expressed as mg% of dry de-fatted bone for CPC precipitable GAG. Data expressed as mg% of dry CPC precipitable GAG for total hexosamine, uronic acid and sulphate. Data expressed as % of total hexosamines for galactosamine and glucosamine. The number of experiments is indicated in parenthesis, ±S.D. = standard deviation.

Table 3
Chemical Analysis of CPC Precipitable GAG from Cortical Bone
of Normal and NaF Treated Rabbits

	Total Hexosa- mines Mean±S.D.	Galactosa- mines Mean±S.D.	Glucosa- mines Mean±S.D.	Uronic Acid Mean±S.D.	Sulphate Mean±S.D.	Molar Ratios** Uronic Acid:hexos- amine	Sulphate Hexosamine	Ratios Galactos- amines: Glucos- amine
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Control (3)	20.8±0.7	57.7±1.5	42.3±1.5	22.8±0.6	15.6±0.5	1.01	1.39	1.36
6 months(3)	27.9±0.4	64.0±4.0	36.0±4.0	29.5±1.2	27.0*±1.0	0.98	1.79	1.78
8 months(3)	25.9±0.5	66.0±3.5	34.0±3.5	26.2±0.4	26.3*±0.6	0.95	1.91	1.94
10 months(3)	21.7±2.9	59.3±1.5	40.7±1.5	25.7±1.5	28.0*±1.0	1.09	2.40	1.46

* Significant at $P < 0.0005$. ** Molar ratios are based on hexosamine = 1.0. Data expressed as mg% of dry de-fatted bone for CPC precipitable GAG. Data expressed as mg% of dry CPC precipitable GAG for total hexosamine, uronic acid and sulphate. Data expressed as % of total hexosamines for galactosamine and glucosamine. The number of experiments is indicated in parenthesis, ±S.D. = standard deviation.

pH 3.5. The strips were stained according to the method of Seno et al. (8) with 0.5% Alcian blue in 3% acetic acid.

Results

The rabbits obtained on CPC precipitable GAG and their chemical composition in both cortical and cancellous bones are reported in Tables 2 and 3. It is evident from the data that among the various constituents of GAG analyzed, the SO_4 content increased significantly both in cortical and cancellous bone. This may be due to increased rate of sulphation, or to increased sulphated GAG content.

The amount of GAG isolated from the iliac crest, the relative amounts of isomeric chondroitin sulphate and hyaluronic acid obtained are reported in Table 4. It is evident from the table that the GAG content of the fluorosed bone is enhanced to twice that of the control. The results on isomeric chondroitin sulphate reveal the presence of chondroitin sulphate A, chondroitin sulphate C and hyaluronic acid in control whereas, in the ex-

Table 4
CPC Precipitable GAG and Chondroitin Sulphate Isomers
in Control and NaF Treated Iliac Crest Bone

	Control	Experimental
CPC-precipitable GAG*	3.53	7.67
Chondroitin Sulphate A	68	60
Chondroitin Sulphate B (Dermatan Sulphate)	-	12
Chondroitin Sulphate C	28	26
Hyaluronic Acid	4	2

*Data expressed as mg% of dry defatted bone. Data expressed as the % of the total unsaturated disaccharides formed by the action of chondroitinase.

perimental samples, the chondroitin sulphate reveals, in addition to the 3 constituents of the control, a fraction of chondroitin sulphate B as well. The occurrence of chondroitin sulphate B (Dermatan sulphate) in fluorosed iliac crest was confirmed by Gel filtration and electrophoresis (Fig. 4-6). Characterizations of the isomers of chondroitin sulphate were carried out by Gel filtration of the samples before and after treatment with chondroitinase ABC and chondroitinase AC. Both control and fluorosed samples of GAG prior to enzyme digestion eluted near the void volume and

Figure 4

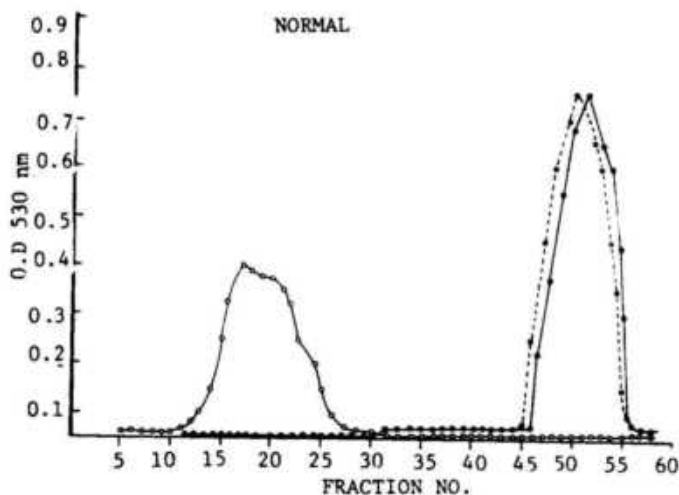


Figure 5

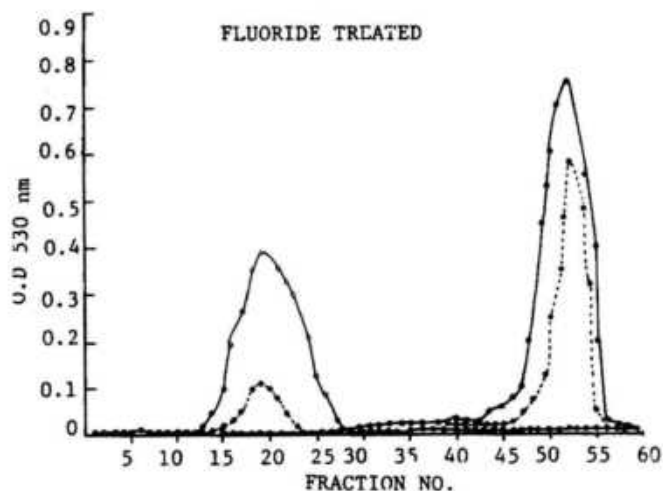
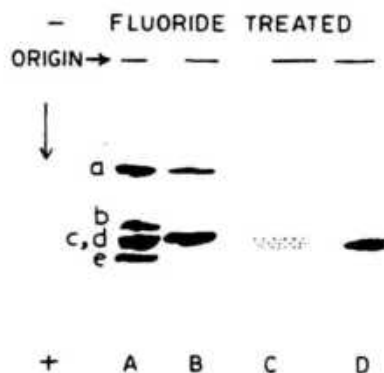


Figure 6



the unsaturated dissacharides produced by the action of chondroitinase ABC eluted between fraction nos. 45 to 55. The controls digested with chondroitinase AC also eluted between fraction nos. 45 to 58 whereas in

FLUORIDE

fluorosed samples an additional small peak between fraction nos. 15 and 25 in addition to the sharp peak between 45 to 58 was obtained. The small peak near void volume after chondroitinase AC digestion represents the dermatan sulphate which is not digested by chondroitinase AC. The presence of chondroitinase AC resistant material revealed by electrophoresis also supports the presence of dermatan sulphate in fluorosed samples. The presence of dermatan sulphate may possibly be one of the reasons that the newly formed bone remains unmineralized during fluoride treatment.

Acknowledgement

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FLUORIDE BRIEF

This investigation demonstrates that intraperitoneal injections of NaF (15 mg F/kg) results in hyperglycemia, inhibition of glycolysis, and an increase in tissue cAMP concentrations. Sodium fluoride significantly increases the cAMP concentration in liver, submaxillary gland, lung, heart, and kidney within 60 minutes following the injection.

Allmann, D.W., and Kleiner, H.S.: Effect of NaF on Rat Tissue cAMP Levels in vivo. *Pharmacol. Ther. Dent.*, 5:73-8, 1980.

ON THE SIGNIFICANCE OF SIALIC ACID AND GLYCOSAMINOGLYCANS
IN THE SERUM OF FLUOROSSED HUMAN SUBJECTS

by

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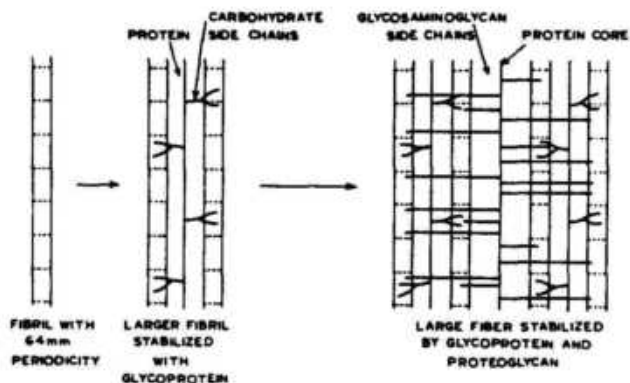
SUMMARY: The levels of sialic acid and glycosaminoglycans (GAG) have been explored in the serum of fluorosed human subjects. The changes observed in the level of these chemical constituents in the serum possibly reflect the changes occurring in cancellous bone, cortical bone and in other tissues due to fluoride ingestion. The sialic acid content versus GAG revealed a 50% reduction in serum from fluorosed subjects. The possibility of developing a sensitive prognostic test for fluorosis is discussed.

Introduction

GAG and glycoproteins form an integral part of the organic matrix of bone which is constituted predominantly of collagen fibers (Fig. 1).

Figure 1

Collagen Fibrils and Fiber Constituting Bone Matrix



Note the relationship of collagen fiber with glycoproteins and GAG. (Gould, B.S.: Biology of Collagen. Treatise on Collagen, Vol. 2, N.Y. Academic Press, 1968.)

From the Fluorosis Research Laboratory, Dept. of Anatomy, All India Institute of Medical Sciences, New Delhi, India. Presented at the 12th I.S.F.R. Conference, May 16 - 18, 1982, St. Petersburg Beach, Florida.

At the 12th International Conference of Fluoride Research a detailed analysis of GAG and its status due to fluoride ingestion in both cancellous and cortical bone of rabbit was reported (1). The authors have also investigated one of the pathological lesions that occurs in the trabeculae of cancellous bone and have evaluated the extent of accumulation of GAG in those sites, both by quantitative and qualitative methods (2-4).

Besides GAG, the glycoprotein profile especially that of sialic acid (N-acetyl-neuraminic acid) both in cancellous and cortical bone have also been investigated. According to the authors (5) sialic acid, the only parameter among the various parameters investigated, is present in the same amount in both cancellous and cortical bone. However, the response of sialic acid content to fluoride ingestion differs significantly between cancellous and cortical bones; it is significantly enhanced in cancellous bone and decreased in cortical bone. The changes observed in GAG and glycoprotein content in tissues of rabbit have led the investigators to explore the status of these constituents in the sera of both rabbits and human subjects afflicted with fluorosis. The present report provides the data on GAG and sialic acid in the sera and elucidates the significance of the study.

Material and Methods

Normal and fluorosed human sera were collected from patients of endemic regions of India. Moreover, rabbit sera both from normal and from those which have been fed daily 10 mg NaF/kg body weight for 8 months, have also been collected and investigated.

Estimation of GAG in Sera: GAG in human and rabbit sera were estimated according to the method of Gold (6). To a known volume of serum (0.2 ml), 1.2 ml of Alcian Blue reagent* was added and after 10 min. optical density was measured at 488 nm. Chondroitin sulphate was used as the standard. Results are expressed as mg/100 ml of serum.

Sialic Acid Estimation: Sialic acid in sera of human and rabbit were estimated according to the method of Winzler (7). To 0.2 ml of serum 4.8 ml of 5% trichloroacetic acid was added and placed in a boiling water bath for 15 minutes. The samples were centrifuged at 2000 rpm for 10 min. Aliquot of the supernatant was used for color development with diphenylamine (DPA) reagent**. Correction for nonspecific color development was applied. Optical density was taken at 530 nm. Results are expressed as mg/100 ml sera.

Results

The results on sialic acid and GAG contents of rabbits and human sera of both normal and fluorosed subjects are given in Table 1. It is ob-

* Alcian Blue reagent was prepared in 0.5 M sodium acetate to produce a final dye concentration 1.4 mg/ml.

** 1 gm of diphenylamine dissolved in glacial acetic acid and concentrated sulfuric acid mixed in the ratio of 9:1.

vious from the data that the sialic acid content decreased whereas GAG increased significantly due to fluoride toxicity. In the ratio of sialic acid versus GAG, fluorosed sera were reduced more than 50%. The results on human sera resemble that of rabbit sera.

Table 1
Serum Sialic Acid and GAG Content of Normal
and Fluorosed Human Subjects and Rabbits

		Sialic Acid (SA)	Glycosaminoglycans (GAG)	SA/GAG
Rabbit	Normal(5)	61.97±1.89	6.98±1.21	10.19
	Fluorosed(5)	42.71±4.78*	13.27±1.57*	3.22
Human	Normal(9)	61.09±2.72	9.45±0.53	6.47
	Fluorosed(9)	45.39±2.68**	12.20±1.51**	3.73

Data expressed as mg/100 ml of serum. Numbers in parenthesis indicate the number of experiments carried out. * P <0.05; ** P <0.01.

Studies on the serum of fluorosed patients and of fluorosed rabbits have clearly shown that the sialic acid and GAG contents are altered. Sialic acid and its status in serum as a result of changes occurring in cancellous and cortical bone are of particular interest. The sialic acid content reveals a significant reduction in circulating levels after fluoride ingestion which is true even in sera of fluorosed humans. This observation is also consistent with the results reported for other protein-bound carbohydrates such as hexosamine and fucose in fluoride poisoning (8,9).

As the ratio of sialic acid content versus GAG revealed a 50% reduction in sera of fluorosed humans, it appears that this test could be employed as a sensitive prognostic test for fluorosis.

Acknowledgement

One of the authors (AKS) wishes to acknowledge grants-in-aid from the Department of Environment (Government of India) and the International Development Research Centre, Canada.

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ADENYL CYCLASE ACTIVITY AND CYCLIC AMP LEVELS FOLLOWING F⁻ INGESTION IN RABBITS AND HUMAN SUBJECTS

by

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SUMMARY: Fluoride is known to activate adenylyl cyclase in vitro and in intact cells. The present report describes the effect of fluoride on tissue adenylyl cyclase activity and cyclic AMP levels in vivo.

The adenylyl cyclase activity increased significantly in bone, liver and kidney following ingestion of 10 mg fluoride per kg body weight. The increased activity paralleled elevated tissue fluoride levels. Among the various tissues investigated, bone tissue showed the highest increase in activity which approximated 193%. Further, the plasma cyclic AMP levels increased by 45% and 114% in animals given fluoride for 6 and 12 months respectively. In view of the above findings, cyclic AMP levels have been investigated in various tissues of the rabbit as well as urine and plasma of human subjects afflicted with fluorosis. The significance of the data with respect to pathological changes occurring in fluorosis is discussed.

From the Fluorosis Research Laboratory, Dept. of Anatomy, All India Institute of Medical Sciences, New Delhi, India. Presented at the 12th I.S.F.R. Conference, May 16-18, 1982, St. Petersburg Beach, Florida.

Introduction

While exploring the derangement in collagenous and noncollagenous constituents in fluoride toxicity and fluorosis (1,2), the possibility that the adenylyl cyclase-cyclic AMP system plays a major role in collagen metabolism and calcification has been explored. It is established that fluoride activates adenylyl cyclase in broken cell preparations and in intact liver cells (3,4). However, the effect of fluoride on the activity of adenylyl cyclase *in vivo* has not been investigated. Adenylyl cyclase is known to catalyze the conversion of ATP to cyclic AMP (cAMP) which is a "second messenger" of numerous hormones and an important metabolic regulator (5). An increase in the cellular concentration of cyclic AMP is known to alter a number of metabolic processes namely, glycogenolysis, muscle gluconeogenesis, plasma glucose and steroidogenesis (5). This report deals with the effect of fluoride ingestion on adenylyl cyclase activity and cAMP levels in various tissues of the rabbit and of fluorosed human subjects as indicated below:

1. Adenylyl cyclase activity in calcified and noncalcified tissues of rabbits.
2. a: Cyclic AMP levels in calcified and noncalcified tissues and plasma of rabbits.
b: Cyclic AMP levels in plasma of patients afflicted with fluorosis.

SECTION I - ADENYLYL CYCLASE ACTIVITY FOLLOWING FLUORIDE INGESTION (6):

Eight female albino rabbits, weighing 880-1100 g each, were divided into groups of 4 and fed a balanced diet obtained from Hindustan Lever (Bombay). The animals in the first group were incubated daily with 10 mg NaF/kg body weight (one dose) for 6 months whereas those in the second group served as controls. After treatment with sodium fluoride, the animals were sacrificed; bone (cortical), skeletal muscle (quadriceps), liver and kidney were removed and analyzed for adenylyl cyclase activity.

Adenylyl Cyclase Activity: Enzyme activity in whole homogenate was assayed by the method of Krishna et al. (7). The incubation medium contained Tris-HCl buffer pH 7.5 ($4 \times 10^{-2}M$), $MgSO_4$ ($3.3 \times 10^{-3}M$) theophylline ($10^{-2}M$), ^{14}C ATP (sp act. mCi/m mol; $1-2 \times 10^{-3}M$) and tissue (5 mg) in final volume of 0.3 ml. After incubation at $37^\circ C$ for 15 minutes, unlabelled cAMP (0.5 ml of 5 mg/ml) was added to each tube and these were then plunged into boiling water for 3 minutes. The tubes were cooled, centrifuged at 3000 rpm for 5 min. and the supernatant was passed through a column of aluminum oxide (almina) to separate cAMP by the method of Ramachandran (8).

The radioactivity of the fractions containing cAMP was counted in a Packard Tricarb Liquid Scintillation Spectrometer (Model 3200) using a mixture of PPO and POPOP in toluene as the scintillation fluid. Efficiency of the counter was determined using automatic standardization procedure. Total protein was determined by the method of Lowry et al. (9) using bovine serum albumin as the standard. Enzyme activity is expressed as the amount of cAMP recovered/mg protein/15 min at $37^\circ C$.

Results

The adenyl cyclase activity of bone, skeletal muscle, liver and kidney treated with fluoride is reported in Table 1. Following fluoride ingestion, enzyme activity was significantly increased in bone, liver and kidney, whereas it remained unaltered in skeletal muscle. The increase in adenyl cyclase activity was highest in calcified tissue (bone).

Table 1

Effect of F^- Ingestion on Adenyl Cyclase Activity in Rabbits
(p mole cAMP recovered/mg protein/15 min.)

Tissue	Control Mean \pm S.D.	F^- Treated Mean \pm S.D.
<u>Calcified Tissue</u>		
Cortical bone	30.0 \pm 7.2	88.0 \pm 25.0*
<u>Noncalcified Tissue</u>		
Skeletal muscle	27.8 \pm 2.5	28.7 \pm 4.0
Liver	14.1 \pm 2.6	27.8 \pm 5.2 *
Kidney	14.2 \pm 2.9	25.6 \pm 3.6 *

* Significant difference ($P < 0.005$)

S.D. = Standard deviation

SECTION II - CYCLIC AMP LEVELS FOLLOWING FLUORIDE INGESTION:

Material and Methods

Ten female albino rabbits weighing 800-1050 g each, divided into two groups (5 each) were fed a balanced diet obtained from Hindustan Lever (Bombay). The animals in the first group were incubated with 10 mg NaF/kg body weight daily (one dose) for six months, those in the second group served as controls. After the treatment with NaF, the animals were sacrificed under ether anesthesia and blood was drawn from the heart. Besides cancellous bone (iliac crest region of the pelvic girdle), cortical bone (shaft of the femur), skeletal muscle (quadriceps), liver and kidney were also dissected out and processed as follows for assay of cyclic AMP.

1) Blood was drawn into test tubes containing 100 μ l of 0.5 M EDTA (pH 7.7) per 10 ml blood which acted as both anticoagulant and phosphodiesterase inhibitor. Samples were centrifuged immediately at room tem-

perature and plasma separated and stored until used. The plasma samples were assayed for cyclic AMP levels by the method of Tovey et al. (10).

2) Noncalcified Tissues: Samples of fresh tissue were homogenized at a concentration of 100 mg/ml in 0.1 M HCl using a polytron homogenizer. The homogenate was heated at 100°C for 2 min. After cooling the suspension was centrifuged, the supernatant taken and adjusted to a pH 7.5 with 1 M NaOH, after which it was further diluted with assay buffer. The cyclic AMP levels were determined by Tovey et al. (10), using cyclic AMP Assay Kit supplied by Amersham Radiochemicals (U.K.).

3) Calcified Tissues: The method of Shanfeld et al. (11) was used for assay of cAMP in bone tissue. Bone samples were split with a chisel and residual soft tissues were removed. Thus, bone fragments of 400 to 500 mg (wet weight) which were obtained were pulverized in a heavy porcelain mortar. Frozen pellets of water (1-10 ml/100 mg of bone weight) were then introduced into the mortar and thoroughly triturated. The powdered mixture was transferred into a thick walled test tube, immediately immersed into a hot salt bath (125°C.), brought to a boil within 30-60 seconds and boiled for 3 minutes. The mixture was then cooled and brought to -40°C. in an ultra low refrigerator and homogenized by crushing in a mortar and pestle. The homogenate was reconstituted to original volume and then centrifuged at 4000 g for 15 minutes. An aliquot was taken and cAMP assayed by the method of Tovey et al. (10) using cyclic AMP Assay Kit supplied by Amersham Radiochemicals. The protein content was estimated by the method of Lowry et al. (9). The concentration of cyclic AMP in tissues was expressed as picomole cAMP/mg protein and in plasma as picomol/ml plasma.

The significance of the data was evaluated by Student's "t" test.

Results

1) cAMP Levels in Calcified and Noncalcified Rabbit Tissues: The results obtained on cyclic AMP levels are shown in Table 2. It is evident from the table that all tissues showed elevated cyclic AMP levels following fluoride ingestion. In particular, the increased cyclic AMP levels were very pronounced in calcified tissues; the increase measured 96% in cortical bone and 134% in cancellous bone. Among the noncalcified tissues, liver showed the maximum percentage increase in cAMP levels followed by kidney, whereas muscle showed the least change.

2) cAMP Concentration in Plasma Samples of Fluorosed Human Subjects and Rabbit: The data obtained on cyclic AMP levels of normal and fluorosed patients is given in Table 3. Of three patients analyzed for plasma cyclic AMP levels only two showed an increase in cyclic AMP levels as compared to the mean of normal subjects. The increase in F₁ was appreciable and measured about 46% whereas the increase in F₃ was marginal and only approximated 30%.

In the animal models the plasma cyclic AMP levels increased significantly (45%) following fluoride ingestion.

Table 2

Effect of F^- on Rabbit Tissue
and Plasma cAMP Level

Tissue	Control Mean S.D.	F^- Treated Mean S.D.
<u>Calcified Tissue</u>		
Cancellous bone	4.8±0.6	11.2±1.6
Cortical bone	3.2±0.5	6.3±0.9
<u>Noncalcified Tissue</u>		
Liver	15.7±2	28.6±3.1
Kidney	10.2±1.2	16.4±1.9
Skeletal muscle	4.1±0.6	6.4±0.8
<u>Plasma</u>	33.0±5	48.0±6.2

Table 3

Plasma Cyclic AMP Levels in Normal
and Fluorotic Human Subjects

	Code No.	cAMP Level (p mol/ml)
Normal	N ₁	27
	N ₂	23
	N ₃	17
Fluorosed	F ₁	32
	F ₂	23
	F ₃	29

Mean = 21

Values are significant at $P < 0.005$ Discussion

Although the number of human subjects investigated is less than in animals the results obtained clearly indicate that tissue levels of cyclic AMP and adenyl cyclase activity increased after prolonged ingestion of fluoride. The results also reveal a correlation between the increased cyclic AMP levels in tissues and increased plasma cyclic AMP levels.

It can be seen from the data on fluoride levels (presented earlier) that serum and tissue fluoride levels were significantly elevated; calcified tissue showed the highest percentage increase in fluoride content, skeletal muscle the lowest. Bone revealed the highest percentage increase in adenyl cyclase activity and cAMP levels, whereas muscle showed a negligible change in adenyl cyclase activity and a moderate increase in cyclic AMP levels. However, the increased fluoride content of liver and kidney was higher than that of muscle, and enzyme activity and cAMP was significantly elevated in these tissues.

It is evident from these studies that increased cyclic AMP levels are due to the direct stimulatory effect of fluoride on adenyl cyclase activity. In the case of skeletal muscle, which showed increased cAMP levels but not increased adenyl cyclase, we believe that fluoride may inhibit 3',5' - cyclic AMP phosphodiesterase - an enzyme which catabolizes cAMP (converts cAMP to AMP). In fact, various kinds of phosphodiesterases are known to be inhibited by low concentrations of fluoride *in vitro* (12).

Although elevated fluoride content in tissues paralleled increased adenyl cyclase activity and cyclic AMP levels, it remains to be demon-

strated whether the activation is due solely to the direct effect of fluoride on adenylyl cyclase or whether there are other contributory factors.

Excessive fluoride ingestion is known to result in secondary hyperparathyroidism leading to increased levels of parathyroid hormone in plasma (13,14). This polypeptide hormone mediates its action by activating adenylyl cyclase activity thus stimulating cAMP production in kidney and bone (15,16). Further this hormone is known to activate adenylyl cyclase in liver also (17). It is, therefore, possible that the increased fluoride content of tissues and enhanced hormone levels might account for the increase in adenylyl cyclase activity which in turn explains increased cAMP levels following fluoride ingestion.

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ULTRASTRUCTURAL OBSERVATIONS ON THE EFFECTS OF FLUORIDE INGESTION ON THE PARATHYROID GLAND OF THE RAT

by

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SUMMARY: The parathyroid glands of rats given distilled water, to which 150 ppm fluoride was added, for 10 weeks contain abundant lamellar arrays of rough endoplasmic reticulum and multiple dilated Golgi complexes. Secretory granules are released within cytoplasmic projections and aggregations of glycogen granules are seen within the chief cell cytoplasm as well as within enlarged intercellular spaces. Fluoride ingestion appears to result in increased functional activity of the rat parathyroid gland.

Introduction

The parathyroid glands of the rat consist of one parenchymal cell type, the chief cell (1-5). Within this single cell category, light and dark cells have been described as representing inactive and active stages of secretory activity respectively (6-8). The structure of the rat parathyroid gland (6) is similar to that of other species (9, 10) except for a sparse amount of glycogen and the presence of few secretory granules (11).

The ingestion of waterborne fluoride has been reported to affect calcium homeostasis in both humans and animals. However, the exact mechanism leading to the involvement of the parathyroid glands in skeletal fluorosis is unclear. Although much evidence suggests that fluoride ingestion causes hyperactivity of the parathyroid glands in humans (12-14) and in some animals (15,16), contradictory findings are common, particularly when the rat is used as the experimental animal. The objective of this study was to describe the morphological appearance of the rat parathyroid gland following short-term fluoride ingestion.

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Materials and Methods

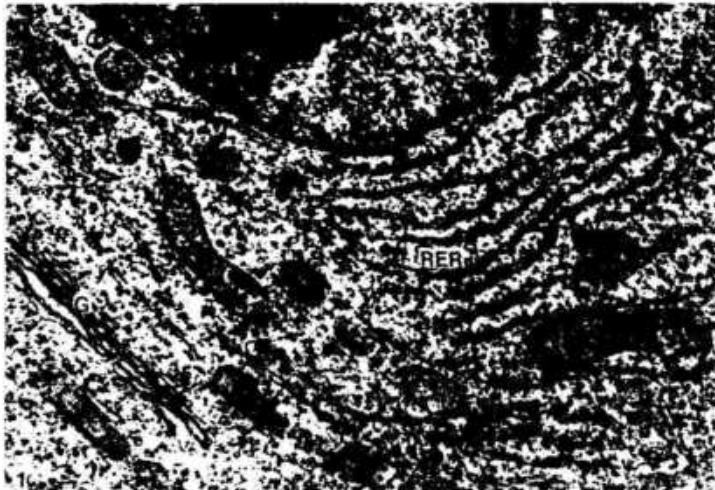
Young adult male Sprague-Dawley rats were divided into control and experimental groups of 12 animals each. Both groups were given distilled water ad libitum, to which 150 ppm fluoride as sodium fluoride was added for the experimental group. At the end of the 10-week experimental period, all animals were anesthetized with ether and perfused with Karnovsky's fixative (17) buffered to pH 7.3. The parathyroid glands were then freed from the surrounding thyroid tissue, placed in fresh fixative for 2 hours, and rinsed in two changes of phosphate buffer at pH 7.3 (18). After postfixation for 1 hour in buffered 2% osmium tetroxide, the tissues were dehydrated in increasing concentrations of ethyl alcohol, followed by propylene oxide, and embedded in Epon 812 (19). Thin sections were stained with 3% aqueous uranyl acetate, poststained with lead citrate (20) and examined with a Zeiss EM-9 electron microscope.

Results

In contrast to those of the control rats, the predominant cell type in the parathyroid glands of rats given fluoride in the drinking water is the dark chief cell. The organelles concerned with protein synthesis and packaging of the secretory product are abundant and more developed than those in the dark cells of control rat parathyroids. The rough endoplasmic reticulum is increased and aggregated into large lamellar arrays. The Golgi complexes are also enlarged and consist of dilated cisternae (Fig. 1). Moreover, the Golgi complexes often appear as multiple complexes in several parts of the cell.

Figure 1

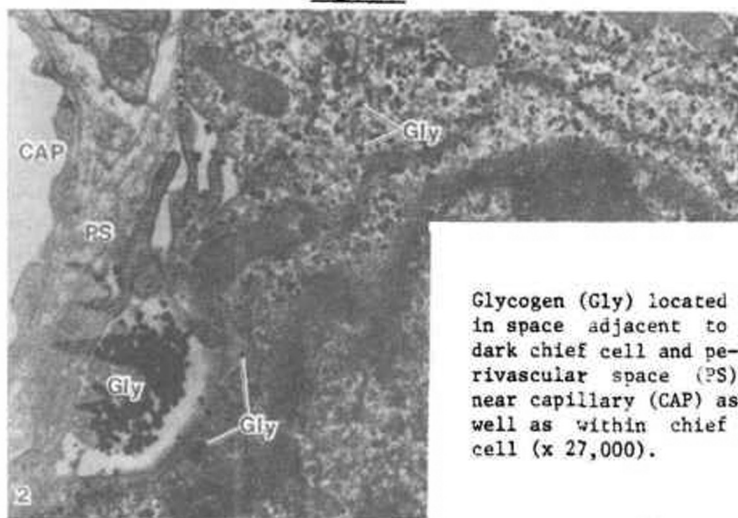
Dark Chief Cell Containing Extensive Lamellar Array
of Rough Endoplasmic Reticulum (RER)



and a Large Golgi Complex (G) (x 27,000)

Many dark cells form complex interdigitations with adjacent cells while enlarged intercellular spaces are frequently seen between other dark cells. Large aggregations of glycogen granules are often seen within the widened spaces as well as within spaces subjacent to perivascular spaces (Fig. 2). In addition to the extracellular accumulation of glycogen, many dark cells contain large numbers of glycogen granules randomly scattered throughout the cytoplasm (Fig. 3).

Figure 2



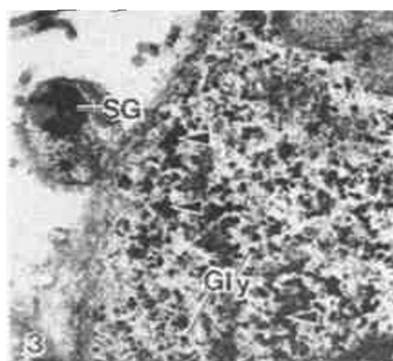
Glycogen (Gly) located in space adjacent to dark chief cell and perivascular space (PS) near capillary (CAP) as well as within chief cell (x 27,000).

Numerous secretory granules can be seen in various stages of release from the chief cells. At the perivascular surface of the cell, the secretory granule presses against the plasma membrane and causes the surface to bulge outward (Fig. 4). The connecting stalk of the cytoplasmic projection narrows and finally becomes detached from the chief cell by pinching off as a result of plasma membrane rupture and refusion (Fig. 5).

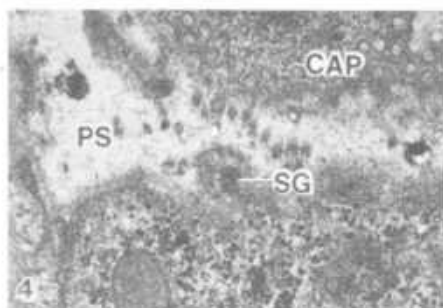
Discussion

It has been shown that there is direct feedback control by calcium (21) of parathyroid hormone. Experimental studies on rat parathyroid glands have demonstrated ultrastructural evidence of parathyroid stimulation and increased cellular activity under conditions of low serum calcium concen-

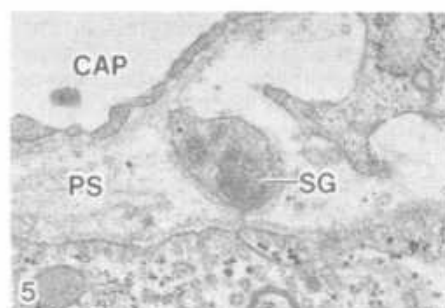
Figure 3



Scattered glycogen granules (Gly) and small clumps of glycogen (arrowheads) within dark chief cell. Note Secretory granule (SG) within cytoplasmic bleb (x 27,000).

Figure 4

Cytoplasmic projection containing secretory granule (SG) projecting from chief cell into perivascular space (PS) adjacent to capillary (CAP) (x 27,000).

Figure 5

Cytoplasmic projection containing secretory granule (SG) lying free in perivascular space (PS) adjacent to capillary (CAP) (x 27,000).

tration (4,6). These studies show that chronic stimulation leads to an increase in plasma membrane tortuosity, multiple cisternae of rough endoplasmic reticulum and large Golgi complexes. The structure is similar to that described in the parathyroid glands of rats following nephrectomy (1,2,22) or given a diet deficient in calcium (23), as well as in sheep (16) and rabbits (24) following fluoride ingestion.

The present study shows that the ingestion of 150 ppm fluoride in the drinking water for 10 weeks also appears to result in increased cellular activity in the parathyroid glands of the rat. Rat parathyroid cells undergo asynchronous cyclic changes in protein synthesis and hormone secretion (6) similar to those described in man (9). However, as a result of fluoride ingestion, there is a change in the predominantly light chief cell population in control rats to a predominantly dark chief cell population in fluoride-treated rats. The dark chief cells are considered to represent those cells in the stage of the secretory cycle involved in the synthesis of parathyroid hormone (25). Rough endoplasmic reticulum, aggregated into parallel arrays and stacks, and large Golgi complexes associated with vacuoles and vesicles, such as that seen in the present study, are all associated with increased parathyroid hormone synthesis (11,26,27). Moreover, these morphological characteristics of the dark chief cells in the fluoride-treated rat are never seen in the normal rat parathyroid gland, but are similar to the dark chief cells of the human parathyroid gland (9).

The widened intercellular spaces between adjacent chief cells in the fluoride-treated rats appear to be the result of reduced cytoplasmic area. A similar increase in intercellular spaces has been reported in young cats with experimental hyperparathyroidism (28). The marked increase in tortuosity of the plasma membranes and in the amount of interdigitation with

the plasma membranes of adjacent cells has been previously associated with hyperactivity of the rat parathyroid gland (29).

The parathyroid gland of the normal rat contains relatively small amounts of glycogen as compared to that of other species (11). In the normal rat, there is an inverse relationship between glycogen deposits and parathyroid secretory activity, where inactive chief cells contain abundant glycogen, and active chief cells sparse glycogen (6). This is in contrast to the present study and to studies of hyperparathyroidism in man (30) wherein glycogen increases with secretory activity. Not only is the amount of glycogen within the chief cells in control rats less than in fluoride-treated rats, but glycogen granules are never seen in the extracellular compartment of the normal rat parathyroid.

The increased amount of glycogen in the fluoride-treated rat suggests a closer correlation between glycogen accumulation and increased secretory activity than has been previously reported in the rat (30) and that an inverse relationship between the two is not found with hyperactivity. Consequently, secretory cycles associated with hyperparathyroidism in the rat closely parallel those reported in man.

It is not clear whether this abundance of glycogen is significant relative to the production and storage of energy for increased hormone synthesis, or whether it is the result of abnormal control mechanisms of cellular metabolism associated with overstimulation of these cells.

A number of studies have suggested that hormone secretion in avian (31), bovine (25) and human (32) parathyroid glands occurs by exocytosis. An alternative apocrine-like mechanism has been suggested for the extrusion of secretory material into the perivascular space in normal (33) and hyperactive parathyroid glands of pigs (34). The present study readily shows that secretory granules can also be released from the chief cells within cytoplasmic projections. This alternative mechanism may be an expression of the hyperactivity of the gland in response to fluoride administration. Since apocrine-like secretions are observed in both the hyperactive as well as the normal parathyroid of pigs, this form of hormone secretion may also occur in the normal rat parathyroid.

The results of the present study suggest that the ingestion of high doses of fluoride in the rat induces a type of secondary hyperparathyroidism wherein increased protein synthesis occurs in glycogen-rich chief cells. Although no evidence of either hyperplasia or hypertrophy was found, the alterations in morphology of the stimulated parathyroid glands are similar to those previously seen in other fluoride-treated animals.

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COMPARATIVE METABOLIC STUDIES IN FLUORIDE-TREATED AND STREPTOZOTOCIN-DIABETIC RATS

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SUMMARY: Rats consuming distilled water containing fluoride as NaF in various concentrations (0, 25, 50 ppm) were treated with streptozotocin (65 mg/kg body weight), i.p. 3 days prior to being sacrificed. Increased and longterm fluoride intake promoted diabetic hyperglycemia. Serum alkaline phosphatase activity increased in all diabetic groups as well as non-diabetic groups consuming 50 ppm. Weight loss of the submandibular and parotid glands was most apparent in the diabetic group treated with 50 ppm fluoride. In both fluoride consuming diabetic groups, the plasma ionized fluoride level was about one-half that of the fluoride consuming non-diabetic controls.

Introduction

Many studies have been carried out on the adverse effects of high and

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chronic fluoride intake on bones and teeth (1-5). Other tissues, however, as possible affected sites, and their relation with some metabolic disorders have received less attention. Szymanska et al. (6) reported an increased frequency in abnormalities of blood glucose curves and an enhanced activity of the enzyme phosphohexoseisomerase in the sera of humans exposed to fluoride for a prolonged period.

During the period from 1965 to 1975, while the use of fluoride for both prophylactic and therapeutic purposes was increasing (7,8), the incidence of diabetes increased by 6%/year (9). The present study was designed to examine the effects of longterm fluoride loading on experimental diabetes and to investigate the fluoride metabolism in diabetic conditions.

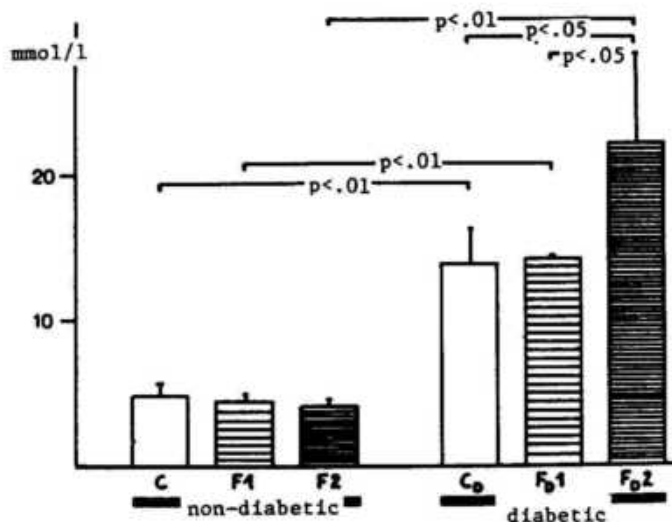
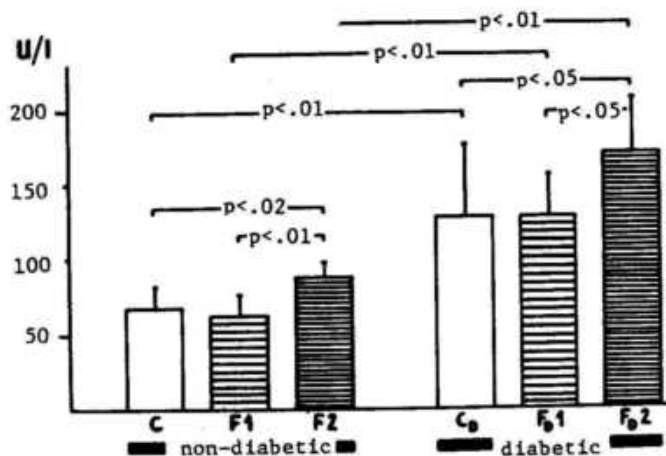
Materials and Methods

Female Wistar rats whose body weights averaged 220-250 grams at the beginning of the experiment were used. Animals were kept in a temperature-controlled 23°C. room, with a fixed 12 h artificial light cycle (8:00 a.m.-8:00 p.m.). They were fed a standard laboratory chow (LATI, Hungary) ad libitum. The rats, which were divided into three groups of ten each, were treated as follows: The control group (C) was offered distilled, deionized water for four weeks. The groups F1 and F2 consumed sodium fluoride in deionized water at concentrations of 25 and 50 ppm, respectively. Three days before the end of the experimental period, five rats from each group (groups C_D, F_D1, and F_D2) received streptozotocin (SERVA, Heidelberg, West Germany) in a dose of 65 mg/kg b.w.i.p., dissolved in isotonic saline set to pH 4.2 with citric acid. On day 28, after overnight starvation with free access to water, rats were sacrificed under nembuthal anesthesia. Blood samples were collected from the femoral vein into centrifuge tubes. After deproteinization and centrifugation at 4°C, glucose was determined by the glucose oxidase method (Boehringer kit, Mannheim, West Germany). The serum alkaline phosphatase (E.C. 31.1-3.1.) activity was estimated at 25°C by a colorimetric method using sodium p-nitrophenylphosphate as substrate (10). The plasma ionized fluoride level was determined at 37°C using fluoride sensitive microcapillary electrode (RADELKIS OP 262, Hungary). All data were expressed as mean \pm SD and significance was calculated by Student's t-test.

Results

Under the experimental conditions no considerable differences were seen in the average body weights of the rats either in the fluoride-treated or in the diabetic groups (data not shown). Data presented in Fig. 1 show that, on the third day after streptozotocin administration, all groups treated with the drug developed a high blood glucose level. It was also observed that fluoride treatment alone did not result in any changes of blood glucose concentration. However, mean blood glucose level in the diabetic group that consumed 50 ppm fluoride in water was extremely high.

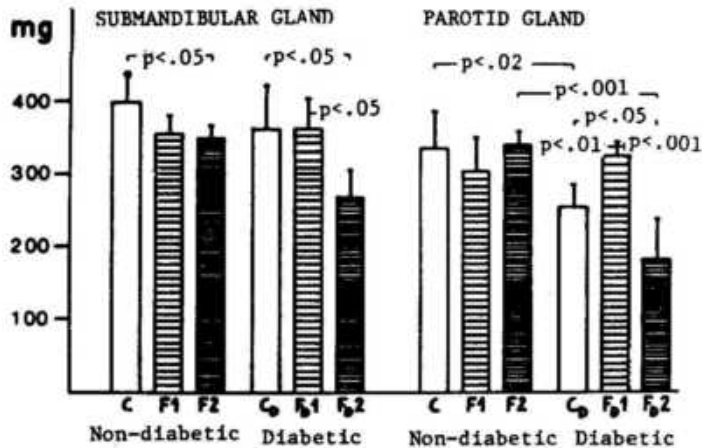
The alkaline phosphatase activity in the serum is illustrated in Fig. 2. In the nondiabetic F1 group, the enzyme activity did not differ from the control but activity was enhanced in the fluoride consuming F2 group. Streptozotocin administration produced a significant increase in the enzyme activity in all of the diabetic groups; no essential differences were observed between the diabetic groups with or without fluoride loading.

Figure 1Blood Glucose Concentrations Following F^- in Drinking Water**Figure 2**Serum Alkaline Phosphatase Activity Following F^- in Drinking Water

Groups: C = control, F1 = 25 ppm F^- , F2 = 50 ppm F^- , C_D = control-diabetic, F_D1 = 25 ppm F^- and diabetic, F_D2 = 50 ppm F^- and diabetic. Data are means \pm SD.

Figure 3

Changes in Wet Weight of Submandibular and Parotid Glands Following F^- in Drinking Water



Groups: C = control, F1 = 25 ppm F^- , F2 = 50 ppm F^- , Cd = control-diabetic, Fd1 = 25 ppm F^- and diabetic, Fd2 = 50 ppm F^- and diabetic. Data are means \pm SD.

Fig. 3 illustrates wet weights of salivary glands in various groups; a reduction in the weight of the submandibular gland in group F2 was more apparent in group F2. Fluoride administration had no considerable effect on the weight of the parotid gland. In contrast to the human results, in the diabetic animals the weight of the gland was decreased and weight loss in group Fd2 was more significant.

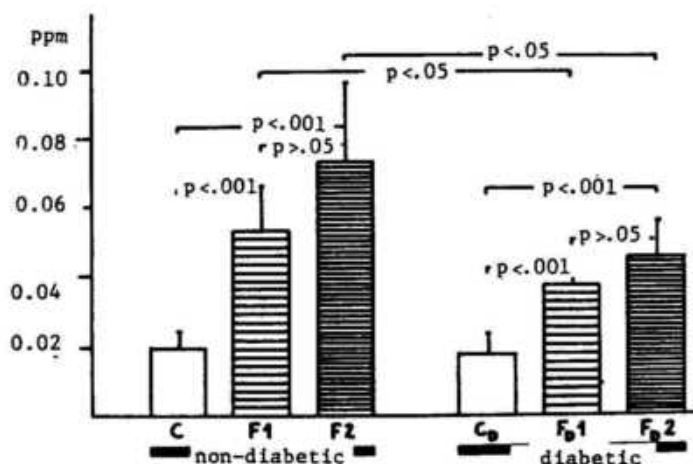
Animals receiving fluoride in drinking water always developed higher plasma ionic levels than those solely receiving distilled water (Fig. 4).

It should be noted that the mean of the ionic fluoride in the plasma of the diabetic animals (Cd) was practically the same as that of the untreated control (C). In the diabetic animals consuming drinking water with 25 or 50 ppm F^- , ionic fluoride concentration in their plasma was less enhanced than in the animals of groups F1 or F2.

Discussion

Streptozotocin is known to produce selective necrosis of the pancreatic islet cells and diabetes (11) and it is widely used in animal experiments. The defect in carbohydrate metabolism is a significant component of fluoride toxicity. Dost et al. (12) reported that the defect in carbohydrate metabolism in rats which occurred during continuous infusion of fluoride, is the blockade of glucose entry to cells, presumably through failure of phosphorylation. It is also reported (13) that insulin should

Figure 4

Plasma Concentrations of Ionic F^- Following F^- in Drinking Water

Groups: C = control, F1 = 25 ppm F^- , F2 = 50 ppm F^- , Cd = control-diabetic, Fd1 = 25 ppm F^- and diabetic, Fd2 = 50 ppm F^- and diabetic. Data are means \pm SD.

relieve certain manifestations of fluoride intoxication. These findings call attention to the fact that fluoride loading, whether derived from medical or surgical use, or from industrial or environmental sources, has been shown to elevate in humans the serum inorganic fluoride well into the range high enough to produce a depression in glucose utilization. Therefore, it seemed worthwhile to study interrelationships that might exist between diabetes and prolonged and increased fluoride intake. The current studies were undertaken to determine whether the development of experimental diabetes and the diabetic status of rats could be influenced by longterm pretreatment of fluoride in drinking water. In addition, we were interested in determining whether or not any changes in blood glucose are related to the fluoride intake per se. Under normal circumstances in the intact non-diabetic rats, no differences were observed in the blood glucose concentrations between the control and the fluoride-treated groups, which agrees with the findings reported with regard to several fluorotic patients and animals (14). In the control-diabetic rats the blood glucose concentration averaged 13.54 ± 2.19 mmol/liter, but the hyperglycemia was more apparent (21.83 ± 6.1 mmol/liter) in the diabetic group that consumed water containing 50 ppm fluoride showing that under fluoride loading a more severe diabetes developed.

Few data are available on *in vivo* effects of fluoride intake on enzymatic activities. Mechanisms of inhibition by fluoride of several enzyme

systems have been reported but the fluoride concentrations used were several times higher than those present in normal body fluids. In most cases, the changes which developed were shown to be secondary to a primary effect of dietary fluoride on the pattern of food intake (15). Ferguson (16) reported that 10 ppm fluoride in drinking water for 12 weeks induced a decrease in serum alkaline phosphatase activity in rats without any changes in the activity of the enzyme in the liver or intestinal tissue. In our experiment, the activity of this enzyme in the serum of the intact but fluoride consuming F2 group was significantly elevated compared with the control. It is suggested that the change in the enzyme activity could be associated with the toxic effect of fluoride on the liver. Iwase et al. (17) reported mild to severe degeneration in the myocardium and liver in chronic fluorosis. In the diabetic rats, the serum alkaline phosphatase activity was enhanced in all of the drug-treated groups but no difference between either C_D and F_{D1} groups or between C_D and F_{D2} groups was noted. These changes are due to the slight focal necrosis in the liver induced by streptozotocin 3-4 days after its injection (18).

It is well known that the salivary glands are commonly affected by endocrine dysfunction. Sialosis is the term which is used to describe the enlargement of salivary glands unaccompanied by signs of inflammation. In the majority of cases, the swelling occurs principally in the parotid gland (19). The bilateral parotid enlargement has also been described in diabetes mellitus (20) with reduced flow rate values (21). Recently, Weiss et al. (22) reported marked changes in the parotid gland of rats caused by diabetes with pronounced ultrastructural changes in the acinar and striated duct cells. The secretory granules were reduced in size and number and the acinar cells accumulated large cytoplasmic lipid droplets. The authors concluded that the normal gland function and structure may in part be insulin-dependent. The submandibular glands of mice exhibit a hormone-dependent sexual dimorphism of size and structure which is also affected by diabetes (23).

According to our results, in contrast to human findings under experimental conditions of diabetes, the enlargement of salivary glands did not occur. In the C_D group, the weight of the parotid gland was decreased and a further weight loss was observed in the F_{D2} group. On the contrary, change in weight of the submandibular gland caused by diabetes was not pronounced but a significant decrease occurred in the F_{D2} group. It appears that prolonged and increased fluoride intake may aggravate the structural and functional alterations of salivary glands, characteristic of diabetes. It should be noted that 50 ppm fluoride in drinking water given to intact, non-diabetic rats also affects the submandibular gland.

Factors which might influence fluoride metabolism, i.e. fluoride intake, distribution and excretion are well known. However, the metabolism of this ion in the diabetic organism has not been investigated. It was supposed that the diabetic polydipsia and polyuria might be important factors in determining the fluoride loading of the organism. In addition, the kidney, as the major excretory organ, appears to be one of the organs showing early changes in function following treatment with high doses of fluoride. Recent reports from Reynolds et al. (24) and Whitford et al. (25)

indicate that the acid-base status of rats is an important factor influencing sensitivity to acute fluoride toxicity. There are apparent species differences in the distribution of fluorine between the ionic and bound forms in the plasma of the rat as compared with human plasma. In rats, if total fluorine increases, it is in ionic rather than bound form, the latter remains fairly constant (26). For this reason in our study the plasma ionic fluoride was estimated. An aqueous fluoride intake at a concentration of 25 or 50 ppm in drinking water to rats for 4 weeks produced an elevation of plasma levels of ionic fluoride which was more marked in the non-diabetic F1 and F2 groups than in the Fp1 or Fp2 groups. To elucidate the mechanisms responsible for this phenomenon, a continuous monitoring of the daily fluoride intake and the urinary fluoride excretion is planned in the future.

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FLUORIDE BRIEFS

Solutions of sodium fluoride at pH 3 to 4 inactivated enteroviruses, whereas other sodium salts had little or no effect on virus infectivity. Solutions of potassium fluoride also inactivated viruses under similar conditions. Light, temperature, and the presence of organic compounds such as detergents and fecal matter did not affect inactivation of virus by 0.4 M solutions of sodium fluoride at pH 3 to 4.

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THE URACIL-FLUORIDE INTERACTION: AB INITIO
CALCULATIONS INCLUDING SOLVATION

by

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(Abstracted from J. Chem. Soc., Chem. Commun., 476-478, 1982)

The crystalline 1:1 complex between the RNA component uracil and potassium fluoride reported by Clark and Taylor (J. Chem. Soc., Chem. Commun., 466-468, 1981; abstr. FLUORIDE, 15:48-49, 1982) is shown by an ab initio calculation method described previously (J. Am. Chem. Soc., 103:24-28, 1981) to be thermodynamically stable even when hydrated. Under such conditions the N-H-F bond energy at N-3 in uracil is estimated to be 42 kJ/mol more stable than uracil N(3) + HF. At N-1 it is predicted to be 7 kJ/mol less stable than N(1) + HF. Because N-3 and not N-1 is involved in connecting uracil to adenine in RNA, the authors propose that substitution of F⁻ for the adenine nitrogen in the normal hydrogen bonding pairing "could play a disruptive role towards RNA and DNA; thymine in the latter should hydrogen bond equally as well as uracil with F⁻."

In considering reports relating low levels of fluoride to "birth defects, allergic responses, and even cancer", the authors suggest that fluoride interference with hydrogen bonding at the NH group in uracil and thymine "may provide the fluoride ion with a mechanism and the necessary energy to cause fundamental biochemical changes, given the right environment."

A.W.B.

(Reprints: Dept. of Chem., King's College, Strand, London WC2R 2LS, U.K.)

A REVIEW OF CLINICAL RESEARCH ON THE USE OF PRENATAL FLUORIDE
ADMINISTRATION FOR PREVENTION OF DENTAL CARIES

by

W.S. Driscoll

(Abstracted from Drug Metab. Dispos., 9:19-24, 1981)

The author recommends additional research on placental transfer of fluoride and the mechanisms of its action and states that permanent teeth are unlikely to benefit from dietary fluoride supplements. Since conclusive clinical evidence that administration of dietary fluoride supplements to pregnant women reduces dental caries in the teeth of offspring is lacking, the procedure cannot be recommended at this time.

Correction: Substitute this table for table 5 in FLUORIDE, 15:62, 1982.

Table 5

Fluoride ($\mu\text{g g}^{-1} \pm \text{standard error}$)⁺ in Body Tissues
and Estimated Diets from the Dam Surface

Tissue	<u>Microtis agrestis</u>	<u>Sorex araneus</u>
Femur	554 \pm 43 (10)	1282 \pm 252 (7) *
Pelvic Girdle	585 \pm 63 (9)	1298 \pm 245 (8) *
Skull	517 \pm 37 (10)	714 \pm 120 (7) NS
Kidney	22.7 \pm 2.9 (13)	22.5 \pm 3.6 (9) NS
Liver	15.7 \pm 1.0 (13)	15.9 \pm 1.4 (9) NS
Muscle	41.2 \pm 7.0 (12)	27.6 \pm 4.3 (8) NS
Total body concentration	139 \pm 11 (9)	259 \pm 42 (8) *
Estimated Diet**	332	1063

+ Values expressed on a dry weight basis.

* Significant ($p < 0.05$) or NS not significant differences between species.

** Estimated Diet: M. agrestis (ground-cover vegetation);
S. araneus (invertebrates) based on components of the
diet and ratios described by Godfrey (11), Chitty, et
al. (12) and Rudge (10).
Number of replicates in parenthesis.

FLUORIDE BRIEF

Monofluorophosphate (MFP), usually sodium-MFP, $\text{Na}_2\text{PO}_3\text{F}$, now widely used in dentifrices has been suggested for the treatment of osteoporosis. Its PO_3F^{--} ions are hydrolyzed to F^- and orthophosphate ions by phosphatases. In experiments on rats, no splitting was observed in the stomach; hydrolysis was rapid in the small intestine and in the liver, and slower in the blood. In neither rats nor humans was there any evidence of direct absorption of PO_3F^{--} into the blood. The authors state that " PO_3F^{--} ions resemble sulphate ions," that they have "little antienzymatic or other toxic potential" and that "any parenteral occurrence of these ions would have little physiologic or toxicologic effect."

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Table 2

Percentage of Bone Trabeculae in Cancellous Bone

I	Control	31.8
II	0.5 mg NaF	37.0
III	5.0 mg NaF	29.9
IV	0.3 mg Borax	30.0
V	0.5 mg NaF + 0.3 mg Borax	32.5
VI	5.0 mg NaF + 3.0 mg Borax	32.7
VII	5.0 mg NaF + 6.75 mg metasilicate	31.4
VIII	5.0 mg NaF + 2.25 mg MgO	32.3

High fluoride ingestion resulted in slight enlargement of the thyroid gland and a reduction in height of the follicular cells. The results on the thyroid gland are preliminary and require further investigation.

Table 3

Parathyroid Activity

Groups	Cut Faces of Nuclei
I Control	20.18/ μm^2
II 1.5 mg NaF	18.98/ μm^2
III 18 mg Borax	20.48/ μm^2
IV 15 mg NaF + 18 mg Borax	20.85/ μm^2
V 15 mg NaF + 0.5 A ₁ (Boric Acid)	23.82/ μm^2
VI 15 mg NaF + A ₂ (Borax)	22.27/ μm^2
VII 15 mg NaF + 20 mg Vit. C	22.60/ μm^2
VIII 15 mg NaF + 1.25 g Al ₂ (SO ₄) ₃	21.88/ μm^2

Conclusion

Feeding 5 mg/kg NaF daily for a period of 1 year to pigs resulted in thicker cortices of long bones. On the other hand, in cancellous bone, osteopenia resulted. The effect of NaF on bone cortices could be reduced by

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