From: The Chemical Laboratory of the Roya
Veterinary and Agricultural College
(Head: Professor A. Tovborg Jensen
Ph. D.) and the Royal Dental College
Copenhagen, Denmark

AN X-RAY DIFFRACTION STUDY OF OSSEOUS AND DENTAL TISSUES FROM RATS WITH EXPERIMENTAL CHRONIC FLUOROSIS[1]

by
Gertrud Lindemann

In the literature dealing with histological changes in teeth and bones in chronic experimental and spontaneous fluorosis, in man and in animals, it is frequently stated that a certain proportion of the calcium phosphate content of the hard tissue is replaced by calcium fluoride.

This view is based upon the fact that on histological examination, fluorotic bones and teeth often prove to contain a number of peculiar granules, which assume a deep blue colour in conventional hematoxylin-eosin staining. These dark blue granules, the "fluoride granules", are regarded as calcium fluoride deposits.

The first statement that calcium fluoride had been found was made by *Brandl & Tappeiner* (1891), who inferred from a crystallographic examination of the bones of an experimental dog that the luminescent grains found in undecalcified osseous tissue consisted of calcium fluoride. Since then no other authors have described a phenomenon quite identical with that reported by *Brandl & Tappeiner*. On the other hand, many investigators have found "fluoride granules" in decalcified osseous and dental tissue. *Westin* (1935) and *Bauer* (1945) consider it probable that the "fluoride granules" are actually composed of calcium

[1] This work was supported by a grant from Evers and Co., and published in the Danish language in Odontologisk Tidskrift *64*, 1956: 17—29.

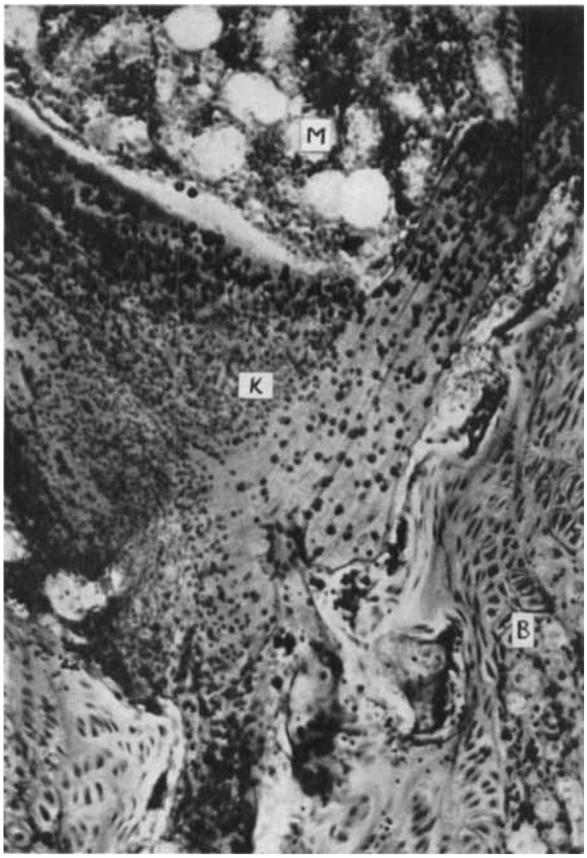

Fig. 1. "Fluoride granules" in bone and cartilage. Section through a decalcified rat femur. K = bone, B = cartilage, M = bone marrow.

fluoride, while *Sutro* (1935) and *Kellner* (1939) do not commit themselves with regard to the chemical nature of the granules. *Roholm* (1937) found these granules both in animals and in man and examined samples of bone tissue by crystallographic methods. He obtained no indication of the presence of calcium fluoride. In 1938 the University of Leipzig published a number of theses on the examination of fluoride-poisoned animals with a view to ascertaining the possible presence of calcium fluoride in osseous, dental, and soft tissues. The results of this teamwork were summarized by *Hiller* (1938), who found, using dark field microscopy, some glistening particles in liver and kidney tissue.

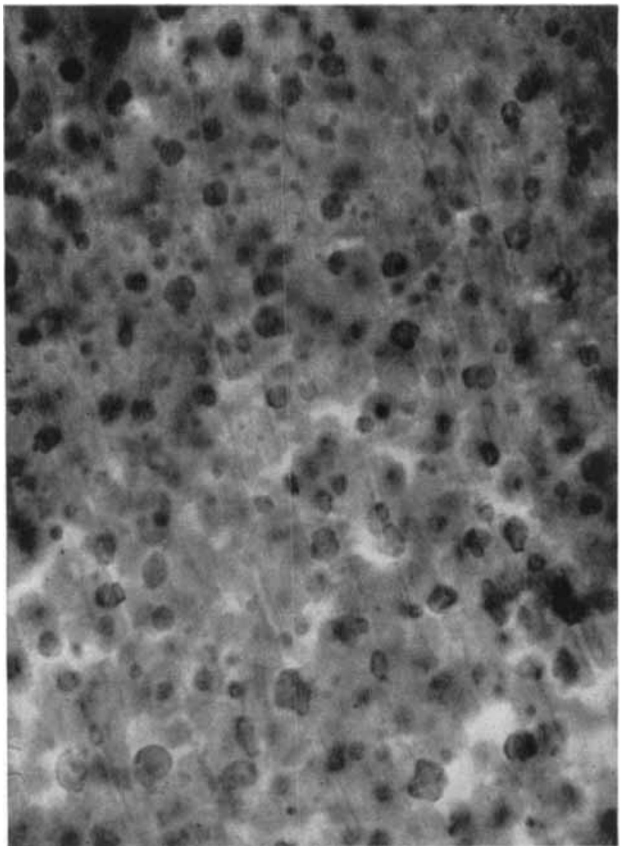

Fig. 2. "Fluoride granules" in dentine. Section through a decalcified rat incisor.

Hiller claims to have proved the presence of calcium fluoride in these tissues.

Pande (1945) detected similar granules in dentine and in bone tissue from spontaneously fluoride-poisoned rats, but did not discuss the problem of their composition.

Euler & Eichler (1942) felt that the granules do not contain calcium fluoride since they were able to produce such granules by feeding their experimental animals an organic fluorine compound which is not, according to the statements of these authors, metabolized to form fluoride ions. In their opinion the granules represent merely a pathological calcification.

Eichler (1950) considered the fluoride granules in enamel to be the result of a disturbance in the formation of the enamel matrix caused by some action affecting the cells involved.

In an experimental study of regeneration of osseous and dental tissues after chronic fluorosis the present writer (1950) found these granules in greater or lesser quantities in decalcified tissues from some of the experimental animals, particularly in those from rats which were continually given sodium fluoride in their diets, see Figs. 1 and 2.

On the basis of this finding and in view of the disagreement in the literature concerning the nature of the granules, an investigation of the latter was considered useful. X-ray diffraction analysis seemed to be a suitable tool for this purpose and was therefore preferred. As evidenced by the literature very few X-ray diffraction studies of similar material had been undertaken previously.

PREVIOUS X-RAY DIFFRACTION STUDIES

In 1938 a study was published by *Reynolds, Corrigan, Hayden, Macy* and *Hunscher,* who had examined dental and osseous tissue from normal and from fluorotic rats. They arrived at the conclusion that all enamel from normal rats showed apatite structure, but that it could not be concluded that the material was hydroxyapatite. Comparison of the diagrams from fluorotic *teeth* with that of calcium fluoride showed that probably calcium fluoride had been deposited. On the other hand, unashed *bone* from normal rats yielded a diagram identical with that of unashed bone from rats which had received a diet containing 0.3 per cent NaF.

Geroulds (1945) used the electron microscope for a study of the mechanism of deposition of fluorine in teeth. He states that when fluorotic enamel and dentine are etched with hydrochloric acid a new fine type of background structure appears in the electron microscope. This suggest an extraordinarily fine dispersion of a new mineral phase, which is more resistant to hydrochloric acid. Theoretically it seems probable that a new mineral phase arising in fluorotic teeth will be either calcium fluoride or fluorapatite. *Geroulds* compared the dissolution rates in hydrochloric acid of these two minerals with that of ordinary hydroxyapatite, see Table 1.

Table 1
(After Geroulds, 1945).

Loss in weight of calcium hydroxyapatite, calcium fluorapatite, and calcium fluoride after treatment with N/1 HCl for 2 hours.

Calcium hydroxyapatite	80 % loss in weight
Calcium fluorapatite	56 % loss in weight
Calcium fluoride	Negligible loss in weight

A severely fluorotic tooth was then examined by means of electron diffraction (surface polished and etched, and electron beam reflected from this surface).

In these studies a strong apatite pattern was found by *Geroulds*. No trace of calcium fluoride could be detected. If there had been a trace of calcium fluoride, the lines originating from this practically insoluble mineral should have been very strong *Geroulds* concluded.

Brandenberger & Schintz (1945) studied a considerable number of bones from normal animals and humans and from animals and humans with various bone diseases. With regard to the results of their examination of fluorotic rabbit bones with the aid of X-ray diffraction, these two investigators write as follows (p. 44):

"The bones of rabbits fed fluorine compounds, after ignition at 950° C. show certain differences in interference patterns in the range of very large scattering angles, as compared with the X-ray diagram of ignited, normal rabbit bone. No differences can be ascertained between non-ignited normal bones and non-ignited bones of rabbits fed fluorine compounds. The observation in the case of the ignited bones of a somewhat changed interference pattern may possibly indicate the formation of mixed crystals of the hydroxyapatite originally present, with fluorapatite. This is supported in particular by the perfect identity of the X-ray diagrams of ignited bones from fluorotic rabbits with that of an ignited shark tooth (these are known to have a comparatively high fluorine content). It should be stressed, however, that the diagrams of the bones in question (including that of the shark tooth) are in general far more similar to the diagram of hydroxyapatite than to that of fluorapatite. Moreover, it remains an open question whether this incorporation of a few fluorine atoms in place of OH groups in the lattice of hydroxyapatite has already taken place in the living bone, or whether merely an *adsorption* of fluoride occurs. In the latter case it would be a secondary process induced by the ignition, when the increased mobility of the lattice elements at the higher temperature permits the incorporation of the F atoms".

THE WRITER'S X-RAY DIFFRACTION STUDIES

Working Hypothesis.

The starting point of this study was the hypothesis that calcium fluoride may be formed in fluorotic bone. The problem then was to attempt to identify this substance in fluorotic teeth and bones. At present, a vast majority of investigators regard the mineral phase of osseous and dental tissue as consisting mainly of hydroxyapatite. If in addition suitable quantities of calcium fluoride are present, the X-ray diagrams obtained will be hydroxyapatite diagrams with superimposed calcium fluoride lines.

As mentioned elsewhere (*Lindemann* 1956) the limiting amount of a single crystal component which can be identified in a mixture, varies considerably from one kind of crystal to another, and in some cases it is not even a constant for the same kind of crystal. A knowledge of this limiting amount and of the variation in its dependence on the other components of the mixture is, therefore, indispensable in ascertaining the presence or the absence of a given kind of crystal.

Apparatus and Methods.

The camera used was a 19 cm Bradley camera with a rectangular slit. Cobalt K_α radiation, $\lambda = 1.78890$ Å, was used. Filter: 0.017 mm iron foil. Ilfex double coated film was exposed for ca. 6 hours. The camera was kept filled with hydrogen in order to minimize the exposure time. Only powder specimens were used. The powder was fixed with Canada balsam to a glass fiber, which was then placed at the center of the camera and was rotated throughout the exposure. The voltage applied to the

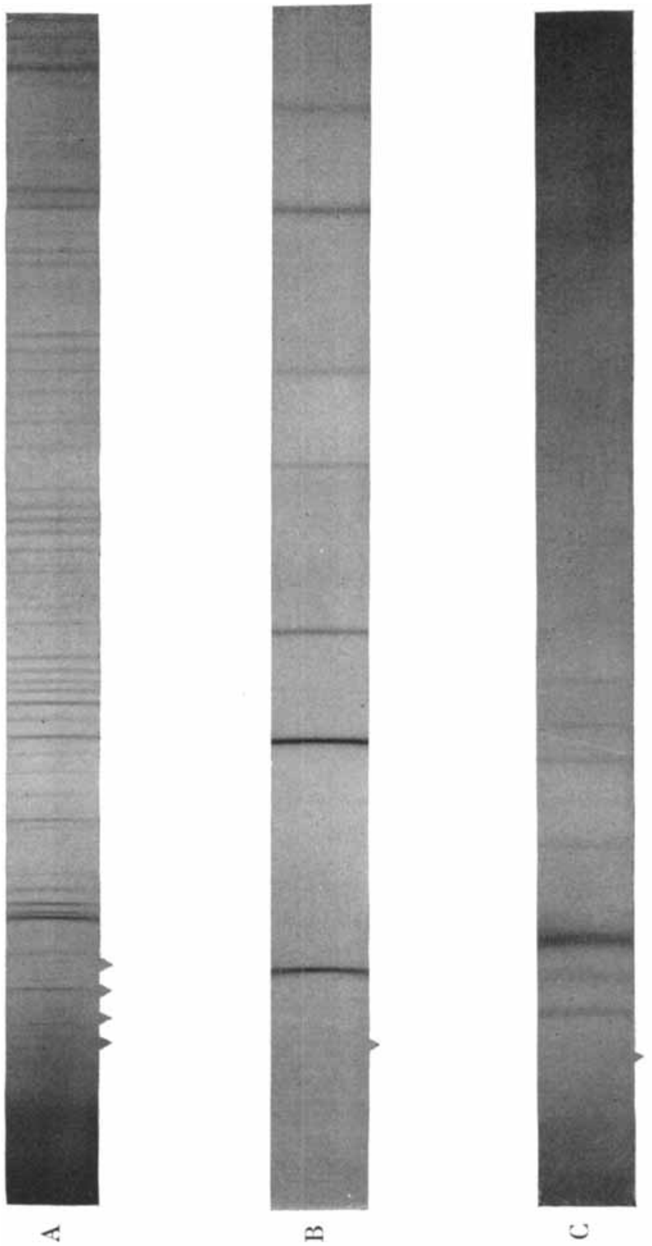

Fig. 3. X-ray diffraction diagrams given by: A, hydroxyapatite; B, Calcium fluoride; C, normal, non-ignited bone powder.

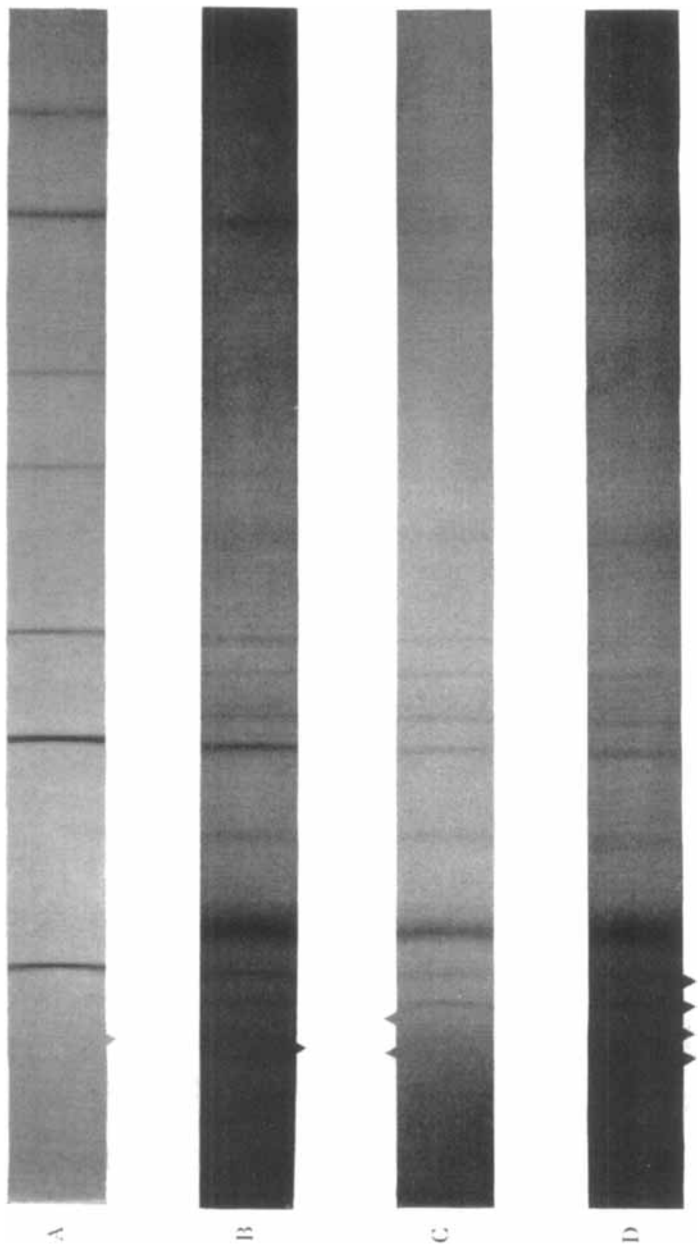

Fig. 4. X-ray diffraction diagrams given by: A, calcium fluoride; B, normal non-ignited bone powder and 5% calcium fluoride; C, normal non-ignited bone powder and 2% calcium fluoride; D, normal non-ignited bone powder and 1% calcium fluoride.

X-ray tube was 30 kV constant potential and the current 15 mA. In the computations of lattice spacings corrections were introduced both for the thickness of the specimen (absorption) and for the shrinkage of the film during development, fixation, and drying.

Material.

Pure calcium fluoride and recrystallized synthetic hydroxyapatite were available. X-ray diagrams were taken of both of these powders (Figs. 3 A and 3 B). The lattice spacings were calculated from the lines of the calcium fluoride diagram. They were found to agree well with those given in the card index published by the American Society for X-ray and Electron Diffraction.

Bone tissue was procured in the following manner: From three rats which had been kept on a stock diet[1], osseous tissue was taken from various parts (see Table 2). It was fixed in a 4 per cent formaldehyde solution. The bones were dissected free of muscle and tendon, were dried at 37° C., and were ground in a metal grinding mill. The powder was sifted through a metal sieve with a mesh of ca. 0.7 mm. Fat was extracted by shaking with ether for ten to fifteen minutes. Mixtures containing 5 %, 2 % and 1 % calcium fluoride were prepared by grinding 10 mg of CaF_2 with 190 mg of normal bone powder (Table 2). Aliquots of this mixture were further diluted with bone powder. The mixtures were ground finer still in an agate mortar.

Table 2

The origin of the bone powder used for X-ray diagrams of normal bone.

Rat No.	Sex	Weight/grams	Age/days	Type of bone
Fl 269 c ...	♀	228	512	tibia
Fl 269 c ...	♂	360	535	tibia
Fl 269 f ...	♀	370	512	tibia, humerus, radius, and scapula

[1] Stock diet No. 8 of the firm A/S Medicinalco, composed of: Dry skimmilk powder, 35 %; rye meal, 35 %; wheat bran, 11 %; dry yeast 8 %; arachis oil, 11 %; shark liver oil, 3000 I. U. Vitamin A per kg.

X-Ray Diagrams of the Mixture.

Fig. 3 C shows an X-ray diagram of normal, non-ignited bone powder. Fig. 4 B shows the diagram of the 5 % mixture, Fig. 4 C that of the 2 %, and Fig. 4 D that of the 1 % mixture. Inspection of the original films proves that a content of 2 % CaF_2 in bone powder is easily recognized. It is even possible to detect a content of 1 %, but with some difficulty.

A PRELIMINARY EXAMINATION OF FLUOROTIC RATS

So far hard tissues from three fluorotic rats have been examined. All of these rats had been kept on the above mentioned stock diet No. 8 to which 0.05 % NaF was added. They all showed marked histological changes in their bone tissue as well as in their dental tissues. (*Lund* 1950). Table 3 shows the data of the animals used. Table 4 shows from where the hard tissues were taken.

Table 3

Data of the fluoride poisoned rats used in the present study.

No. and sex	Diet	Age in days at start of exp.	Exp. period in days	Weight at start of exp.	Weight at end of exp.	Total F dose in mg ca.
Fl 189 ♂	Stock diet + 0.05 % NaF.	67	103	165	241	372
Fl 194 ♂		67	131	163	250	515
Fl 196 ♂		67	201	153	219	597

Table 4

Sources of hard tissue examined.

Fl 189	Enamel + dentine of incisors
Fl 194	Alveolar bone, dentine + enamel, and femur
Fl 196	Alveolar bone

The result of the examination

None of the X-ray diagrams indicated the presence of calcium fluoride. Since it was possible to demonstrate the presence of 1 % calcium fluoride added artificially, ½ % calcium fluoride was added to the bone powder of one of the fluoride-poisoned rats. If from ½ to 1 % calcium fluoride were present, this addition of a further half per cent would increase the total content to 1 % or more, which it should be possible to detect. However, the diagram thus obtained was in no way different from the diagram of normal bone powder.

DISCUSSION

The method employed permitted detection of one or even one half per cent of calcium fluoride in bone powder. However, no calcium fluoride was found in bone powder from rats that had been kept for 103 to 201 days on a diet containing 0.05 % NaF.

In the introductory review of the literature reference has been made to several authors who have conducted similar studies. *Reynolds, Corrigan, Hayden, Macy & Hunscher* (1938) found calcium fluoride. They found it in rat incisors, but not in bone tissue. They conclude that because the incisors grow continuously they will reflect pathological conditions more readily. It should be noted, however, that the granules which some histologists consider to be composed of calcium fluoride, are found in osseous as well as in dental tissue. If these granules do consist of calcium fluoride it should be possible to detect them in X-ray diagrams of bone tissue. Moreover, these authors state that they have observed only one line that can be attributed to calcium fluoride while the remaining calcium fluoride lines may have been masked by apatite lines. In view of this, and of the difficulties inherent in exact determination of the positions of the lines, the present writer does not feel convinced that calcium fluoride was actually present in the incisors examined.

On the other hand, the writer does not wish, on the basis of this preliminary study, to express any definite opinion concerning the possibility of deposition of calcium fluoride in osseous and dental tissue after ingestion of large doses of NaF. It is conceivable that at more sensitive method involving *e.g.* strictly monochromatic radiation might lower the limiting proportion considerably and thus make it possible to demonstrate the presence of CaF_2 in osseous and dental tissue from fluoride poisoned rats.

SUMMARY

To detect calcium fluoride in bones and teeth of rats with chronic fluorosis the powder method was employed with iron filtered cobalt Kα radiation. In this way it proved possible to detect calcium fluoride which had been added to bone tissue in the proportion of 1 per cent. Upon examination of teeth and bone tissue from rats kept for 103 to 201 days on a diet containing 0.05 per cent sodium fluoride, no calcium fluoride was detected. The method was modified so that it would have been possible to detect ½ per cent calcium fluoride, but still none was found. It is suggested that it might be possible to detect smaller quantities if a more sensitive method could be devised, *e.g.* by employment of absolutely monochromatic radiation.

RÉSUMÉ

L'EXAMEN RADIOCRISTALLOGRAPHIQUE DE TISSU OSSEUX ET DENTAIRE FLUOROTIQUE PROVENANT DE RATS

La méthode des poudres, avec une anode de Cobolt donnant des rayons K_α a permis de déceler une quantité de 1 % de fluorure de calcium ajoutée artificiellement à du tissu osseux. Une méthode modifiée permettrait de déceler une proportion de ½ % de fluorure de calcium.

L'examen de tissu osseux et dentaire fluorotique provenant de rats soumis pendant une période de 103 à 201 jours à un régime contenant 0.05 % de fluorure de sodium, n'a pas permis de déceler la présence de fluorure de calcium. L'auteur indique qu'il est possible qu'une méthode plus sensible, comme par exemple l'emploi de rayons absolument monochromatiques, permette de déceler des quantités encore plus petites.

ZUSAMMENFASSUNG

RÖNTGENOGRAPHISCHE KRISTALLUNTERSUCHUNGEN DES KNOCHEN- UND ZAHNGEWEBES VON RATTEN MIT EXPERIMENTELLER CHRONISCHER FLUOROSE

In der Literatur über chronische Fluorose ist nicht geklärt, ob im Knochen- und Zahngewebe ein Teil des Calciumphosphates durch Calciumfluorid ersetzt wird. Bei der experimentellen Fluorose findet man im Zahn- und Knochengewebe eigentüm-

liche Körnchen, die sich im histologischen Schnitt mit Hämatoxylin-Eosin dunkelblau verfärben. Mehrere Forscher meinen, dass diese Körnchen aus Calciumfluorid bestehen. Verf. hat diese Körnchen ebenfalls in entkalkten Schnitten von Knochen und Zähnen von Ratten gefunden, deren Kost 0,05 % NaF hinzugesetzt gewesen war.

Es wird über den Versuch berichtet, im pulverisierten Hartgewebe dieser Tiere Calciumfluorid durch röntgenographische Kristalluntersuchungen nachzuweisen. Mit Hilfe der Kα-Strahlung einer Kobaltanode liess sich Calciumfluorid, das normalen Knochenpulver zugesetzt war, bis zur Konzentration von 1 % nachweisen. Im Hartgewebe obengenannter fluorotischer Ratten fand sich mit dieser Methode kein Calciumfluorid, und das Röntgendiagramm blieb auch unverändert nach Zusatz von ½ % Calciumfluorid, woraus geschlossen wird, dass im Hartgewebe der fluorvergifteten Ratten jedenfalls nicht mehr als ½ % Calciumfluorid vorhanden sein kann.

Durch Verfeinerung der Methode, insbesondere durch Anwendung absolut monochromatischer Strahlung, wird es vielleicht möglich sein, noch geringere Mengen als ½ % Calciumfluorid nachzuweisen.

ACKNOWLEDGEMENTS

The author is greatly indebted to *Erik Jacobsen*, M.D., the Biological Laboratory, Medicinalco Ltd., Copenhagen and to professor *A. Tovborg Jensen*, Ph.D. and *Marianne Danö*, Research Associate, the Chemical Laboratory, the Royal Veterinary and Agricultural College, Copenhagen.

REFERENCES

Bauer, W. H.: Experimental Chronic Fluorine Intoxication: Effect on Bones and Teeth. Am. Journ. Orthod. and Oral Surgery *31*, 1945: 700—719.

Brandenberger, E. & H. R. Schinz: Über die Natur der Verkalkungen bei Mensch und Tier und das Verhalten der anorganischen Knochensubstanz im Falle der hauptsächlichen menschlichen Knochenkrankheiten. Helv.Med.Acta *12*, 1948: Supplementum 16, 1—16.

Brandl, J. & H. Tappeiner: Über die Ablagerungen von Fluorverbindungen im Organismus nach Fütterung mit Fluornatrium. Zeitschr. f. Biol. *28*, 1891: 518—538.

Eichler, O.: Karies und Fluor. Zahn-, Mund- u. Kieferheilk. i. Vortr. Heft *4,* 1950: 135—143.

Euler, H. & O. Eichler: Über die Wirkung von Fluor in organischer Bindung auf das Zahnsystem der Ratte. Arch. exp. Path. u. Pharmakol. *199,* 1942: 179—187.

Geroulds, C. H.: Electron Microscope Study of the Mechanism of Fluorine Deposition in Teeth. Journ. Dent. Res. *24,* 1945: 223—234.

Hiller, G.: Calciumfluoridablagerungen in Organen bei experimenteller Fluorvergiftung. Inaug. Diss. Leipzig 1938.

Lindemann, G.: En orientering i røntgenkrystallografi. Odontologisk Tidskrift *64,* 1956: 1—16.

Lund, G.: Recovery from Experimental Chronic Fluorosis in Rats. Paper read in Helsinki 1950, in London 1952 (XIth International Dental Congress).

Pande, P. G.: Dental and Osseous Changes in Spontaneous Fluorosis in Rats. Ind. Journ. Med. Res. *33,* 1945: 121—128.

Reynolds, L., K. E. Corrigan, H. S. Hayden, I. G. Macy & H. A. Hunscher: Diffraction Studies of Sodium Fluoride and Parathormon upon the Incisors and Tibiae of Rats. Am. Journ. Roentgenol. *39,* 1938: 103—117.

Roholm, K.: Fluorine Intoxication. 185—187, 220—224, 239—240, 249—253, Disp. København 1937. Nyt nordisk forlag, Arnold Busck.

Sutro, C. J.: Changes in the Teeth and Bones in Chronic Fluoride Poisoning. Arch. Path. *19,* 1935: 159—173.

Westin, G.: Die Fluorose der Kiefer und Zähne. Svenska Tandläkaresällskapets Festskrift 1935: 289—333.

Address: *The Royal Dental College,*
4, Universitetsparken,
Copenhagen Ø,
Denmark.