

EFFECT OF FLUORIDE ON MOLECULAR WEIGHT, CHARGE DENSITY AND AGE RELATED CHANGES IN THE SULPHATED ISOMERS OF GLYCOSAMINOGLYCANS OF THE RABBIT CANCELLOUS BONE

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Summary: *The effect of fluoride on the composition, molecular weight and charge density of the glycosaminoglycan isomers in the cancellous bone of the iliac crest region of the pelvic girdle was studied in rabbits treated with fluoride for 3, 9 and 16 months and the corresponding age-matched controls. With advancing age the chondroitin-6-sulphate concentration was found to increase in the fluoride-treated as well as the control animals. The concentration of dermatan sulphate was found to decrease with advancing age in the control groups. However, the fluoride-treated groups showed increasing concentrations of dermatan sulphate as the age and the duration of fluoride treatment advanced. The glycosaminoglycans in the fluoride-treated group showed the presence of low-molecular-weight molecules which were not found in the corresponding control group, and also showed an increased charge-density heterogeneity as compared to the corresponding control group. The data presented provide evidence to suggest that glycosaminoglycans of cancellous bone undergo age-related changes in their isomeric profile. The increase in dermatan sulphate and the presence of low-molecular-weight, highly charged molecules in the adult fluorosed bone may be related to the cartilagenous loci formation as reported earlier.*

Introduction

An intake of excess fluoride over a long period of time leads to abnormal calcification of bone. This results in increased bone mass and density in skeletal fluorosis — a clinical condition known to occur as a result of ingestion of excess fluoride. Glycosaminoglycans (proteoglycans) are associated with the process of calcification (1-3), but their exact role in this process is not known (4). In fluorosed cancellous bone of rabbit Jha and Susheela (5-7) reported an increased sulphate content and an increased ratio of sulphate to

hexosamine. Recently, increased concentrations of non-sulphated and 6-sulphated disaccharides and a reduced 4-sulphated disaccharide content have been reported in rabbit cancellous bone following fluoride administration for a longer duration (8). Moreover, the appearance of dermatan sulphate, one of the isomers of glycosaminoglycans which is normally found in high concentrations in the organic matrix of soft tissue but not in calcified tissues, was also reported. The cancellous bone response to fluoride toxicity is distinctly different from the cortical bone response. Cartilagenous loci, rich in glycosaminoglycans,

were detected only in the cancellous bone (6, 7). Similar changes in glycosaminoglycans (proteoglycans) in rat teeth following fluoride ingestion have been reported by Embery *et al* (9). The presence of high dermatan sulphate content in human teeth in dental fluorosis has also been reported (communicated).

The glycosaminoglycan content of bone and its isomer composition are known to change during bone development (10, 11). Continuous formation and resorption of bone in adults may also lead to changes in the composition of the isomers of glycosaminoglycans.

In the present study, we report the effect of fluoride on the isomeric profile of sulphated glycosaminoglycans and age-related changes in chondroitin sulphate isomers of glycosaminoglycans of cancellous bone of rabbit. The inherent charge and molecular-weight heterogeneity of the glycosaminoglycans have also been compared in the normal and fluorosed cancellous bone.

Materials and methods

Twenty-five age-matched rabbits 3 months old were divided into two groups of 13 and 12 animals. One group was given NaF at 10 mg/kg body weight/day as an aqueous solution by the intragastric route. The other group was pair-fed but given no fluoride. Standard animal diet and water were provided *ad libitum*. A batch of rabbits from the fluoride-treated group was sacrificed after 3 months, while the remainder were sacrificed at 9 and 16 months' intervals. Age-matched control rabbits were sacrificed with each experimental batch. Cancellous bone (iliac crest region of the pelvic girdle) was dissected out and stored at -40°C .

Purification of glycosaminoglycans. Bone was cleaned of the adhering tissues, cut into pieces, defatted for 3 days with a daily change of diethyl

ether: acetone (1:1, v/v) mixture and dried in ether for one day. Dry defatted bone was ground and subjected to digestion with 0.2 M di-sodium EDTA; 0.05 M cysteine hydrochloride, pH 7.0 (40 ml/gm tissue powder), containing 1.7 mg papain per 40-ml digestion mixture for 6–10 h at 65°C . Glycosaminoglycans were precipitated as cetylpyridiniumchloride (CPC) complex from the supernatant, dissociated with 4% CPC-60% n-propanol and reprecipitated with ethanol and saturated sodium acetate (12).

Quantitation of chondroitin sulphate isomers. Chondroitin sulphate isomers were quantitated by enzyme digestion (with chondroitinase ABC or chondroitinase AC) following by desulphation of disaccharides (with chondro-4-sulphatase or chondro-6-sulphatase) and estimation of desulphated disaccharides by the borate-catalysed Morgan-Elson reaction (13).

Chromatography. Gel filtration chromatography was performed in a Sephadex G-75 column (1.5×70 cm) equilibrated with 0.2 M NaCl at a flow rate of 30 ml/h. Fractions of 4 ml were collected and analysed for uronic acid (14); blue dextran was used for finding the void volume. Ion-exchange chromatography was performed in a DEAE cellulose column (1.5×20 cm). The gel was equilibrated with 0.1 M sodium acetate, pH 5.0. The samples were applied in the same buffer and the column washed with 3 bed volumes of the buffer. Bound glycosaminoglycans were eluted with a linear gradient (total volume 200 ml) of 0.1–0.25 M sodium acetate at a flow rate of 30 ml/h. Uronic acid was estimated by the modified tetraborate-carbazole reaction (14) with D-glucuronolactone as standard. The results of uronic acid estimation are presented as absorption at 530 nm (OD 530 nm).

Statistical analysis. The results were evaluated by using the χ^2 test for the 2×3 contingency table. The comparison of different treatment groups was made between the fluoride-treated and the corresponding controls with respect to the

three chondroitin-sulphate isomers (15).

Materials. Chondroitinase ABC (EC 4.2.2.4), chondroitinase AC (EC 4.2.2.5), chondro-4-sulphatase (EC 3.1.6.9) and chondro-6-sulphatase (EC 3.1.6.10), sephadex G-75 and DEAE cellulose were obtained from Sigma. All other chemicals were either BDH analytical grade chemicals or E-Merck products.

Results

The relative concentrations of chondroitin-sulphate isomers in the cancellous bone of fluoride treated animals for varying durations and their corresponding age-matched controls are shown in Fig. 1. The data obtained are from material pooled from 4 animals in each group,

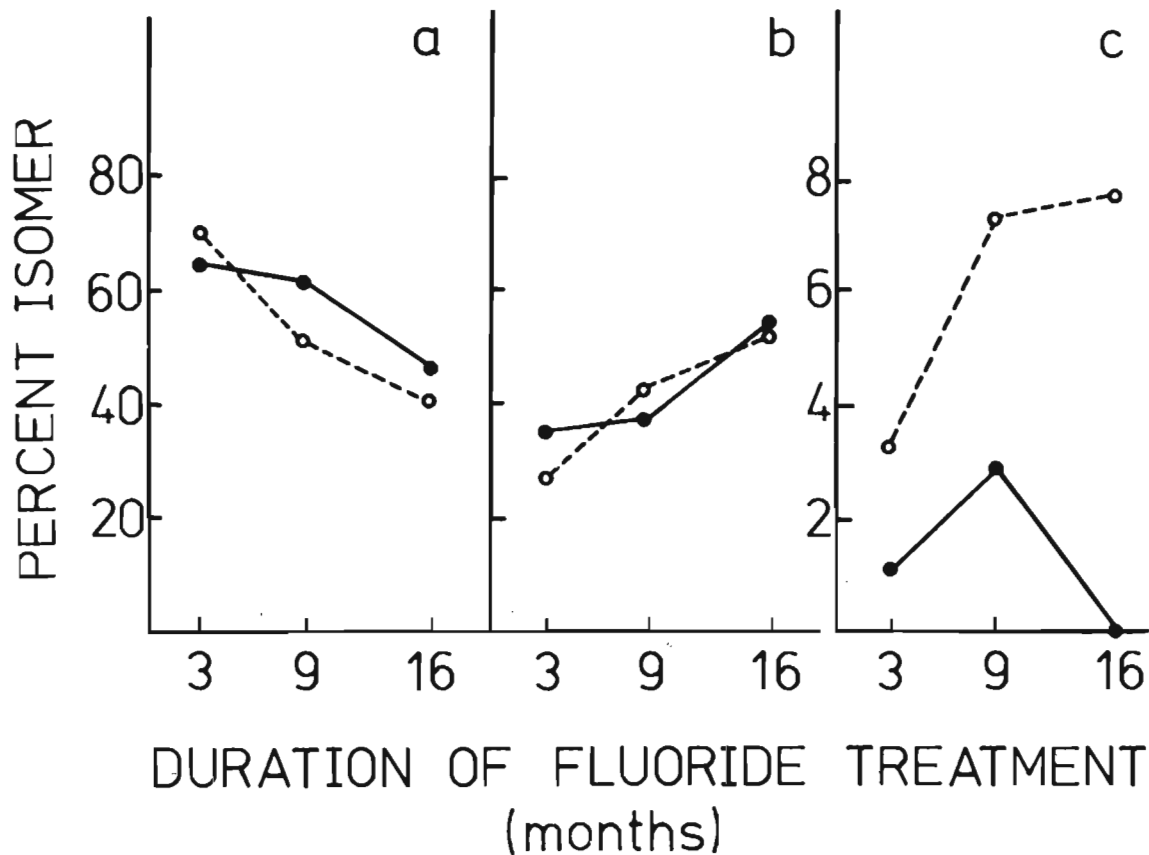


Fig. 1a Chondroitin-4-sulphate isomers (%) in rabbit cancellous bone following NaF administration for 3, 9 and 16 months (o—o) and the corresponding age-matched controls (●—●).
b Chondroitin-6-sulphate isomer (%) in rabbit cancellous bone following NaF administration for 3, 9 and 16 months (o—o) and the corresponding age-matched controls (●—●).
c Dermatan sulphate (%) in rabbit cancellous bone following NaF administration for 3, 9 and 16 months (o—o) and the corresponding age-matched controls (●—●).

except for the 9-month treated group in which there were 5 animals.

In the control groups, chondroitin-4-sulphate decreased from 64.00% to 61.04% to 45.88% over the periods of 6–19 months of age. The pattern of decrease over a period of 3–16 months of fluoride treatment was from 69.77% to 50.61% to 40.18% (Fig. 1a). However, an increase in the concentration of chondroitin-6-sulphate was recorded in both the control as well as the treated groups (Fig. 1b). In the control group, it increased from 34.93% to 36.09% to 54.12% over a period of 6–19 months of age. In the treated group, the increase was from 27.01% to 42.15% to 52.20% for a duration of 3–16 months of fluoride treatment.

Dermatan sulphate was not detected in the 16-month control group, whereas the 3- and 9-month control groups had 1.06% and 2.86% dermatan sulphate respectively. However, among the fluoride treated animals, the 16-month treated group had 7.61% dermatan sulphate, while in the 3- and 9-month treated animals the dermatan sulphate contents were 3.22% and 7.23% respectively (Fig. 1c).

Statistical analysis of the data on chondroitin sulphate isomers (Table I) using the χ^2 test for the 2×3 contingency table revealed a statistically significant ($p < 0.025$) difference in the relative concentration of the isomers in the 16-month fluoride treated rabbit cancellous and the corresponding age-matched control group.

Gel filtration. The Sephadex G-75 (exclusion limit for dextrans m.w. 50,000) chromatograms for the control and fluoride-treated (9-month group) cancellous bone glycosaminoglycans are shown in Figs. 2a and 2b respectively. In the control group, all the glycosaminoglycans (100% uronic acid) eluted at the void volume as a single peak of m.w. 50,000 (Table II). In the corresponding fluoride-treated group, only 40.99% of the glycosaminoglycans eluted at the void volume and the remaining 59.01% eluted much later (m.w. $< 50,000$) in three peaks (Table II).

Table I Effects of age and ingestion of excess fluoride on sulphated isomers of GAG of rabbit cancellous bone.

Isomer	Age months	Percentage isomer		
		Control	Duration of NaF exposure	NaF treated
Chondroitin-4-sulphate	6	64.00	3 months	69.77
	12	61.04	9 months	50.61**
	19	45.88	16 months	40.18
Dermatan sulphate	6	1.06	3 months	3.22
	12	2.86	9 months	7.23**
	19	*	16 months	7.61
Chondroitin-6-sulphate	6	34.93	3 months	27.01
	12	36.09	9 months	42.15**
	19	54.12	16 months	52.20

* Dermatan sulphate was not detectable in the 19-month-old animals used as controls.

** Material pooled from 5 animals; all other samples were pooled from 4 animals in each group.

Ion-exchange chromatography. The glycosaminoglycans eluted as heterogeneously charged molecules both in the controls (Fig. 3a) and the fluoride-treated groups (Fig. 3b) at different ionic strengths of the sodium acetate buffer, viz. 0.16–2.1 M and 0.22–2.21 M respectively. The percentage distribution of uronic acid eluting at different ionic strengths of the elution buffer is shown in Table III. From the data presented, it is evident that in the case of fluoride-treated cancellous bone there is a reduced concentration of glycosaminoglycans with low charge density (0.1 M–0.5 M), and that the high-charge-density glycosaminoglycans (> 0.5 M) are increased in concentration as well as heterogeneity as compared to the normal cancellous bone glycosaminoglycans.

Discussion

The chondroitin-4-sulphate and chondroitin-6-sulphate in the control group revealed distinct

Table II Uronic acid content of normal and fluoride-treated [9 months] cancellous bone eluting in different peaks following Sephadex G-75 chromatography.

	V_o [Void Volume]	Peaks eluting at		
		V_e [After void volume]		
		1	2	3
Normal [n = 5]*	100**	—	—	—
Fluoride Treated [n = 5]	40.99	32.75	19.74	6.52
			59.01	

* The number of animals from which the material was pooled in each group.

** All values represent the % uronic acid content.

differences, viz. chondroitin-4-sulphate was higher in concentration as compared to chondroitin-6-sulphate. Chondroitin-4-sulphate decreased as the age advanced, whereas chondroitin-6-sulphate increased with age. Prince and Navia (16) had found similar changes in the rat cortical bone where no chondroitin-6-sulphate was detected at 0 month but 1- and 2-month-old rats had detectable concentrations and the concentration of chondroitin-4-sulphate was found to decrease.

Dermatan sulphate has been reported to be either absent (5, 9) or present in very low concentrations (16, 17) in calcified tissues. In the present study with the methods employed, dermatan sulphate was not detected in the normal animals which served as controls for the 16-month treated animals. Normal animals which served as control for the 3- and 9-month treated groups showed a detectable amount of dermatan sulphate. The disappearance of dermatan sulphate or reduction to negligible amounts recorded only in the 16-month control animals (aged 19 months) suggests that changes in glycosaminoglycan composition in the cancellous bone probably extend beyond the period of bone growth. The presence of relatively high concentrations of dermatan sulphate in the 3-month (3.22%), 9-month (7.23%) and 16-month (7.61%) treated

groups suggests a retention and stimulation of dermatan sulphate synthesizing capacity in the fluoride-treated cancellous bone. This might be related to the cartilaginous loci, with chondrocyte like cells, seen in the fluoride-treated cancellous bone, as reported earlier (6, 7).

The increase in the concentration of high-charge-density glycosaminoglycans (>0.5M, Table III) of the cancellous bone in the fluoride-treated animals as observed on DEAE cellulose ion exchange chromatography (Figs. 3a and 3b) can be related to an increase in dermatan sulphate, since this isomer has a higher sulphate content as compared to others (4). Sulphation plays an important role in the formation of L-iduronic acid, and Jha and Susheela (5) observed an increase in sulphate content of glycosaminoglycans in the fluoride-treated cancellous bone. The increased charge-density heterogeneity of the highly (>0.5M) charged glycosaminoglycans in the fluoride-treated bone is in agreement with the earlier observations on fluoride-treated rabbit-tooth glycosaminoglycans (18).

The low-molecular-weight (<50,000) glycosaminoglycans (Fig. 2b) are formed possibly due to cartilaginous loci formation in the fluoride-treated cancellous bone. Hjertquist and Vejlens (12) reported that the glycosaminoglycans of dog

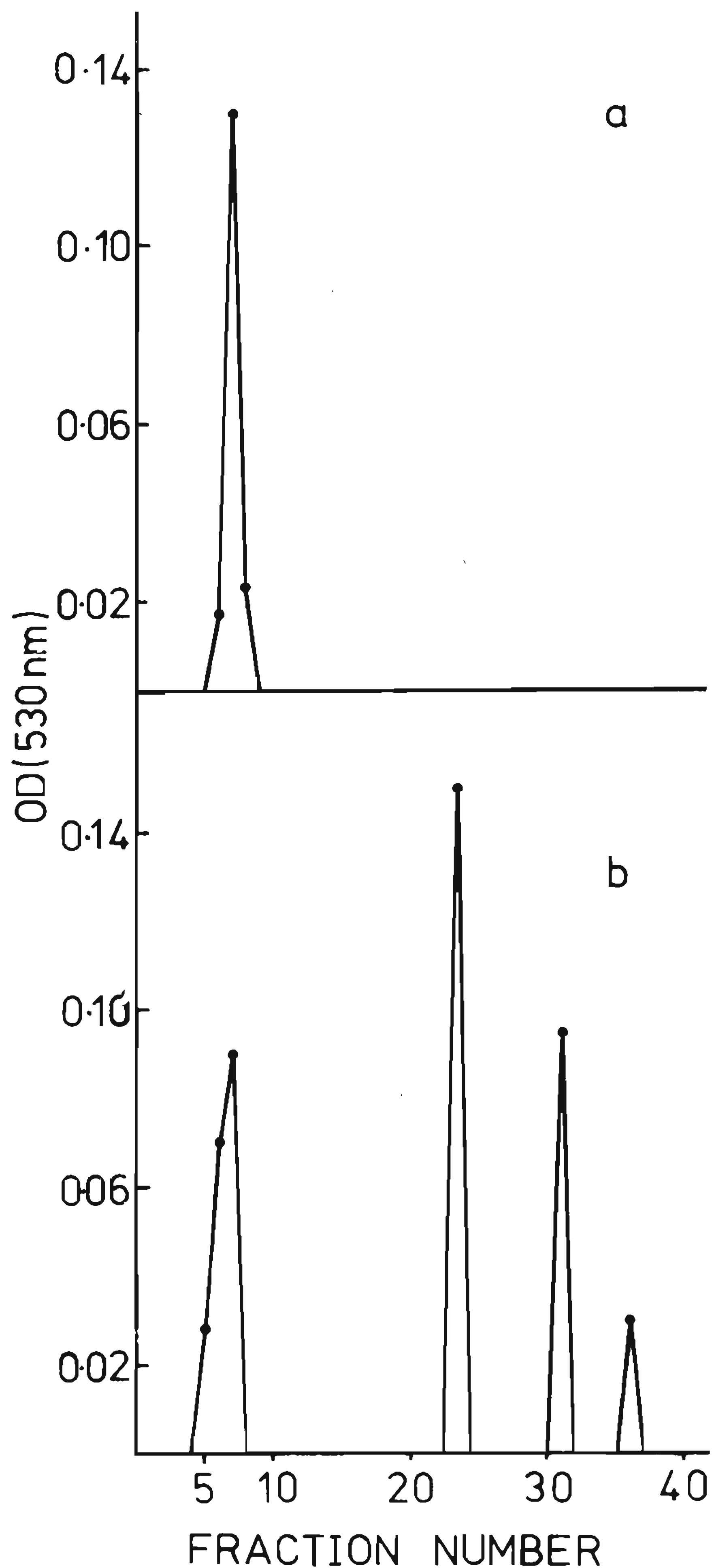


Fig. 2 Gel filtration chromatograms (Sephadex G-75) of glycosaminoglycans of rabbit cancellous bone from age-matched control (a) and 9-month fluoride-treated (b) groups.

In the control group, all the glycosaminoglycans eluted at the void volume, whereas in the treated group the glycosaminoglycans eluted in 4 peaks, one at the void volume and the other three much later in the bed volumes.

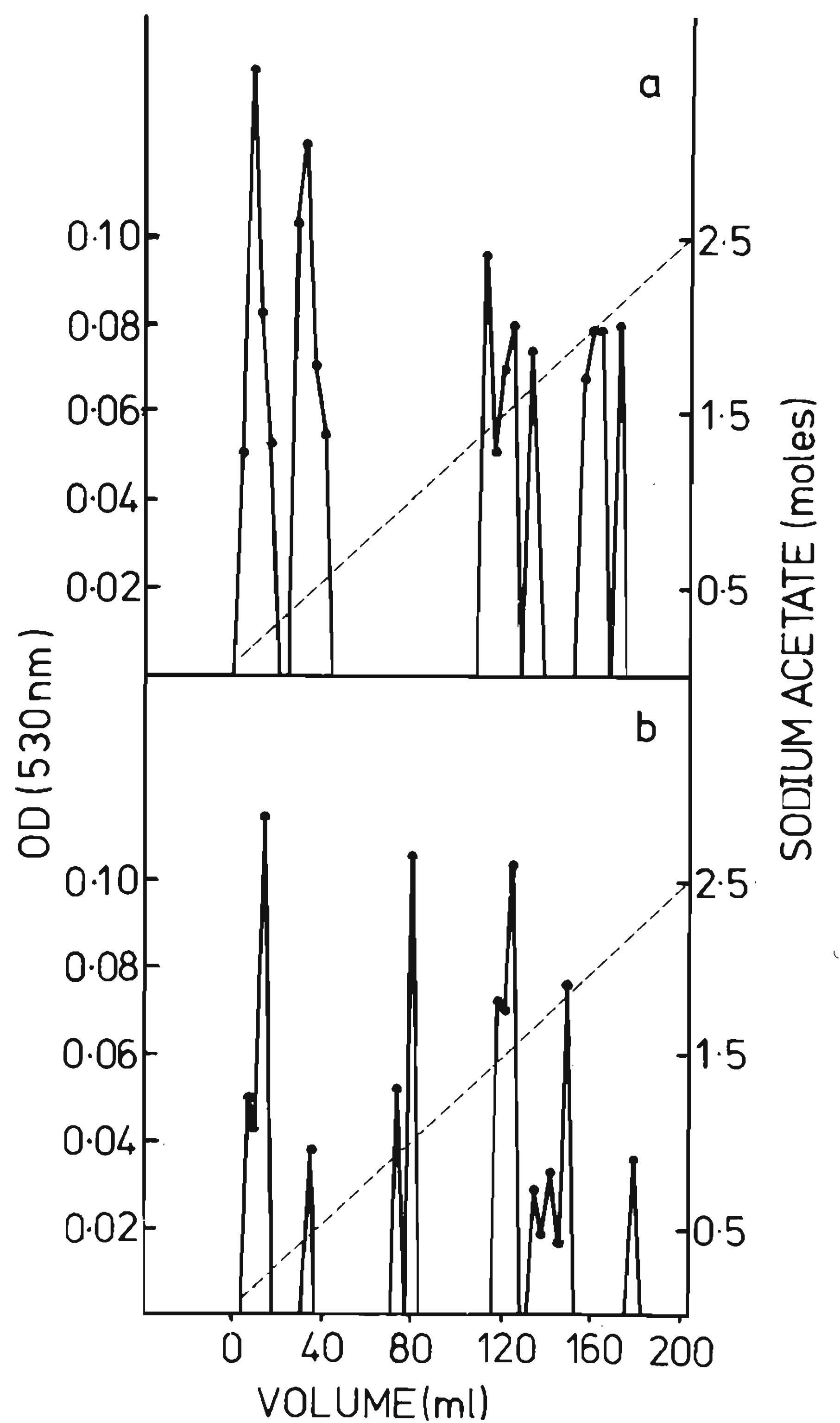


Fig. 3 DEAE cellulose ion-exchange chromatograms of the cancellous bone glycosaminoglycans of the 9-month control (a) and fluoride-treated group (b).

The control group glycosaminoglycans eluted between 0.16M–2.1 M sodium acetate (a) and the treated-group glycosaminoglycans between 0.225 and 2.21 M sodium acetate (b) concentrations.

compact bone have a molecular weight between 45,000 and 56,000 and that of cartilage have a molecular weight between 14,000 and 25,000. High-molecular-weight glycosaminoglycans have

Table III Uronic acid content of glycosaminoglycans of normal and fluoride-treated [9 months] rabbit cancellous bone eluting at different ionic strengths of elution buffer in DEAE-cellulose chromatography.

	Peaks eluting at [molar sodium acetate]			
	0.1 M–0.5 M	0.5 M–1.0 M	1.0 M–1.5 M	1.5 M–2.5 M
Normal [n = 5]*	48.28**	—	9.32	42.40
Fluoride Treated [n = 5]	31.27	8.05	27.40	33.28

* The number of animals from which the material was pooled in each group.

** All are values percentage uronic acid of the total.

been detected in the control as well as the treated group of rabbits in the present study (Fig. 2a). Low-molecular-weight glycosaminoglycans were detected only in the treated group of animals (Fig. 2b).

In conclusion, the present study provides evidence to suggest that glycosaminoglycans of cancellous bone undergo age-related changes in their isomeric profile, in that chondroitin-4-sulphate decreases and chondroitin-6-sulphate increases with age. Dermatan sulphate, although present in early ages, decreases to negligible amounts as the age advances. In the case of fluorosed cancellous bone, the changes in chondroitin-4-sulphate and chondroitin-6-sulphate follow the same trend as in the controls. However, the dermatan sulphate content increases with age, and on exposure to NaF the quantity is greatly enhanced. The increase in dermatan sulphate content and the presence of low-molecular-weight highly-charged molecules in the adult fluorosed cancellous bone may be related to the cartilaginous loci formation (6, 7). These cartilaginous patches are the site for dermatan sulphate synthesis and are rich in dermatan sulphate content.

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References

- (1) Baylink D., Wergedal J., Thompson E. *Loss of protein-polysaccharide at sites where bone mineralization is initiated*. *J. Histochem. Cytochem.*, **20**, 279, 1972.
- (2) Kuettnner K.E., Sorgente N., Croxen R.L., Howell D.S., Pita J.C. *Lysozyme in preosseous cartilage, VII. Evidence for physiological role of lysozyme in normal and endochondral calcification*. *Biochem. Biophys. Acta*, **372**, 335, 1974.
- (3) Chen C.C., Boskey A.L., Rosenberg L.C. *The inhibitory effect of cartilage proteoglycans on hydroxyapatite growth*. *Cal. Tiss. Int.*, **36**, 285, 1984.
- (4) Fisher L.W., Termine J.D. *Non-collagenous proteins influencing the local mechanisms of calcification*. *Clin. Orthop. Rel. Res.*, **200**, 362, 1985.
- (5) Susheela A.K., Jha M. *Effects of fluoride on glycosaminoglycans of cancellous and cortical bone of rabbits*. *Experientia*, **37**, 1097, 1981.
- (6) Jha M., Susheela A.K. *In vivo chondrogenesis and histochemical appearance of dermatan sulphate in rabbit cancellous bone*. *Differentiation*, **22**, 235, 1982.
- (7) Jha M., Susheela, A.K. *Characterization of glycosaminoglycans from normal and fluoride treated rabbit iliac crest*. *Biochem. Biophys. Res. Comm.*, **105**, 711, 1982.
- (8) Sharma Kamal, Susheela A.K. *Fluoride ingestion in excess and its effect on the disaccharide profile of glycosaminoglycans of cancellous bone of the rabbit*. *IRCS Med. Sci. Res.*, **16**, 349, 1988.
- (9) Embery G., Stanbury, J.B., Smalley J.W. *The metabolism of proteoglycans and glycosaminoglycans in dental fluorosis*. In:

Susheela A.K. ed. "Fluoride toxicity". Proceedings of the 13th Conference of the International Society for Fluoride Research, 1985, pp. 65-77.

(10) Engfeldt B., Hjerpe A. *Glycosaminoglycans of dentine and predentine*. Cal. Tiss. Res., **10**, 152, 1972.

(11) Lohmander S., Hjerpe A. *Proteoglycans of mineralizing rib and epiphyseal cartilage*. Biochem. Biophys. Acta, **404**, 93, 1975.

(12) Hjertquist S.O., Vejlens L. *The GAG of dog compact bone epiphyseal cartilage in the normal state and in experimental hyperparathyroidism*. Cal. Tiss. Res., **2**, 314, 1968.

(13) Saito, H., Yamagata T., Suzuki S. *Enzymatic method for determination of small quantity of isomeric chondroitin sulphates*. J. Biol. Chem., **243**, 1536, 1968.

(14) Bitter T., Muir H.M. *A modified uronic acid carbazole reaction*. Analyt. Biochem., **4**, 330, 1964.

(15) Snedecor G.W., Cochran W.G. "Statistical methods," 6th ed. Oxford and IBH Publishing Co., India, 1968, pp. 238-243.

(16) Prince C.W., Navia J.M. *Glycosaminoglycan alterations in rat bone due to growth and fluorosis*. J. Nutr., **113**, 1576, 1983.

(17) Prince C.W., Rahemtulla F., Buller W.T. *Metabolism of rat bone proteoglycans in vivo*. Biochem. J., **216**, 589, 1983.

(18) Susheela A.K., Sharma K. *Fluoride induced changes in the tooth glycosaminoglycans: an in vivo study in the rabbit*. Arch. Toxicol., (in press) 1988.