TOXICOLOGIE EXPÉRIMENTALE

Fluoride poisoning and the effect on collagen biosynthesis of osseus and non-osseus tissues of rabbit

A.K. SUSHEELA and D. MUKERJEE

Fluorosis Research Laboratory
Department of Anatomy
All India Institute of Medical Sciences
New Delhi - 110029

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Summary

Fluoride poisoning is known to cause a debilitating condition clinically referred to as Fluorisis. The present investigation on the experimental animal model has been carried out to collect information on the precise nature of fluoride action, with special reference to collagen biosynthesis.

Rabbits subjected to Fluoride poisoning for varying time intervals were administered with carbon labelled proline. Both osseus and non-osseus tissues were analyzed to measure the rate of incorporation of labelled proline, and index for collagen biosynthesis.

Part I of the article is dealing with ^{14}C proline uptake by Hydrolyzed collagen (obtained by centrifugation at 5000 x g) and residual protein of tissues viz : Bone, Tendon, Muscle, Kidney cortex, Skin, Lung, Pinna and Trachea.

Part II of the article is dealing with 14C proline uptake by different fraction of collagen Vlz : collagenase digested fraction and separated by centrifugation at 9000 x g ; native collagen fibril, acid soluble collagen, alkali soluble collagen and non-collagenous protein. The results obtained in Part I, suggest that in Fluoride poisoning collagen biosynthesis has been greatly impaired both in osseus and non-ossues tissues. This has been further confirmed by the results obtained in Part II of the investigation.

Key-words : Fluoride, Collagen, Bone, Rabbit

Intoxication par le fluor et biosynthèse du collagène dans les tissus osseux et non-osseux du lapin.

Résumé

L'intoxication par le Fluor est connue pour provoquer un délabrement de l'état général, cliniquement rapporté à la fluorose. Ce travail, sur un modèle expérimental animal, a été entrepris pour recueillir des informations sur la nature précise de l'action du Fluor, et plus spécialement vis-à-vis de la biosynthèse du collagène.

Des lapins soumis à une intoxication au Fluor pendant des durées variables ont été traités par le 14C-proline. Dans des tissus osseux et non-osseux, on a mesuré le rythme d'incorporation de la proline marquée, en tant qu'indicateur de la biosynthèse du collagène. La première partie de ce travail est en rapport avec l'incorporation de la 14C-proline par le collagène hydrolysé (obtenu par centrifugation à 500 g) et dans les protéines de divers tissus : os, tendons, muscles, cortex rénal, peau, poumons, et trachée. La deuxième partie de ce travail est en rapport avec l'incorporation de la 14C-proline dans diverses fractions du collagène : fraction digérée par la collagénase et séparée par centrifugation à 9000 g, fibrilles de collagène natif, collagène acido-soluble, collagène alcalo-soluble et protéines non collagéniques. Les résultats obtenus suggèrent que la biosynthèse du collagène a été fortement perturbée à la fois dans les tissus osseux et non osseux par l'intoxication au Fluor.

Mots-clés : Fluor, Fluorures, Collagène, Os, Lapin.

INTRODUCTION

Clinical manifestations in Fluorosis, reveal severe involvement of dental and skeletal tissues (1, 2, 3, 4, 5). However, it has been reported that Fluorosis is not merely a disease of bone and tooth, but it also affects the non-ossues tissues (6, 7, 8, 9, 10). Collagen, one of the structural constituents of both osseus and non-osseus tissues, appears to be severely affected due to Fluoride intoxication (11). The constitution of nascent collagen protein in fluoride toxicity has been reported to be defective. The major defect has been localized in the absence of low molecular weight peptides which normally are known to fabricate the collagen fiber (12). The present investigation has been carried out to elucidate further, the effect of fluoride toxicity on the biosynthetic profile of collagen. The rate of incorporation of ^{14}C proline by rabbit tissues, both osseus and non-osseus tissues, have been reported in this communication.

PART I

MATERIAL AND METHODS

Normal, healthy, young rabbits (Table I) were treated intragastrically with 50 mg of sodium fluoride/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 μc i/ 100 gm body weight ; Sp activity of 125 mCi/mmol, Radiochemical Centre, Amersham). The animals were sacrified after 2 hrs and tissues such as bone, tendon, pinna, trachea, skin, muscle, lung and kidney cortex were dissected out and homogenized in 0.05 M Tris HCLbuffer (pH 7.6) containing 0.005 $Cacl_2$.

TABLE I
DETAILS OF THE RABBITS USED FOR EXPERIMENTATION

Rabbit	Age of the animal when sacrified (in months)	Duration of sodium fluoride 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 the animals used were male rabbits.
N_1 - N_3 = Normal rabbits
F_1 - F_4 = Rabbits administered with NaF

Known volume of tissue homogenates were subjected to collagenase digestion at 37° C (collagenase 140 units/mg ; Worthington Biochemicals) for 6 hrs. 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 (13). The supernatant containing the hydrolyzed collagen was separted. The residual protein was dissolved in 0.5 N NaOH (14, 15). Known aliquots of supernatant (i.e hydrolyzed collagen), and the residual fractions were treated with soluene (sample solubilizer) for 2 hrs at 60° C. Known volume of simple scintillation cocktail containing PPO (5 mg) and POPOP (0.5 gm) in Toluene (1 litre), was added and the rate of uptake of ^{14}C proline was counted using a Packard Tricarb Liquid Scintillation Spectrometer. Counts/minute obtained at 40 % efficiency were corrected for 100 % efficiency. Protein content was measured by Lowry's method (16). 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 DISCUSSION

As the primary structure of collagen protein, is known to vary from Type I to IV, the ^{14}C proline uptake has been assessed with reference to different tissues, known to have different types of collagen. From the results (Table II) reported, it is evident that the rate of incorporation of carbon labelled proline revealed considerable variation among the tissues investigated. In the hydrolyzed collagen fraction of normal tissues, the highest rate of uptake of proline was shown by bone and tendon which are mainly constituted by Type I collagen. The rate of uptake of proline was also high in muscle and kidney cortex, where collagen fibers are predominently Type III and IV respectively. Skin and lung representing predominently Type III collagen have also revealed high rate of uptake. However, the rate of ^{14}C proline incorporation was the lowest in pinna and trachea which are fabricated mainly by collagen Type II .

TABLE II
^{14}C PROLINE UPTAKE (dpm x 10^{-4}/mg protein) IN HYDROLYZED COLLAGEN AND RESIDUAL PROTEIN IN RABBIT TISSUES

		N_1	N_2	F_1	F_3	F_4
Bone	HC	4.8	2.7	0.2	0.4	0.5
	RP	5.3	6.7	0.7	0.7	0.9
Tendon	HC	2.9	3.5	0.2	0.4	0.3
	RP	7.8	9.8	1.0	0.9	1.1
Muscle	HC	4.6	2.7	1.2	0.6	1.4
	RP	1.8	1.5	0.3	0.6	0.6
Kidney (cortex)	HC	5.2	3.3	1.4	1.7	1.6
	RP	3.5	3.5	2.2	1.3	2.6
Skin	HC	3.4	2.5	0.1	0.4	0.5
	RP	5.2	11.7	0.8	0.8	0.6
Lung	HC	2.5	2.4	0.9	1.0	1.8
	RP	3.4	1.2	0.6	0.7	0.6
Pinna	HC	2.3	1.0	0.1	0.3	0.1
	RP	6.6	5.9	0.5	0.7	0.7
Trachea	HC	2.5	1.0	0.2	0.3	0.4
	RP	5.1	4.4	0.6	1.4	1.0

HC = Hydrolyzed collagen
RP = Residual protein

The residual protein although, free of nascent collagen have shown a high rate of incorporation of proline, indicating that within 2 hours of subcutaneous injection, ^{14}C proline has been anabolized and high counts have been obtained in the residual protein fraction. It is also possible that the centrifugation at 5000 x g would not have been adequate to separate smaller peptides from larger polypeptides in enzyme hydrolyzed fraction.

The animal which were on fluoride for 22 (F_1), 80 (F_3) and 83 (F_4) days showed a reduction in the rate of uptake both in the supernatant and residual fraction. It is evident from the tables that bone and tendon, have shown the maximum reduction in ^{14}C proline uptake. There is a significant reduction in the rate of uptake in the rest of the tissues studied. Pinna and trachea, which normally had low uptake of ^{14}C proline, have shown very low counts, even after fluoride intoxication. These results have demonstrated that, in sodium fluoride toxicity the ^{14}C proline incorporation in collagen, in both osseus and non-osseus tissues have been severely impaired.

However, to provide confirmation, we have analyzed the ^{14}C proline uptake in bone and tendon with reference to various collagen fractions viz : (1) Hydrolyzed collagen fraction obtained by collagenase digestion and separated at 9000 x g. (2) Native collagen fibril obtained by thermal reconstitution method (3) Total acid soluble collagen (4) Total non-collagenous protein and (3) Alkali soluble collagen. The details of the experimental procedure and results are reported under Part II.

PART II

Bone and tendon tissues obtained from the animals referred to under the head Material in Part I of this article, was used for the investigations carried out under this section.

1. Hydrolyzed collagen fraction obtained by collagenase digestion and separated at 9000 x g :

Hydrolyzed collagen was obtained by collagenase digestion method of PeterKofsky and Diegelmann (17) as adapted in our laboratory. A known amount of bone and tendon was homogenized in Tris HCL buffer (0.005 M) containing 0.005 M $CaCl_2$ (pH 7.6) at 0-4° C, using a teflon mechanical homogenizer, until a fine homogenate was obtained. A known volume of the homogenate was treated with collagenase (140 units/mg ; Worthington Biochemicals). The collagenase used for digestion was considered as crude collagenase, as it had peptidase and proteinase activity. The crude collegenase (80 μg) was dissolved in 1 ml of 0.05 M Tris HCL buffer containing 0.005 M $CaCl_2$ pH 7.6) and was added to the tissue homogenate and incubated in a metabolic shaker cum incubator at 37°C for 4 hours. At the end of the incubation period the enzyme action was stopped by the addition of 0.5 ml of ice cold water followed by the addition of 0.2 ml of 90 % ethanol. The contents were centrifuged in a Beckman J-21 refrigerated centrifuge at 9000 x g for 30 min. The supernatant (S_1) containing collagen peptides was collected (Flow sheet). The residue (R_1) was saved. A known volume (100 μl) of the supernatant (S_1) was treated with 1 ml of soluene at 60°C for 2 hours. 10 ml of the scintillation cocktail containing PPO (5 gm) and POPOP (0.5 gm) in Toluene (1 litre) was added to the vials and ^{14}C proline uptake counted in a liquid scintillation spectrometer (Packard model : 3301). The counts are expressed as dpm/mg protein. Known volumes of the supernatant was used for protein estimation (16).

2. ^{14}C proline uptake in native collagen fibril obtained by thermal reconstitution method :

The supernatant (S_1) saved from the previous experiment was kept at 4°C for 36 days to separate out the native collagen fibrils by thermal reconstitution process (18, 19, 20). The reconstituted fibrils were separated by centrifugation at 9000 x g at 0-4°C for 15 min. The surpernatant was discarded. The residue containing the native collagen fibrils, was gelatinized in known volume of distilled water at 70°C for 30 min. Known volumes of the getalinized collagen were taken and 1 ml of soluene was added. The samples were digested at 60°C for 2 hours. At the end of the digestion, the samples were maintained at room temperature for overnight. The samples were counted for ^{14}C proline uptake, in a Liquid Scintillation Spectrometer after adding 10 ml of the scintillation cocktail, to each vial. The counts obtained are expressed in dpm/mg protein. Protein estimation was carried out by Lowry Method (16).

3. ^{14}C proline uptake by acid soluble collagen :

The residual protein R_1 was dissolved in O.1 N NaOH at 37°C for 5 min. Tris HCl buffer of 0.05 M, pH 7.6, containing 0.005 M $CaCl_2$ was added. The pH of the substrate mixture was neutralized by the addition of 0.1 N HCl. Collagenase (140 units/mg ; Worthington Biochemicals (25 μg/mg of protein was added to the substrate mixture. The substrate enzyme mixture was incubated at 37°C for 3 hours in a metabolic shaker cum incubator. Following digestion, the contents were centrifuged at 5000 x g for 10 min. The residue (R_2) was saved for further analysis as it contained insoluble collagen. The supernatant (S_2) thus obtained was collected and the protein content precipitated by the addition of equal volume of TCA and Tannic acid mixture (10 % and 0.5 % respectively). The tubes were kept at 0°C for 5 min and then centrifuged in a Sorvall (GLC-1) centrifuge, at 400 x g for 5 min. The supernatant (S_3) was stored. The residue thus obtained was treated with 5 % TCA and 0.25 % Tannic acid and the solution resuspended for 5 min. The supernatant was pooled with S_3 fraction. The residue thus obtained (R_3) was used for studies on non-collagenous protein. To a known volume of the supernatant S_3 which contained acid soluble collagen, 10 ml of the scintillaton cocktail was added and ^{14}C proline uptake counted in a Liquid Scintillation Spectrometer. The results obtained are expressed as total dpm.

4. ^{14}C proline uptake by total non-collagenous protein :

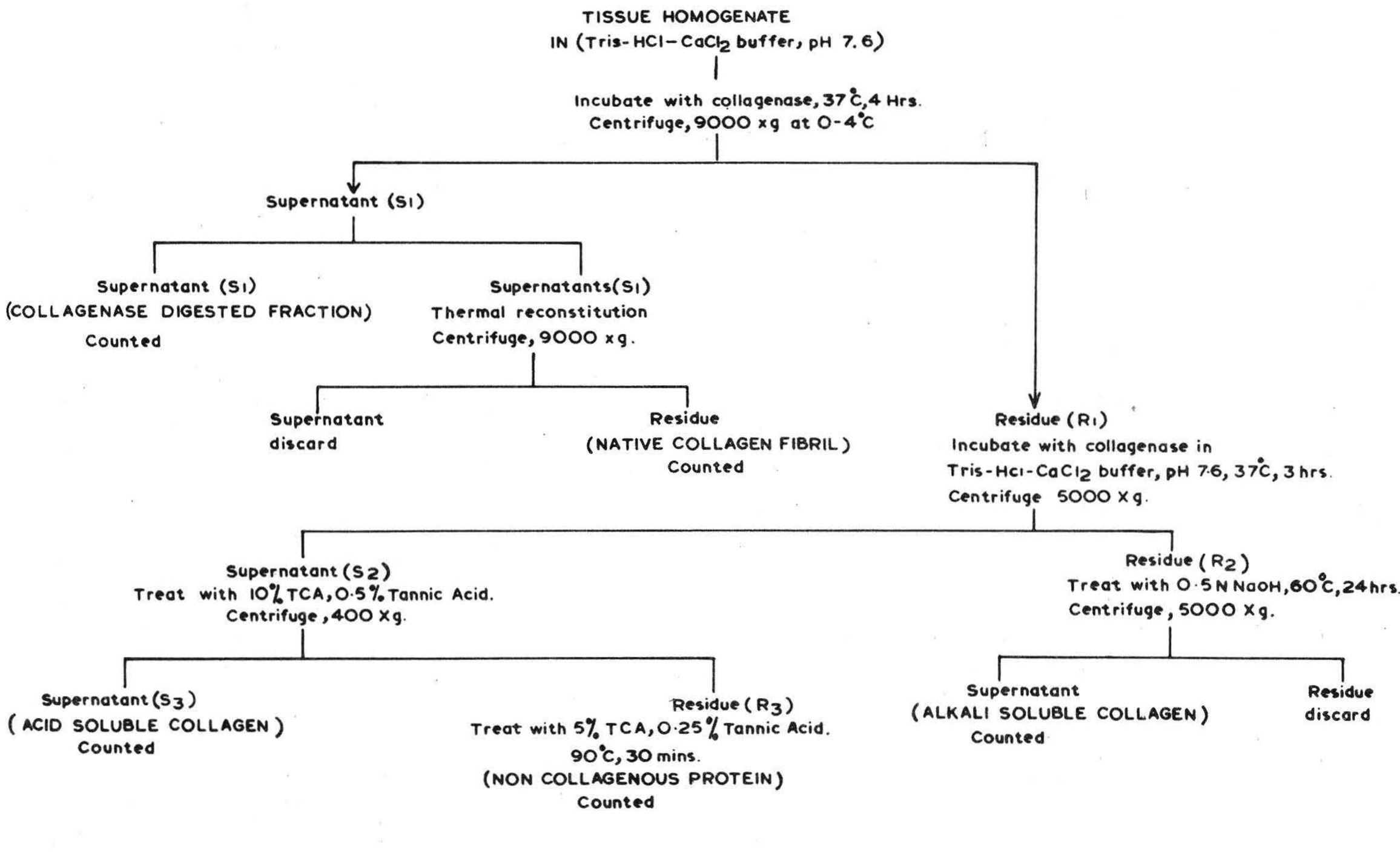
The residue R_3 was treated with 5 % TCA and

0.25 % Tannic acid mixture and were heated upto 90°C for 30 min. The digested non-collagenous protein was transferred to scintillation vials. The tubes were rinsed with 0.5 ml of TCA-Tannic acid mixture and the contents added to the scintillation vials. 10 ml scintillation cocktail was added and counted in a scintillation spectrometer. Results are expressed in total dpm.

5. ^{14}C proline uptake by alkali soluble collagen :

The residue (R_2) was treated with 2 ml of 0.5 N NaOH at 60°C for 24 hours. The alkali extract was centrifuged at 5000 x g for 10 min. 200 μl of the aliquot was used for counting after adding scintillation cocktail. Protein estimation was carried out by the method of Lowry et al (16). The results are expressed in dpm/mg protein.

FLOW SHEET SHOWING COLLAGEN FRACTIONS COLLECTED FOR ^{14}C PROLINE INCORPORATION



RESULTS AND DISCUSSION

From the data obtained for the collagenous and non-collagenous fractions of Tendon and Bone (Tables III, IV), it is evident that in the 3 control animals studied the highest rate of uptake for ^{14}C proline has been revealed by the collagenase digested fraction. The counts obtained for native collagen fibril is of significance, as this fraction is the reconstituted fibril from the collagenase digested fraction and is a more specific index for ^{14}C proline uptake and rate of collagen biosynthesis.

Acid and alkali soluble collagen have also revealed high counts. It is interesting tonote that the non-collagenous protein in all the 3 control animals have shown proline uptake.

TABLE III
EFFECT OF SODIUM FLUORIDE ON THE INCORPORATION OF ^{14}C PROLINE IN COLLAGENOUS AND NON-COLLAGENOUS PROTEIN OF TENDON IN RABBITS
(Results expressed as 10^{-4} x dpm/mg protein)

	CONTROL			NaF TREATED			
	N_1	N_2	N_3	F_1	F_2	F_3	F_4
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
Non collagenous 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

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

TABLE IV
EFFECT OF SODIUM FLUORIDE ON THE INCORPORATION OF ^{14}C PROLINE IN COLLAGENOUS AND NON-COLLAGENOUS PROTEIN OF BONE IN RABBITS
(Results expressed as 10^{-4} x dpm/mg protein)

	CONTROL			NaF TREATED			
	N_1	N_2	N_3	F_1	F_2	F_3	F_4
Collagenase 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
Non collagenous 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

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

The animals which have been subjected to fluoride intoxication for varying time intervals viz : 22 (F_1), 24 (F_2), 80 (F_3) and 83 (F_4) days, have shown a significant reduction in the rate of uptake of ^{14}C proline in all the 5 fractions studied, which includes the collagenous and non-collagenous proteins. However, it is pertinent to point out that the rate of uptake of ^{14}C proline of native collagen fibril, of both tendon and bone have 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 attribute to a 50 mg dose. The extent of intoxication is also being revealed, by the reduction in body weight by 150 to 300 mg over a period of 22 to 83 days.

The non-collagenous 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 non-collagenous proteins.

This aspect has been further explored by carrying out yet another set of experiments on ^{14}C proline uptake on rabbits by administering a low dose of NaF (i.e. 10 mg/kg body weight).

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

TABLE V
EFFECT OF SODIUM FLUORIDE ON THE INCORPORATION OF ^{14}C PROLINE IN COLLAGENOUS AND NON-COLLAGENOUS PROTEIN OF BONE AND TENDON IN RABBIT* ADMINISTERED WITH 10 mg NaF DAILY FOR A PERIOD OF 175 DAYS
(Results expressed as 10^{-4} x dpm/mg protein)

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
Non-collagenous 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
Non-collagenous protein**	1.6	2.7
Alkali soluble collagen	2.0	4.4

* 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

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It is evident that in the NaF treated animal, the first 3 fractions i.e. collagenase digested, native collagen fibril and acid soluble collagen of both bone and tendon have shown reduced rate of ^{14}C proline uptake compared to the control animal. The nature of reduction in ^{14}C proline uptake in the animal administered with the low dose of NaF has been in the same pattern as those animals on high dose of NaF except for the non-collagen protein fraction which has shown an increased rate of ^{14}C uptake both in the bone and tendon. This observation further confirms our finding

that in high degree of intoxication other proteins are likely to be affected. However, in low dose, it is more specifically involving the collagen protein. It may also be added that in low dose of sodium fluoride administration 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 reduced.

This report therefore provides convinsing evidence to believe that in fluoride poisoning both osseus and non-osseus tissues are adversely affected. It is also apparent that collagen protein is one of the elements seriously affected in the present investigation add a new dimension in the understanding of the etiology and pathogenesis of Fluorosis.

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