THE EFFECTS OF FLUORIDE FEEDING ON THE ORGANIC MATRIX OF BONES AND TEETH OF PIGS AS OBSERVED BY AUTORADIOGRAPHY AFTER IN VITRO UPTAKE OF CA^{45} AND S^{35}

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Plates 171 and 172

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INTRODUCTION

Demineralized sections of normal bones of different species of mammals, including man, show a preferential uptake of Ca^{45} at the level of the epiphyseal plate after *in vitro* incubation in a solution of $Ca^{45}Cl_2$. Though demineralized sections of prebone and predentine also take up Ca^{45} under similar circumstances, they do so to a considerably lesser extent (Bélanger (1)). By this technique, age differences of animals can be ascertained when tracheal cartilage is used. Sections of the costochondral junctions from rachitic infants differ from similar sections from normal infants. In all instances the Ca^{45} uptake appears to be related to the ground substance and possibly to its mucopolysaccharide content. In contrast to the results with Ca^{45} are the results when a dilute solution of $H_2S^{35}O_4$ is used in which the S^{35}-sulfate is bound by the perichondrium and the organic matrix of bone and dentine. So far this uptake of S^{35}-sulfate is unexplained (Bélanger, Burke, Tremblay, and Bélanger (2)).

The results of similar experiments on the hard tissues from pigs fed fluoride are reported here.

Materials and Techniques

In this study, tissues from 12 Hampshire male castrate pigs, 6 weeks of age and averaging 65 lb. were used (Visek *et al.*, 1954[1]). The animals, which had been raised on a normal farm ration, were paired and one member of each pair was placed on a diet containing 1000 p.p.m. sodium fluoride; thereafter the animals were pair fed so as to ensure equal intakes except, of course, for fluoride. After 30 days of feeding, each animal was injected intravenously with 6 mc. of $S^{35}O_4$. Pairs were sacrificed at 10 minutes, 5 hours, 24 hours, 10 days, 30 days, and 60 days afterwards.

[1] Visek, W. J., *et al.*, UT—AEC Agricultural Research Program, Semi-Annual Report, December, 1954.

The teeth were removed with portions of the jaw. The tissues of the first three pairs were quick frozen and then fixed in a mixture of 1 part neutral formaldehyde and 3 parts 95 per cent ethanol. The tissues of the 10, 30, and 60 days animals were fixed without previous freezing. All the teeth were shipped to Ottawa in the fixative for further processing and study. Ribs and portions of long bones consisting of the head of metatarsals were fixed in formaldehyde, demineralized in nitric acid, and sectioned in paraffin blocks.

The incisors and molars were demineralized in a mixture of formic acid and sodium citrate of a pH of 4.9 (Greep, Fischer, and Morse (3)) and were then cut at 10 μ in low viscosity nitrocellulose by the technique of C. Bélanger (Boyd (4)). Paraffin-embedded specimens of bones demineralized in 5 per cent nitric acid were obtained from Oak Ridge at a later date. These were also sectioned at 10 μ. At the time of the presently reported utilization of all this material, the residual S^{35} introduced *in vivo* had decayed to insignificant levels as could be ascertained by the Geiger-Mueller counter and control autoradiographs.

Deparaffinized sections of the bones and celloidin-embedded sections of the teeth were soaked for periods of 1 to 3 hours in a solution of $Ca^{45}Cl_2$ (enriched, United States Atomic Energy Commission) of approximately 10 μc./ml. (Bélanger (1)). After washing in several baths of distilled water, the sections were dehydrated in graded ethanol, coated with 1 per cent celloidin, and covered with fluid photographic emulsion in order to obtain integrated autoradiographs (Bélanger and Leblond (5); Bélanger (6), (7)).

For purposes of comparison, other sections were soaked in a bath of carrier-free $H_2S^{35}O_4$ (Atomic Energy of Canada Ltd.) of similar radioactive concentration and autoradiographed.

OBSERVATIONS

Autoradiography of in Vitro Uptake of Ca^{45}.—In the long and short bones of endochondral origin, the cartilage of the epiphyseal plate in the control animals (Text-fig. 1) showed a relatively strong autoradiographic picture. The Ca^{45} uptake at that level appeared exaggerated in the fluorinated animals. This condition was difficult to ascertain in view of the dense picture in all instances.

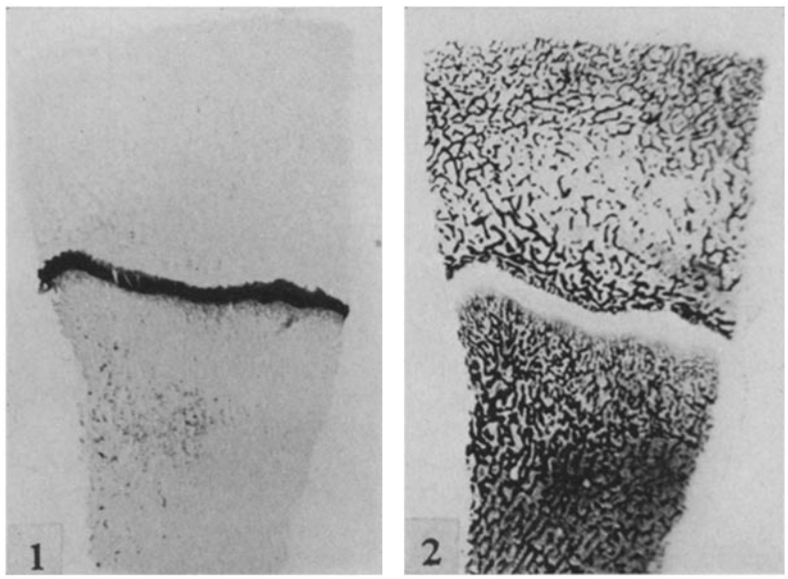
The cartilaginous cores of the bone spicules produced also an autoradiographic record of Ca^{45} (Text-fig. 1).

Considerably longer exposures were required to obtain pictures at the level of *bone* and *tooth* tissue. In the control teeth, the strongest reaction was found over predentine. Images of lesser intensity were recorded at the level of precementum, at the dentino-cementum junction and over the growth lines. Normal bones showed only a weak picture at the outer border of the trabeculae (prebone).

The fluorinated teeth at the 30 day stage (Fig. 1) have shown the localizations described above and also strong spot reactions over the globular masses. The autoradiographic reaction over predentine was stronger than that of controls. This condition was more evident in the younger portion of the tooth, at the root (Fig. 2) where the autoradiographic picture followed the scalloped image of predentine and where the globular masses were more abundant. In this area, concentrations of Ca^{45} were also recorded over the distended tubules (Fig. 2, *DT*).

In the long bones, after prolonged exposures, very little Ca^{45} uptake was recorded except over the globular masses (Fig. 3). The alveolar bone along with the teeth were fixed in formaldehyde-ethanol, thus retaining better than the above mentioned specimens, its polysaccharide content. This better treated bone produced an autoradiographic picture over prebone of formative areas and the adjacent periosteal tissue in fluorinated animals (Fig. 4).

Autoradiography of in Vitro Uptake of Radiosulfate.—It could be argued that the Ca^{45} uptake reported above was the result of a non-specific absorptive property of the tissues involved. In order to test this hypothesis, comparable



TEXT-FIGS. 1 and 2. Two adjacent sections from a demineralized metatarsal soaked in dilute $Ca^{45}Cl_2$ (Text-fig. 1) or $H_2S^{35}O_4$ (Text-fig. 2) and autoradiographed (× 3). The radiocalcium is located in cartilage, while the radiosulfate is present in the bone.

sections were soaked in a weak solution of $H_2S^{35}O_4$ instead of the calcium chloride. A glance at Text-figs. 1 and 2 will show that the autoradiographic image of S^{35} uptake (Text-fig. 2) seems to be practically the opposite of the image recorded after Ca^{45} (Text-fig. 1): the epiphyseal plate is negative and the spicules are all intensely positive. The over-all Geiger counts were very high after radiosulfate treatment as compared to those of the Ca^{45} series.

Regional differences in the long bones have also been observed. At the periosteal surface of the normal bones (Fig. 6), ideally exposed autoradiographs have revealed the presence of S^{35} over the outer periosteum while the inner periosteum (cambium) was apparently negative. The newly formed bone then appeared weakly positive at first and progressively more active as the organic substance of the bone matured. Autoradiographs of the bone growing on top of the epiphyseal plate have revealed a similar progressive concentration of radioactive material accompanying maturation (Fig. 7): the central trabecular reaction, weak near the plate, has become progressively more intense away from the formative surface.

Comparative studies of normal bone and bone formed under the influence of fluorine have revealed remarkable differences: the modified organic matrix has picked up considerably more radiosulfate (Fig. 8). The radioactive material was diffusely distributed throughout the trabeculae. Wherever globular masses were present, they produced a more intense record than that of the diffuse trabecular distribution.

The teeth showed comparable differences. While normal dentine has shown practically no uptake of radiosulfate, fluorine-modified dentine has provided a diffuse image of radiosulfate uptake, over which the globular masses could be recognized as spots of concentrated radioactivity (Fig. 5).

DISCUSSION

A conclusion that can be drawn from these experiments is that specific constituents in the demineralized sections have an affinity for Ca^{45} and different but also specific constituents have an affinity for S^{35}-sulfate. Calcium-45 for instance has already been shown to deposit preferentially in cartilage (Bélanger (1)). In the present material, this property has again been observed (Text-fig. 1) in both normal and fluorinated cartilage. It has also been reported that chondroitin sulfate might be, in cartilage, the chemical entity responsible for retaining *in situ* the radioactive calcium, since the autoradiographic picture after treatment with that isotope, coincides with the metachromasia and with the distribution of radiosulfate after *in vivo* incorporation; also because the radiocalcium could be removed from the section by incubation with hyaluronidase.

With these data in mind, it is interesting to observe that in the present series of experiments, Ca^{45} has been localized in predentine, precementum (Figs. 1 and 2) and prebone (Fig. 4), all areas that also concentrate radiosulfate *in vivo* (Bélanger, Visek, Lotz, and Comar (8); Bélanger (2)). Such is also the rate of radiosulfate synthesis in the same condition (Bélanger, Visek, Lotz, and Comar (8)). The neonatal line and the weaning line, areas of low content of mineral salts (id), have shown a specific Ca^{45} affinity (Fig. 1). This feature along with the intense metachromasia of these bands seems to indicate a large concentration of chondroitin sulfate at that level.

The globular masses have also shown a relatively high radiocalcium uptake (Figs. 1 to 3). Since, however, it has been previously postulated that these masses, on account of their solubilities (id), might be aggregates of CaF_2, it is possible that the Ca^{45} has been deposited in this case by the mechanism of atomic interchange.

The affinity of the globular masses for radiosulfate (Fig. 5) remains totally unexplained. The affinity of radiosulfate for organic bone tissue is intriguing but so far also unexplained. The fact that it is progressively increasing at the periosteal (Fig. 6) and at the epiphyseal (Fig. 7) areas of growth, and also the definite centro-trabecular concentration of radioactivity (Figs. 6 and 7) are indications that the uptake of radiosulfate is a function of maturation in normal bone tissue. On the other hand, while the fibrous outer periosteum (Fig. 6, *OP*) shows an uptake of S^{35}, the inner region of the cambium (Fig. 6, *C*) is negative. So also is the cartilage of the epiphyseal plate (Text-fig. 2). Furthermore, it appears that the organic matrix of bone and dentine formed under the influence of fluorine, becomes progressively depleted of polysaccharides (id). Under these conditions, the tissues of fluorinated animals show a high S^{35} *in vitro* uptake (Figs. 5 and 8). All these facts seem to indicate that the *in vitro* uptake of radiosulfur is inversely proportional to the concentration of sulfated polysaccharides. The Ca^{45} appears to be directly proportional under similar conditions, to the chondroitin sulfate content of the tissues. Consequently differences encountered in the present experiments between fluorinated animals and controls could well represent differences in the concentration of that substance. The present data would indicate an accumulation of the sulfated polysaccharide fraction in non-mineralized portions of cartilage, bone, dentine, and cementum, either by hyperproduction of this substance in fluorosis, or retarded utilization or both. A previous series of histological, histochemical, and autoradiographic studies of *in vivo* uptake of sulfate (Bélanger, Visek, Lotz, and Comar (8)) shows increased synthesis and apparently decreased utilization.

The present observations of *in vitro* uptake of radioactive sulfate cannot yet be ascribed to a specific chemical entity. However, they demonstrate considerable differences between fluorinated tissues and the controls. When these results are compared with that of classical staining such as the staining of mucus by the Masson technique and toluidine blue metachromasia, it appears that an increase of radiosulfate uptake *in vitro* is concomitant with a decrease of mucus content and a decrease of metachromasia (Bélanger, Visek, Lotz, and Comar (8)).

Finally, the results herein described show that the autoradiographic method when coupled with the *in vitro* uptake of isotopes can be of value to the histologist in studies of normal and pathological material. This method is, of course, available for the study of human material as well as for experimental pathology.

SUMMARY

Demineralized sections of fluorinated bones and teeth have been studied by autoradiography following *in vitro* uptake of Ca^{45} or $S^{35}O_4$.

The portions of tissue which do not become mineralized (cartilage, prebone, predentine, and precementum) show an increased Ca^{45} uptake apparently related to an increase in chondroitin sulfate content in fluorosis.

The tissues from the fluoride-fed animals show an increase of *in vitro* uptake of sulfur in the tissues which become mineralized (bone, dentine, cementum).

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REFERENCES

1. Bélanger, L. F., Autoradiography visualization of Ca^{45} intake by normal and pathological cartilage *in vitro*, *Proc. Soc. Exp. Biol. and Med.*, 1955, **88,** 150.
2. Bélanger, L. F., Burke, P., Tremblay, P. C., and Bélanger, C. L., Autoradiographic visualization of calcium intake *in vitro* by sulfopolysaccharides present in cartilage and arteries, *J. Histochem. and Cytochem.*, 1954, **2,** 467.
3. Greep, R. O., Fischer, C. J., and Morse, A., Alkaline phosphatase in odontogenesis and osteogenesis and its histochemical demonstration after demineralization, *J. Am. Dent. Assn.*, 1948, **36,** 427.
4. Boyd, G., Autoradiography in Biology and Medicine, New York, Academic Press Inc., 1955.
5. Bélanger, L. F., and Leblond, C. P., A method for locating radioactive elements in tissues by covering histological sections with a photographic emulsion, *Endocrinology*, 1946, **36,** 8.
6. Bélanger, L. F., A method for routine detection of radiophosphates and other radioactive compounds in tissues. The inverted autograph, *Anat. Rec.*, 1950, **107,** 149.
7. Bélanger, L. F., Improvements to the melted emulsion technique of autoradiography, *Nature*, 1952, **170,** 625.
8. Bélanger, L. F., Visek, W. J., Lotz, W. E., and Comar, C. L., Autoradiographic visualization of the effects of fluoride feeding on radiosulfate incorporation and turnover in the bones and teeth of pigs, data to be published.

PLATES

EXPLANATION OF PLATES

PLATE 171

FIG. 1. Autoradiograph of a demineralized section of the upper root portion of an incisor from a pig on fluorine for 30 days. The section was soaked in weak $Ca^{45}Cl_2$ for 2 hours. The radiocalcium appears to have been retained by predentine (*PD*), the globular masses, a band which might represent both the weaning line (*W*) and the neonatal line. There are weaker reactions at the dentino-cementum junction (*DC*) and at the outer border of cementum (*PC*). Integrated autoradiograph × 57.

FIG. 2. Autoradiograph of Ca^{45} uptake by the region of the root tip of an incisor of pig fed fluorine for 30 days. The radiocalcium is localized to the wide, scalloped predentine, to the globular masses and to the distended dentinal tubules (*DT*). Integrated autoradiograph × 57.

FIG. 3. Autoradiograph of the periosteal portion of a demineralized bone of a 30 day fluorine pig, after *in vitro* uptake of Ca^{45}. Only the globular masses are positive. Integrated autoradiograph × 57.

FIG. 4. Autoradiograph of the newly grown alveolar bone of a fluorinated pig after *in vitro* Ca^{45} uptake. The radiocalcium is restricted to prebone (osteoid) and the immediately adjacent connective tissue. Integrated autoradiograph × 57.

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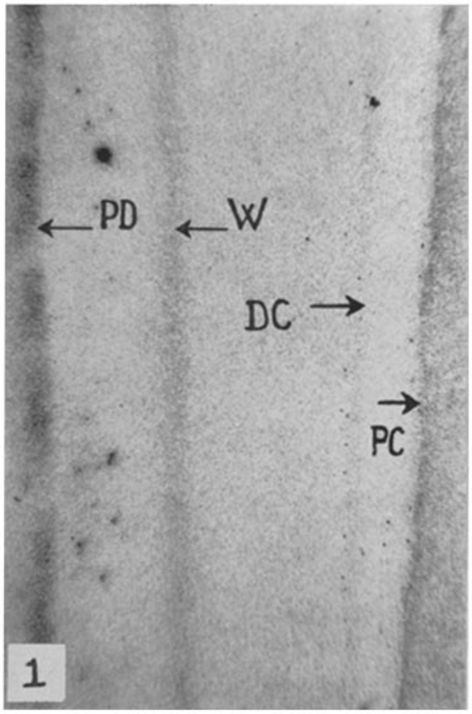

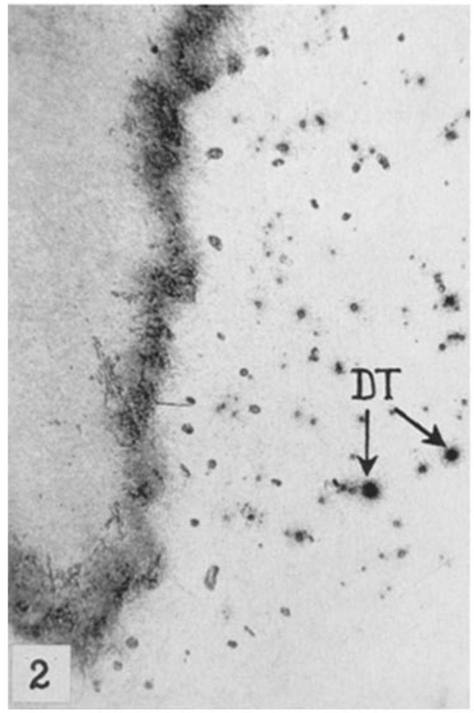

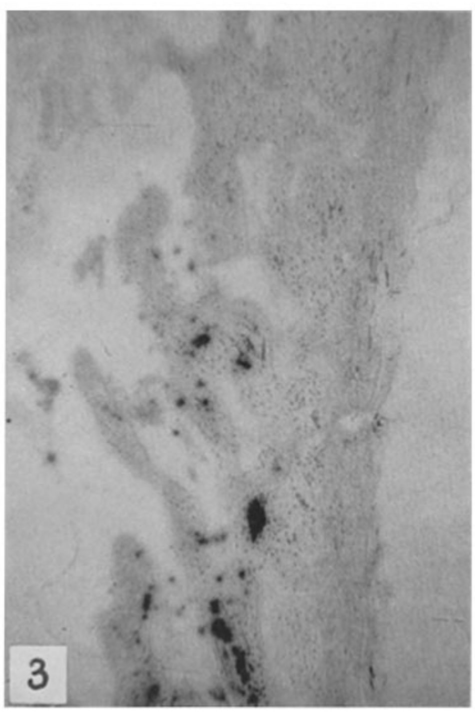

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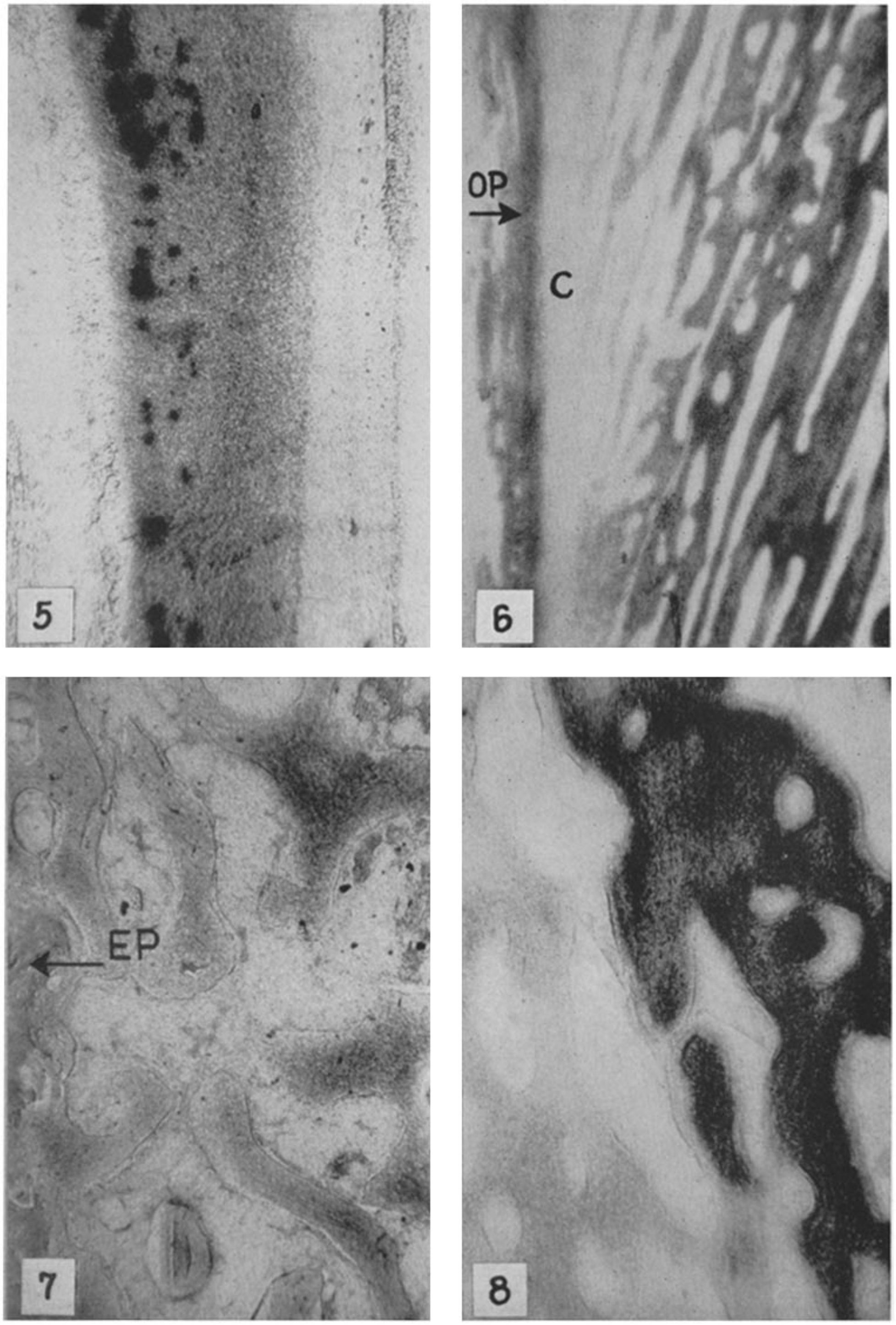
Plate 172

Fig. 5. Autoradiograph of a demineralized section of dentine after *in vitro* treatment with a weak solution of $H_2S^{35}O_4$. The radiosulfur appears localized to the modified portion of organic dentine and to the globular bodies. Integrated autoradiograph × 57.

Fig. 6. Autoradiograph of radiosulfate uptake at the site of periosteal bone growth in a demineralized section of a normal long bone of pig. There has been apparently selective uptake by the outer periosteum (*OP*) while the inner cambium (*C*) is negative. The actual bone tissue shows uptake proportional to maturation. Integrated autoradiograph × 57.

Fig. 7. Autoradiograph of S^{35} *in vitro* uptake by a demineralized section of the epiphysis of a normal pig. From the epiphyseal plate (*EP*) upwards (on the right), the bone trabeculae show progressive uptake and also centro-trabecular localization of the radioactive element. Integrated autoradiograph × 112.

Fig. 8. Autoradiograph of *in vitro* uptake of S^{35} by the alveolar bone of a fluorinated pig. The newly formed modified bone on the right shows a much greater uptake as compared to the bone formed previous to fluorine feeding (on the left). Integrated autoradiograph × 112.



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