INT. J. TISS. REAC. ***VI****(3) 255-261 (1984)*

SCANNING ELECTRON MICROSCOPIC AND ELECTRON MICROPROBE X-RAY ANALYSIS OF CORTICAL BONE OF FLUORIDE-TREATED RABBITS.

JHA MOHAN, SUSHEELA A.K.

Department of Anatomy, All-India Institute of Medical Sciences, New Delhi, India.

Summary: *Sodium fluoride was administered to rabbits through the intragastric route at the rate of 10 mg/kg every day for a period of 8 months. Cortical bone from the diphyseal region of the femur was studied morphologically with a scanning electron microscope, and significant structural changes in collagen fiber were observed in the fluoride-treated animals as compared to normal bone. Similar bone samples were assessed physically for their CaKα/PKα ratio by electron microprobe x-ray analysis, and chemically for their calcium and phosphorus content. The bone from the rabbits to which sodium fluoride had been administered showed a higher Ca/P ratio than that from untreated control animals by both of the methods of assessment. Possible explanations for the increased Ca/P ratio in relation to the observed structural changes are discussed.*

Introduction

Light-microscope studies of fluorosed cortical bone reveal a periosteal bone deposition (1, 2). In addition, an endosteal deposition has also been described, which in some rabbits obliterates the marrow cavity. In human skeletal fluorosis it has been observed that the long bones are nearly twice the normal weight, while the vertebrae and the pelvic girdle are several times heavier. However, it is not fully understood whether the newly laid down bone at the periosteal and endosteal surfaces is adequately mineralized or not. Microscopically the newly laid down bone appears to be more radio-opaque than the original bone. Increased radio-opacity was observed by Leslie (3) who suggested that the effect was due to massive accumulation of incompletely mineralized and poorly structured bone rather than increased mineralization or failure of resorption. With the same experimental material Malcolm (4) indicated that microhardness values for fluorotic bone were lower than those for control bone. Hypermineralization of cortical bone in endemic and industrial fluorosis has also been reported by various authors (5, 6).

In view of the above controversy on the mineralization of newly deposited endosteal and periosteal bone, it was of interest to explore the structural and chemical composition of both the surfaces. Observations with the scanning electron microscope, along with the semi-quantitative results obtained from electron-microprobe x-ray analysis of the corresponding areas reported in the present communication, provide information on the mineral deposits and the mineral matrix relationship.

Address for reprints: Dr. A.K. Susheela, Fluorosis Research Laboratory, Department of Anatomy, All-India Institute of Medical Sciences, New Delhi - 110029, India.

0250-0868/84/0300255 + 7 $02.00/0

Materials and Methods

Rabbits weighing 600 to 800 gm were fed sodium fluoride at 10 mg/kg daily by the intragastric route for a period of 8 months and killed thereafter. Cortical bone from the diphyseal region of the femur was dissected out and freed from the marrow. The bone pieces were divided into two groups, one of which was used for scanning electron microscopy and electron microprobe x-ray analysis, and the other for chemical analysis of calcium and phosphorus. Animals of the same age group but deprived of sodium fluoride were also investigated for information on control material.

Scanning electron microscopy. Organic material was removed from small pieces of femur by treating them with 5.25% sodium hypochlorite solutiion for 1 to 4 h. These bone samples were rinsed briefly with distilled water and dehydrated through a graded series of acetone, and were finally air-dried. They were then attached to stubs with Duco cement, sputter-coated with gold, and observed in a scanning electron microscope (Philips SEM 501 B) at 15 kv. Three to five samples were examined from each group.

Electron micro-probe x-ray analysis. Samples for scanning electron microprobe elemental analysis were prepared in the same way as for SEM studies except that the gold sputter-coating was replaced by carbon coating. Elemental analyses on carbon-coated samples were carried out in a Cambridge Stereoscan S-150 SEM fitted with a Philips (Model 711) energy dispersive x-ray analyser (EDAX). X-ray spectra were run at 20 kev for a pre-set time of 40–60 sec on five randomly selected areas of endosteal and periosteal samples. X-ray counts were obtained for CaKα and PKα peaks by setting separate windows for each peak. The ratios of CaKα to PKα were calculated. No attempt was made to quantitate Ca and P when using the EDAX.

Chemical analysis. Bone samples were defatted in an ether-acetone mixture (1:1 v/v) and dried in acetone for chemical analysis. Calcium was determined (7) using an atomic absorption spectrometer (Carl Zeiss Model AAS_1). Phosphorus was determined (8) employing spectrophotometric measurement.

Results

The scanning electron-microscope observations on the treated rabbits revealed that the periosteal and endosteal surfaces had been considerably altered in their structure. SEM observations were made on the surface of the bone matrix where the cells and organic matrix have been removed in order to recognize the pattern of the collagen bundles. The periosteal surface of normal bone reveals collagen fibrils smoothly impregnated with bone minerals (Figs. 1 and 2). The smooth collagen bundles represent a resting (mineralized) surface (9). However, no resorption sites were observed on the periosteal surface of either the normal or the fluoride-treated rabbits.

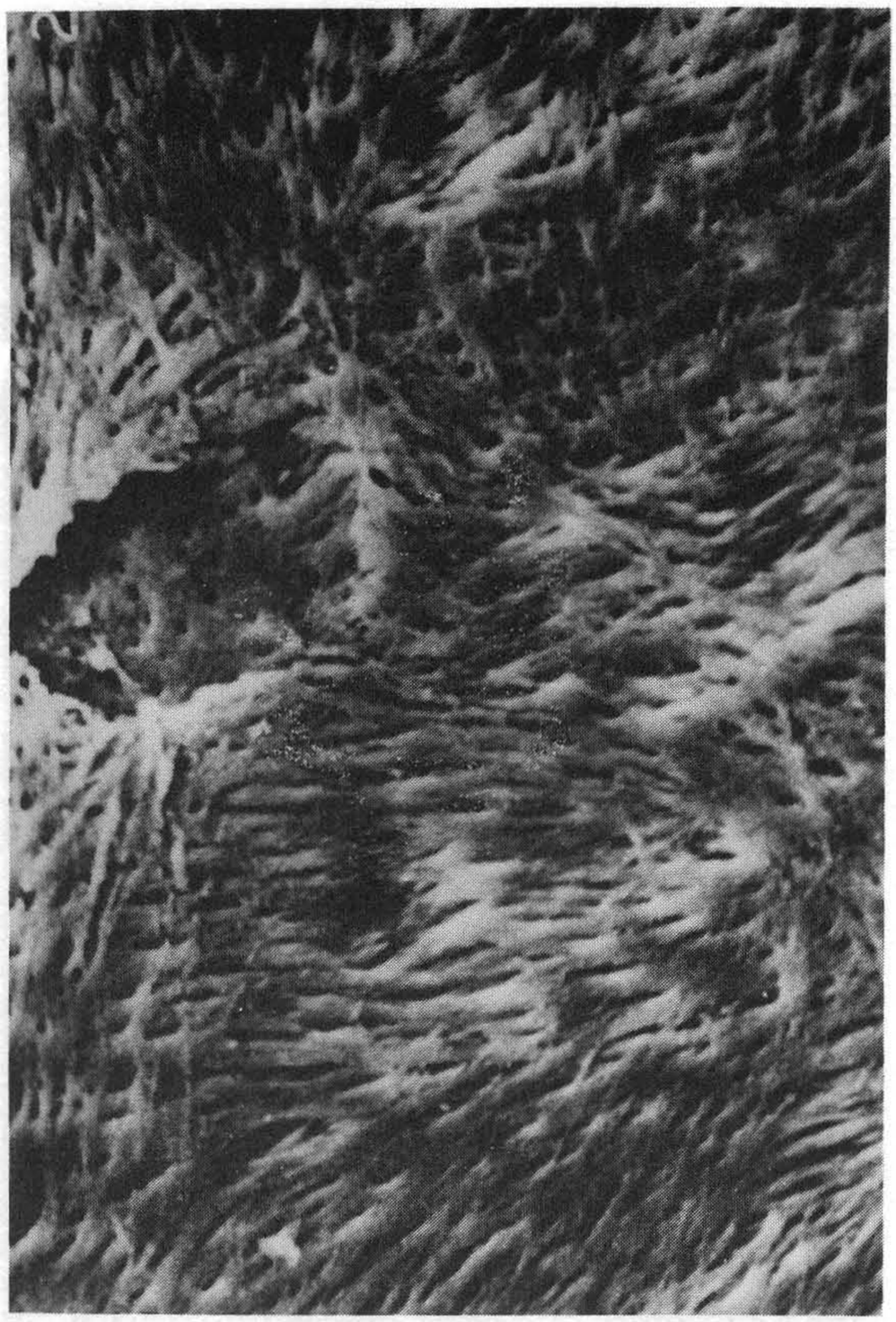
The periosteal surface of the femurs of fluoride-treated animals, after removal of organic matrix and osteoblasts, reveals a roughened bone mineral skeleton and a series of short segments of rough textured and irregularly oriented collagen fibers (Figs. 3–5). These latter represent the mineralizing

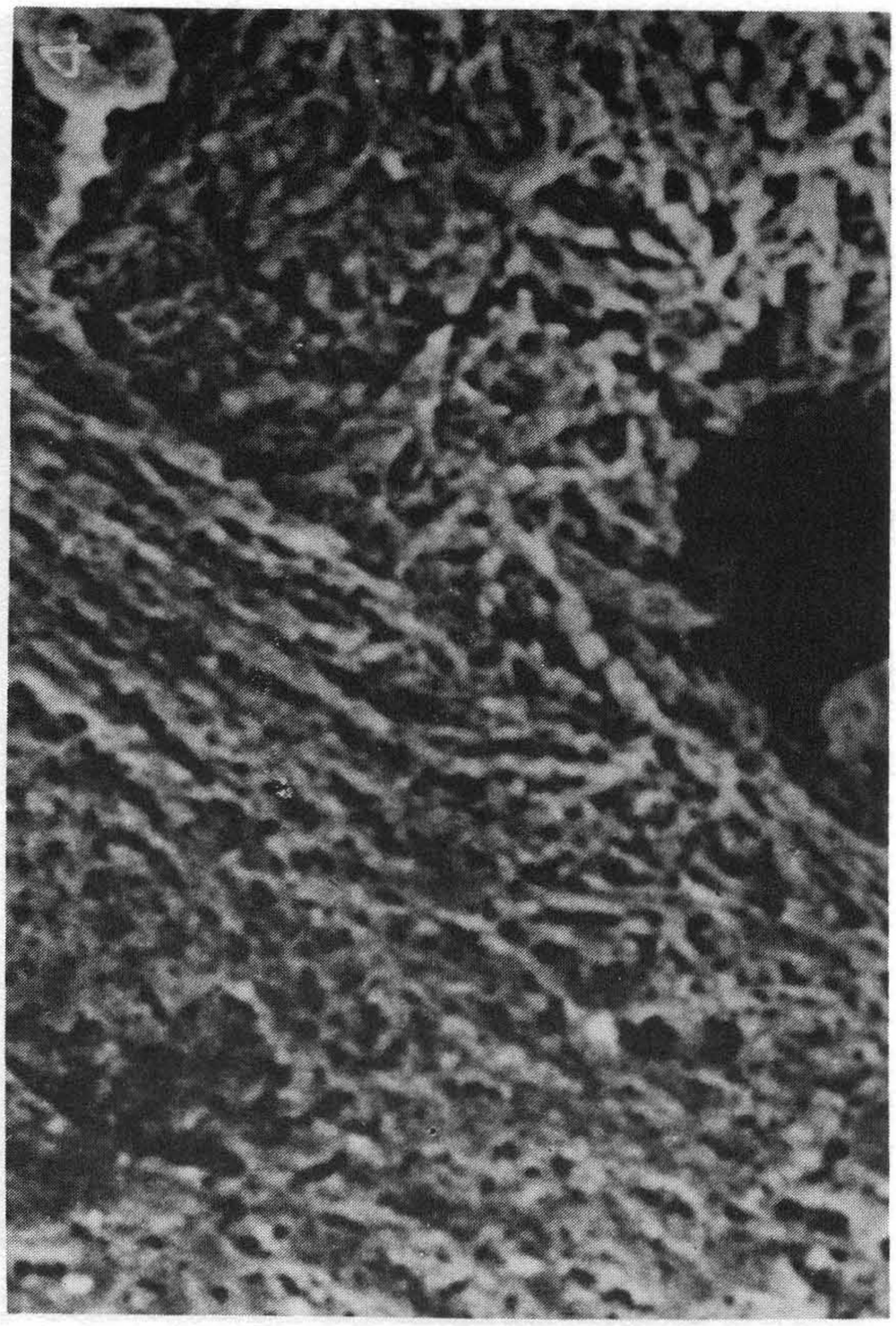
Fig. 1 Low-magnification scanning electron micrograph of normal cortical bone (periosteal surface of rabbit femur) showing smooth surface. x160.

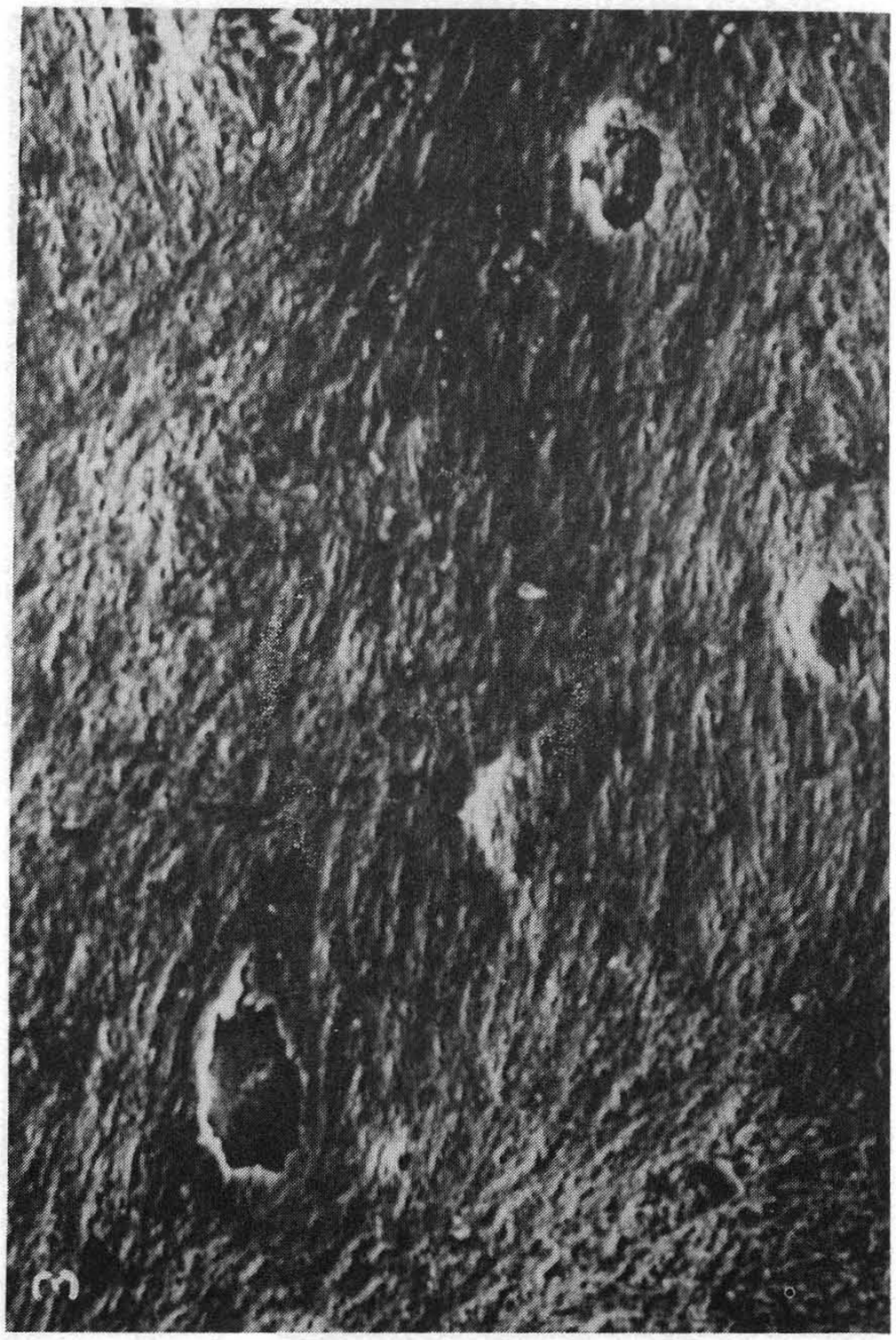
Fig. 2 Scanning electron micrograph of normal cortical bone (periosteal surface of rabbit femur) showing well-organized collagen bundles. x2000.

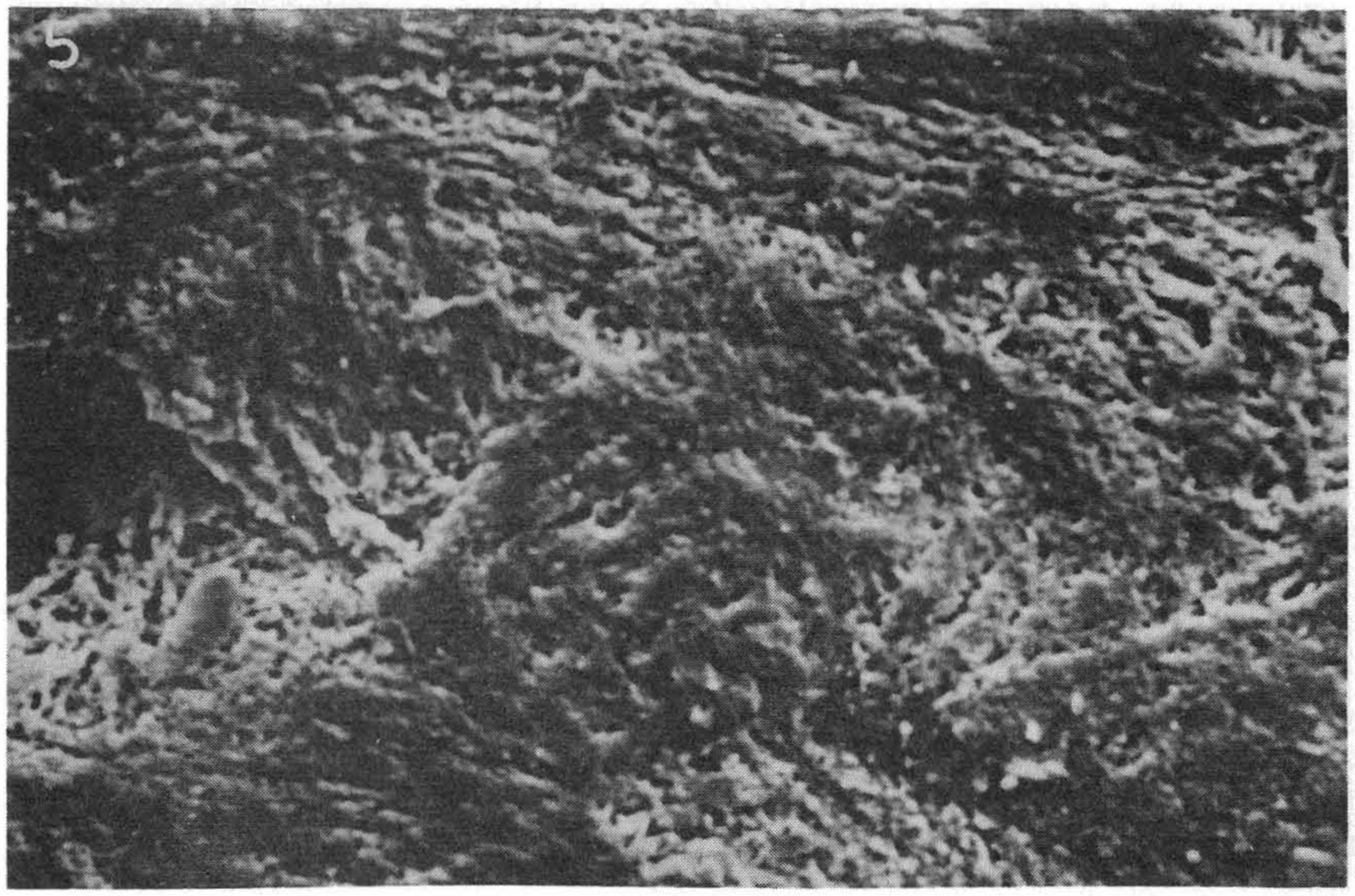
Fig. 3 Low-magnification scanning electron micrograph of cortical bone (periosteal surface) from "fluoride-treated" rabbits, showing rough surface. x160.

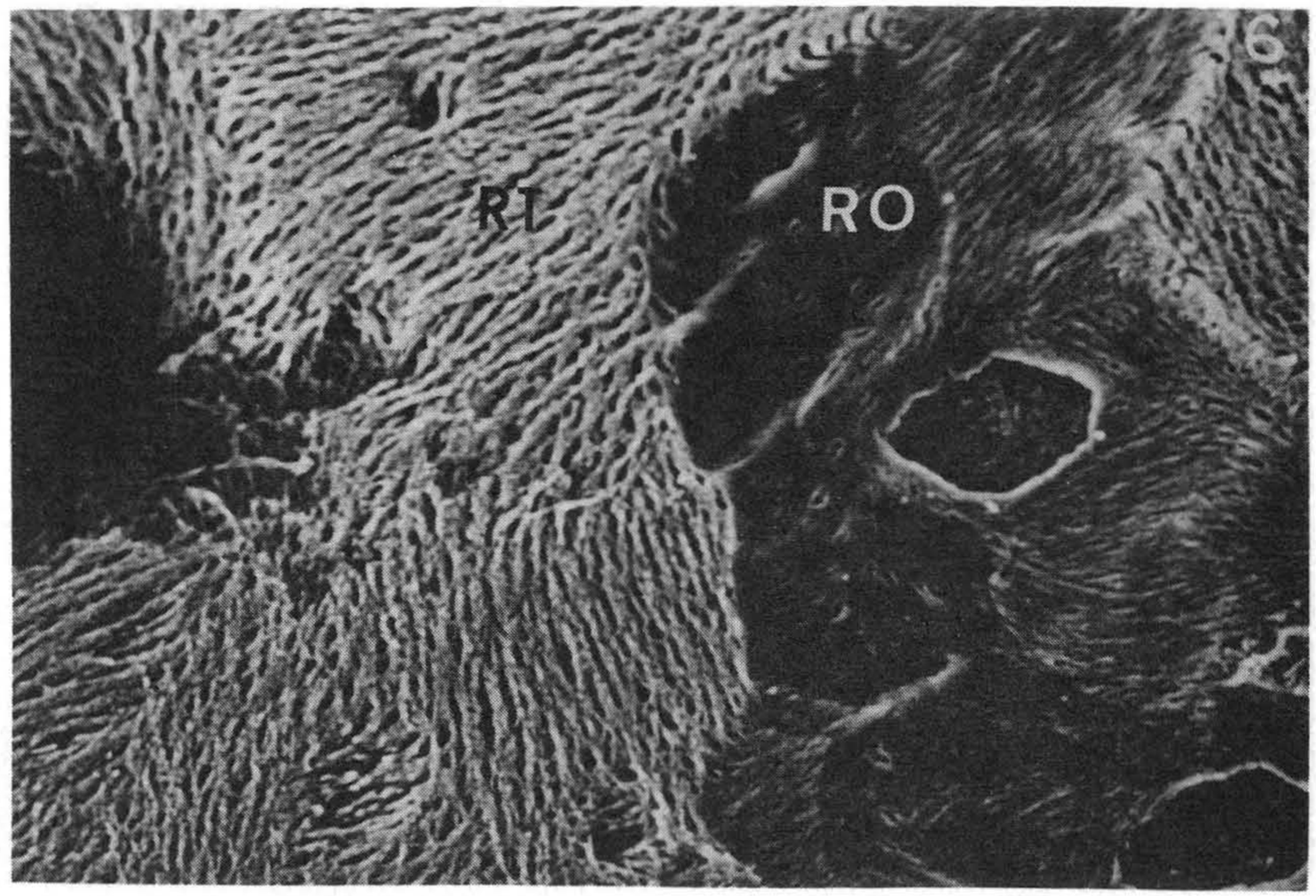
Fig. 4 Scanning electron micrograph of "fluoride-treated" cortical bone (periosteal surface) showing irregularly-arranged collagen bundles with rough texture. x2000.

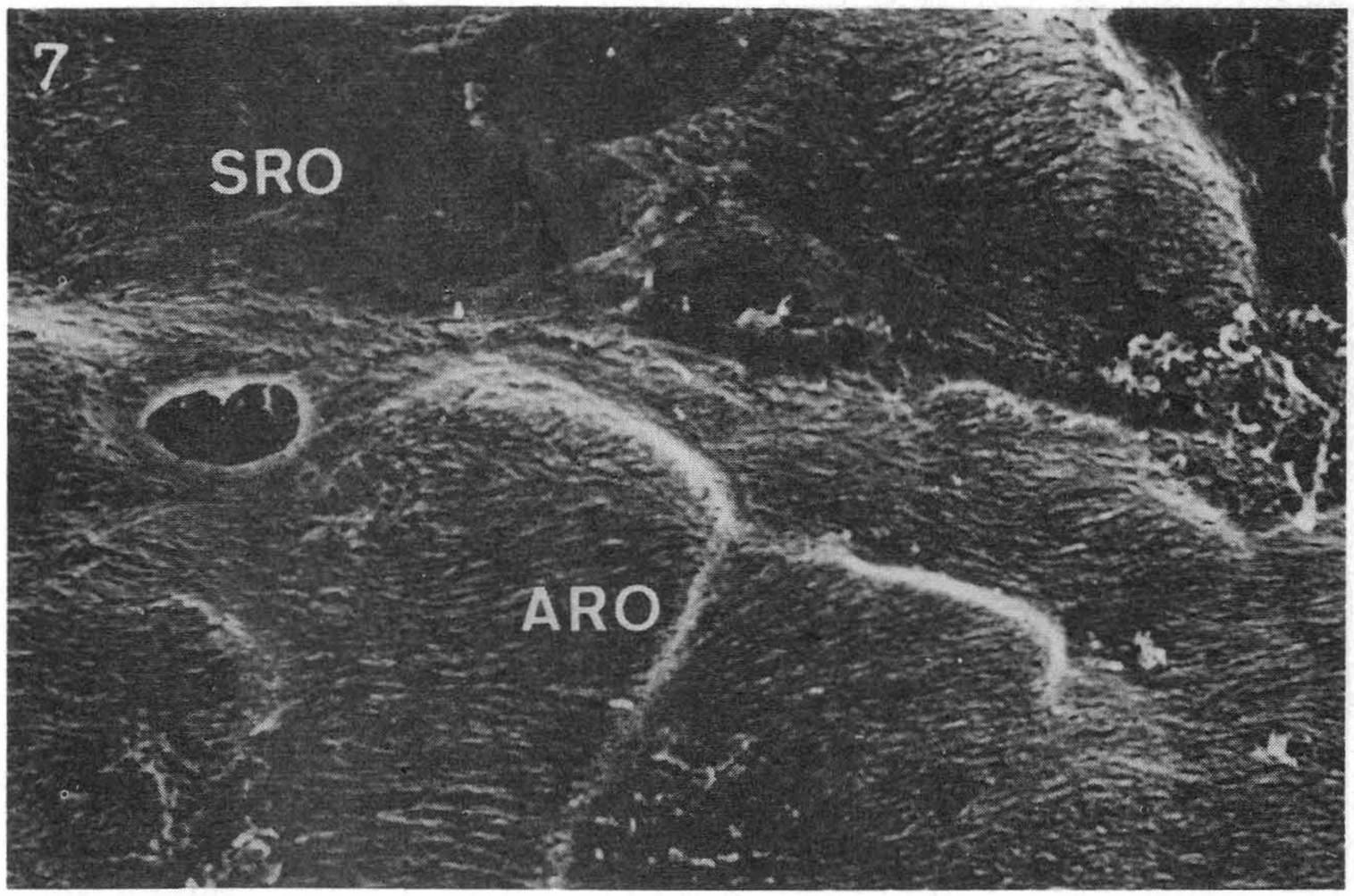


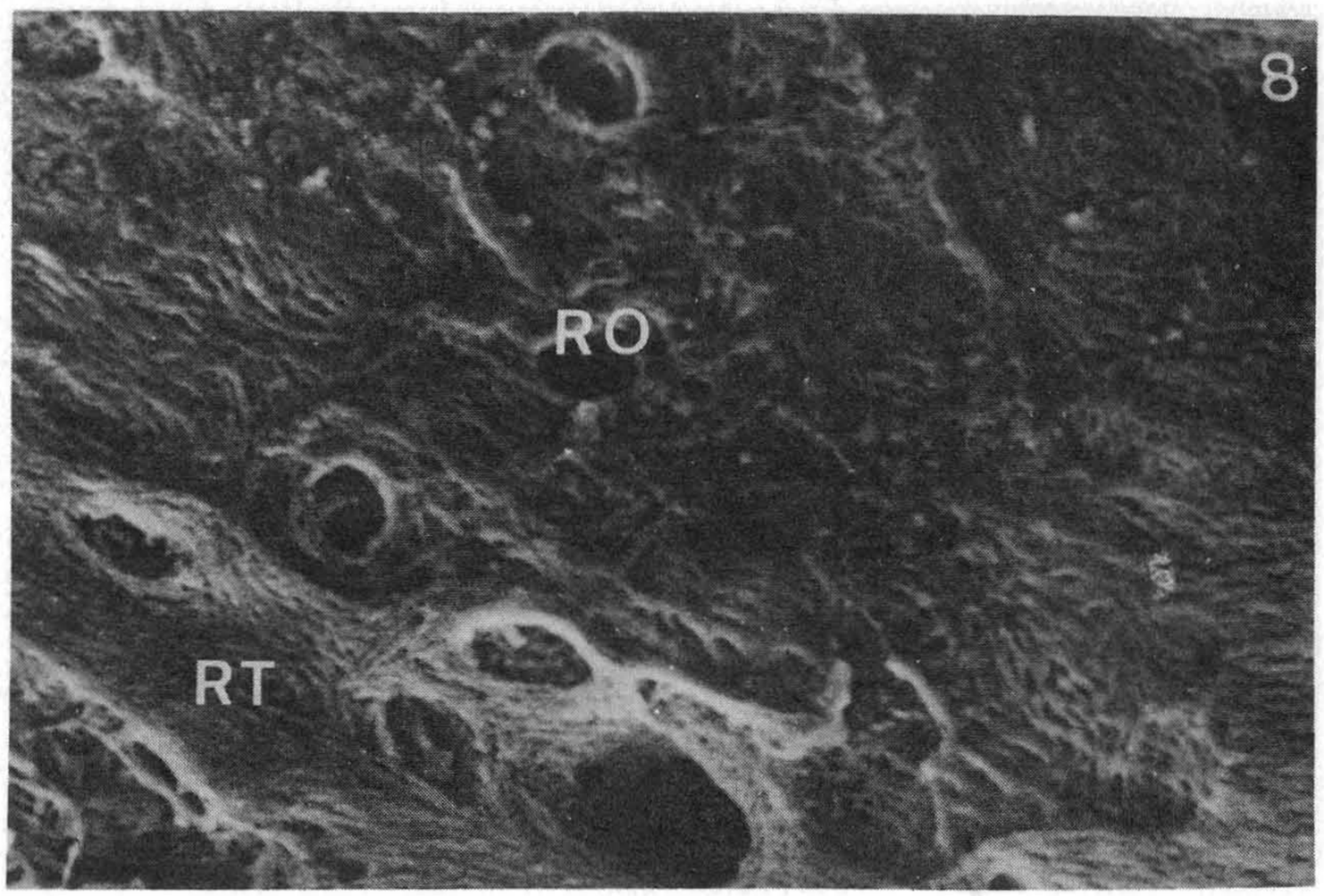





5


RT
RO


7
SRO
ARO


8
RO
RT

front in which mineral deposition on the collagen fibers has not yet proceeded to its limit (9). Another significant observation is that the collagen fibers are of smaller diameter as compared to the normal condition of smooth bundles of large diameter.

The endosteal surface of normal bone shows deep lacunae and sharp edges on the resting surface and in the actively resorbing areas (Fig. 6). Some areas in which resorption has ceased (or is proceeding very slowly) present shallow and rather poorly defined lacunae (Fig. 7). Again in the area of active resorption the skeleton of individual collagen fibrils is seen clearly. In areas in which resorption has ceased the surface tends to be very smooth, and it is therefore not possible to recognize collagen fiber orientation (Fig. 7). The endosteal surface of the bone from fluoride-treated animals is characterized by the obliteration of most of the resorbed areas with newly deposited collagen fibers which are irregularly oriented (Fig. 8).

Electron microprobe x-ray analysis revealed that the periosteal and endosteal surfaces of both normal bone and that from fluoride-treated animals generated an energy spectrum indicating that these regions are composed mainly of phosphorus ($K\alpha = 2.01$) and calcium ($K\alpha = 3.69$). The ratio of $CaK\alpha$ to $PK\alpha$ was determined for each sample, and it was found that the periosteal and endosteal surfaces of "fluoride-treated" cortical bone had a higher $CaK\alpha/PK\alpha$ ratio than the normal control bone (Table I).

Chemical analysis of cortical bone reveals a significant increase in calcium content after 8 months of fluoride treatment (Table II). Whereas the total phosphorus content of "fluoride-treated" cortical bone was not changed by the fluoride in the diet, the Ca/P ratio showed a higher value in "fluoride-treated" than in normal bone. These findings are in agreement with the electron microprobe x-ray analysis of endosteal and periosteal surfaces. Data on the fluoride content of bone showing a significant increase after 8 months of fluoride ingestion have already been reported by us (10).

Discussion

The unmineralized collagen fibers found in the resorbed areas may be due to the presence of high concentrations of glycosaminoglycans. The presence of high concentrations of sulphated glycosaminoglycans, which are potent inhibitors of mineralization, has been demonstrated in bone (9, 10, 11). Removal of glycosaminoglycans is a prerequisite for mineralization of collagen fibers. Hence the presence of high concentrations of glycosaminoglycans may be the reason for poorly mineralized collagen fibers.

The change in the morphology of the collagen fibers and the matrix may possibly be due to reduced cross-link precursors (12) of collagen and collagen biosynthesis (13). Hence reduced collagen cross-links and biosynthesis along with the significant increase in glycosaminoglycans may be one of the reasons for gross morphological changes in "fluoride-treated" bone. However, it is not clear whether these changes in bone surfaces are a compensation for the increased resorption rate induced by fluoride ingestion reported by Weinmann and Sicher (14), or are associated with a markedly increased osteoblastic activity similar

Fig. 5 Scanning electron micrograph of "flouride-treated" cortical bone (periosteal surface) showing irregularly-arranged collagen bundles with rough texture. x2000.

Fig. 6 Scanning electron micrograph of normal cortical bone (endosteal surface) showing both resorbing (RO) and resting (RT) areas. x1088.

Fig. 7 Scanning electron micrograph of normal cortical bone (endosteal surface) showing both actively resorbing area (ARO) and slowly resorbing area (SRO). x2300.

Fig. 8 Scanning electron micrograph of "fluoride-treated" cortical bone (endosteal surface) showing resting (RT) and newly laid down collagen fibers in resorbed areas (RO). x1088.

Table I *Results* of electron microprobe x-ray analysis of cortical bone from rabbits fed sodium fluoride and from untreated controls.*

	Endosteal Surface	Periosteal Surface
Control (5)**	1.67	1.72
Experimental (5)	2.07	1.80

* Values expressed are the ratio of Cakα/PKα

** Numbers in parenthesis indicate the number of experiments carried out.

Table II *Results* of chemical analysis of normal and "fluoride-treated" cortical bone.*

	Calcium Mean ± S.D.	Phosphorus Mean ± S.D.	Calcium / Phosphorus
Control (5)**	25.6 ± 1.6	10.2 ± 1.2	2.51
Experimental (5)	28.9 ± 2.3***	10.5 ± 1.5	2.75

* Data expressed as mg% of dry defatted bone. Numbers in parenthesis indicate the number of experiments carried out.

** $P < 0.05$ significantly differs from control.

to that described by Schenke *et al.* (15).

The increase in CaKα/PKα ratio observed by electron microprobe x-ray analysis of periosteal and endosteal surfaces, and in the Ca/P ratio observed by chemical analysis of whole bone powder, as an accompaniment to the increased fluoride concentration, is in agreement with previous studies (16,17). The increased Ca/P ratio can be explained by the fact that high fluoride concentration favours the transformation of amorphous calcium phosphate, which has a low Ca/P ratio, into crystalline apatite, which has a high ratio (16). Increase in crystal size may also be associated with an increased Ca/P ratio (18,19).

Although the enhanced Ca/P ratio indicate hypermineralization of the matrix, the results obtained from SEM studies suggest hypomineralization of the collagen fibers in animals which have ingested fluoride. This observed hypomineralization could be explained on the basis of the fact that the OH^- position in the crystal lattice is filled by F^- ion, with an equivalent amount of PO^3 ion escaping to balance the charge (20). Another possibility is that electrical neutrality is maintained by a loss of the hydrogen which in low Ca/P apatite is found as a bond between oxygens of adjacent PO_4 groups.

On the other hand, the mere presence of calcium fluoride is likely to cause the abnormally high Ca/P ratio found in "fluoride-treated bone", although the presence and the exact nature of the compound has not been established either by x-ray diffraction or by electron diffraction studies. It could however, exist as a calcium-rich organic complex with proteoglycans and glycosaminoglycans which have been shown to increase the bone of in fluoride-treated animals (10,11). Such a complex may be the explanation for the increase in Ca/P ratio found in the present investigation.

Acknowledgements

We wish to thank Professor C.N.R. Rao, F.R.S. for permitting us to use the EDAX facilities at the Materials Research Laboratory, Indian Institute of Science, Bangalore. We are grateful to Mr. G.N. Subbanna, Materials Research Laboratory, Bangalore, for his valuable guidance during the course of the investigation. We are grateful to Dr. T.C. Anand Kumar of the Experimental Biology Unit of the Department of Anatomy, AIIMS, for providing SEM facilities; to Dr. G.F.X. David, Department of Anatomy for the assistance offered during the course of the SEM studies, and to Mr. S.C. Sharma for his technical assistance.

This research work has been financed by the grants made available by the Department of Environment, Government of India and the International Development Research Centre, Canada.

References

(1) Malcolm A.S., Sterey E. *Osteofluorosis in the rabbit. Microradiographic studies.* Pathology, **3**, 39, 1971.

(2) Weatherell J.A., Weidmann S.M. *The skeletal changes of chronic experimental fluorosis;* J. Path. Bact., **78**, 233, 1954.

(3) Leslie R.A., Malcolm A.S., West V.C. Storey E. *Osteofluorosis in the rabbit*. Calcif. Tissue Res., **4**, 288, 1969.

(4) Malcolm A.S. *Some biophysical aspects of osteofluorosis. J. Dent. Res.* **48**, 1090, 1969.

(5)Baud C.A., Bang S. *Biophysical studies of the bone tissue of fluoride treated mice.* J. Dent. Res., **52**, 589, 1973.

(6) Singer L., Armstrong W.D., Zipkin I., Prazier P.D. *Chemical composition and structure of fluoride bone.* Clin. Orthop., **99**, 303, 1974.

(7) Parker H.E. *Magnesium, calcium and zinc in animal nutrition.* Atomic Abs. News Letter, **13**, 1, 1963.

(8) Fiske C.H., Subba Rao Y. *The colorimetric determination of phosphorus.* J. Biol. Chem., **66**, 375, 1925.

(9) Boyed A. *Scanning electron microscope studies of bone.* In: Bourn, G. H., ed. "Biochemistry and physiology of bone." Academic Press, New York and London, 1972, p. 265.

(10) Susheela A.K., Jha Mohan. *Effects of fluoride on glycosaminoglycan of cancellous and cortical bone of rabbits.* Experientia, **37**, 1097, 1981.

(11) Jha Mohan, Susheela A.K. *Characterization of glycosaminoglycan from normal and fluoride treated rabbit iliac crest.* Biochem. Biophys. Res. Commun., **105**, 711, 1982.

(12) Susheela A.K., Mukherjee D. *Fluoride poisoning and the effect on collagen biosynthesis of osseous and non-osseous tissues of rabbit.* Toxicol. Eur. Res. **3**, 99, 1981.

(13) Susheela A.K., Sharma Y.D. *Effect of fluoride on collagen cross-link precursors of rabbit tissue.* IRCS Med. Sci. Biochem., **9**, 862, 1981.

(14) Weimann J.P., Sicher H. In : Bone and bones, fundamentals of bone biology", 2nd ed. C.V. Marby, St. Lousi, 1955, pp. 301–307.

(15) Schenk R.K., Merz W.A., Reutler F.W. *Fluoride in osteoporosis.* In : "Fluoride in medicine". Vienna, 1970, p. 153.

(16) West V.C. *Observations on phase transformations of a precipitated calcium phosphate*. Calcif. Tissue Res., I, 212, 1971.

(17) West V.C *The* in vivo *effect of fluoride and tetra-cycline on phase transformation of a calciumj phosphate*. Calcif. Tissue Res., **9** 207, 1972.

(18) Posner A.S., Eanes E.D., Harper R.A., Zipkin I. *X-ray diffraction analysis of the effect of fluoride on human bone apatite.* Arch. Oral Biol., **8**, 549, 1963.

(19) Eanes E.D., Zipkin I., Harper R.A. Posner A.S. *Small angle x-ray diffraction analysis of the effect of fluoride on human bone apatit.* Arch. Oral Biol., **10**, 161, 1965.

(20) Amberg C.H., Luk H.C., Wagstoff P.K. *The fluoridation of nonstoichiometric hydroxyapatite. An infrared study.* Can. J. Chem., **52**, 4001, 1974.