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-transtational 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 moities into collagen. It is the carbohydrate moities, 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-HCI 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 vaccuo 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

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

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

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 diphyseal 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
After F^- Ingestion		
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 ^{14}C 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 ^{14}C 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 µCi/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 $CaCl_2$.

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 ^{14}C 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 ^{14}C 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 ^{14}C proline was observed between different tissues (Bar 1-4). The highest rate of ^{14}C 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. $N_1 - N_3$ = Normal rabbits
$F_1 - F_4$ = Rabbits administered NaF.

duction in ^{14}C proline uptake. A significant reduction in the rate of ^{14}C 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 ^{14}C 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 ^{14}C 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 ^{14}C proline in all 5 fractions studied. However, the rate of uptake of ^{14}C 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 ^{14}C 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

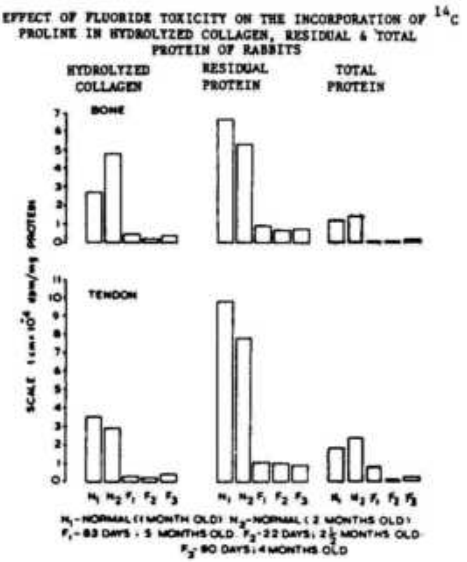

Figure 2

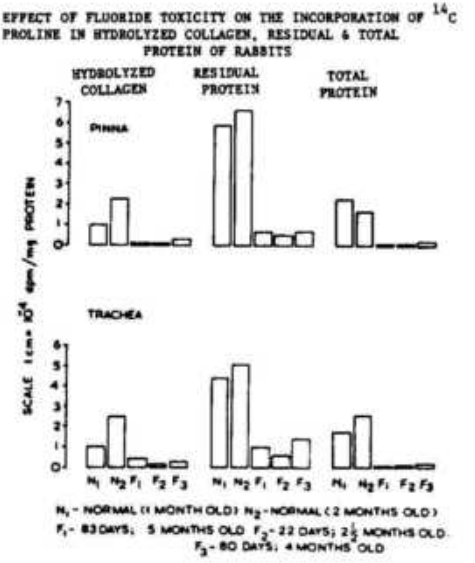

Figure 3

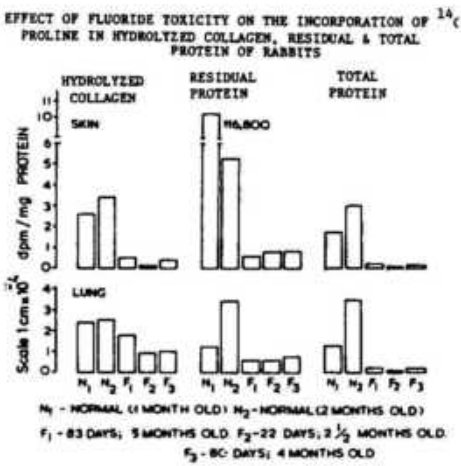

Figure 4

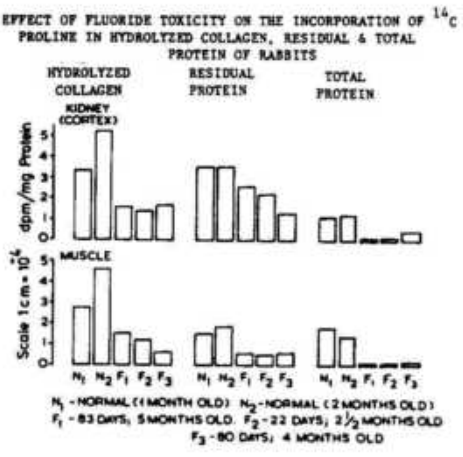

Table 6

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 ^{14}C proline uptake on rabbits by administrating 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 ^{14}C 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 ^{14}C proline uptake compared to the control.

The nature of reduction in ^{14}C 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 Percursors(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 (18) 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±S.D., n=5)

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

Values are expressed as μM 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 $CaCl_2$ 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 catablized.

Table 10

Effect of NaF on Collagen Bound Collagenase Activity in Rabbit Tissues

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