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STUDIES ON MINERALIZED DENTAL TISSUES

X. A microradiographic, autoradiographic and histochemical investigation on dental hard tissues in dogs with experimentally produced vitamin D deficiency.

by

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INTRODUCTION

Where there is an insufficient supply of vitamin D disturbances occur in the mineral metabolism, and hence in the processes of mineralization in the hard tissues. The phenomenon is most pronounced in the young growing subject. After supply of vitamin D the changes in the bone tissue may disappear as a result of the continuous process of rebuilding. In the hard tissues of the teeth, on the other hand, the damage is permanent, since the enamel, the dentine and the cementum once formed are not being rebuilt (*Westin* 1926 a, b).

The dentine is particularly sensitive to disturbances in the vitamin D supply, and long before clinical symptoms of deficiency are evident, the dentine reacts with widening of the predentine zone. The phenomenon of widening of the predentine zone in vitamin D deficiency has been ascribed to abnormally slow differentiation of the odontoblastema. (*Wilton* 1937).

Under normal conditions the odontoblasts build an organic matrix — the predentine — in which calcium salts are precipitated in the form of small globuli which grow by concentric deposition and coalesce to form uniformly mineralized dentine. The odontoblastic processes in the dentine, however, remain unmineralized. The precipitation of the mineral occurs to within

a short distance of the pulp, which is thus lined by no more than a thin layer of predentine.

In vitamin D deficiency the coalescence of the globules is incomplete and there remain areas of unmineralized matrix — the interglobular dentine. When supply of the vitamin is renewed normal dentine is formed on the pulpal side of that which is defectively developed.

The effects on the enamel vary from minor disturbances of mineralization to large hypoplastic defects. This has been established by most workers. *Schour* and *Massler* (1945), however, are of the opinion that the enamel formation is not directly affected by the lack of vitamin D but that other factors are responsible for these defects.

The significance to the dental organ of vitamin D supply has been demonstrated mainly in animal experiments. *May Mellanby* (1929) found pronounced dental defects in the teeth of young dogs fed a diet deficient in vitamin D. A number of other animals have also been used in similar experiments (*Leicester*, 1949). Dental defects similar to those found in animals have also been found in man.

There are still many unsolved problems in connection with the development and mineralization of hard tissues during deficiency of vitamin D. One reason for this is that ordinary histologic techniques give incomplete and limited information when applied to mineralized tissues. Recent years, however, have seen important development and refinement of biophysical methods, and their application to the study of the dental hard tissues has yielded interesting results. The purpose of this investigation is thus to study the defects in the mineralized dental tissues of dogs fed a diet deficient in vitamin D, the essential of the method being the application of autoradiographic (S^{35} and Ca^{45}) and high resolution microradiographic techniques. The results of the autoradiographic study were correlated with the degree of mineralization of the structures in question and these were also studied by histochemical techniques.

MATERIAL AND METHOD

A litter of six pups of a large mixed breed were at the age of seven weeks placed in two spacious cages in a room without

daylight. In one cage were four animals fed a diet deficient in vitamin D (*Mellanby*, 1929). After one month each of the animals was given about 25 cubic centimetres of milk daily for four days as they had fallen ill probably as a result of the experiment. In the second cage were the two other pups. They received the experimental diet together with ten drops of (about 3,000 I.U.) Calciferol daily.

After seven weeks 10 millicurie carrier-free $\text{Na}_2\text{S}^{35}\text{O}_4$ were injected intravenously in one animal from each cage. These animals were sacrificed four days after the injection.

After ten weeks two of the dogs fed the deficient diet (the last three weeks these dogs were also given milk, 15 cc daily) and the second control dog received an intravenous injection of 1.25 mc $\text{Ca}^{45}\text{Cl}_2$. Four days later the dogs were sacrificed.

One dog received the experimental diet for seven weeks, after which vitamin D was supplied daily, the animal then being moved to the control cage. After ten weeks an intravenous injection of 10 mc $\text{Na}_2\text{S}^{35}\text{O}_4$ was given. Four days later the dog was sacrificed.

The upper and lower jaws were taken from all the animals and the soft tissues removed. The mandibles were cut through the median line, and both halves radiographed. The left halves were fixed in absolute alcohol and the right halves as well as the upper jaws in 10 per cent neutral buffered formalin (*Lillie*, 1948).

Ground sections of the left halves of the mandibles were prepared. The halves were cut with a rotating diamond saw into pieces which after complete dehydration were embedded in methylmethacrylate. With the diamond saw the embedded preparations were then cut into slices, about 400 μ in thickness, which were ground to 50—100 μ sections (*Hammarlund-Essler* 1955).

The sections were placed between two sheets of Ilfex x-ray film for autoradiographic study. To ensure good contact between the section and the emulsion they were placed between plane brass plates 0.5 cm in thickness. Some of the sections were covered with stripping film (Kodak Scientific Plate).

The films were developed in Kodak D 19B after an exposure

of 2 to 3 months. After fixing, washing and drying in dust-free air the autoradiograms were mounted on microscope slides in the same way as histologic preparations. The autoradiograms were enlarged by microphotography on Kodak B 40 plates developed in Kodak D 72.

After autoradiography microradiographic examination of the sections was performed, using a Machlett OEG 50 x-ray tube with a tungsten anode and a 0.2 mm beryllium window as the ray-source. The tube was run at 24 kV and 10 mA. With the radiation so obtained at least 97.5 per cent of the absorption was due to the mineral salts in the section. The focus-emulsion distance was 10 centimetres, and for a 100 μ section the geometric unsharpness is approximately 1 μ . The microradiograms were recorded on Eastman Kodak Spectroscopic Plate 649 and developed in Kodak KD 50 for three minutes. As a protection of the emulsion cover glasses were mounted on the microradiograms. These were enlarged by microphotography on Kodak 0 250 plates.

The histologic examination was performed on parts of the right halves of the mandibles and of tooth germs from the upper jaws. The lower right halves were cut in the same way as the left and the pieces decalcified in 5 per cent trichloroacetic acid, embedded in paraffin and cut to give 5—7 μ sections. The germs from the upper jaws were removed and cut with a thin rotating diamond saw into 200 μ slices which without embedding were ground to 20—30 μ sections. The following stains were used on the decalcified and ground sections: Haematoxylin-eosin, Hansen, Bock, Azan, thionin, toluidin blue and PAS reaction.

RESULTS

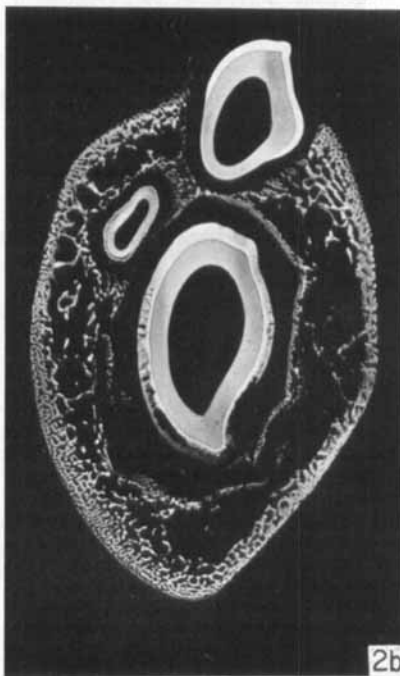
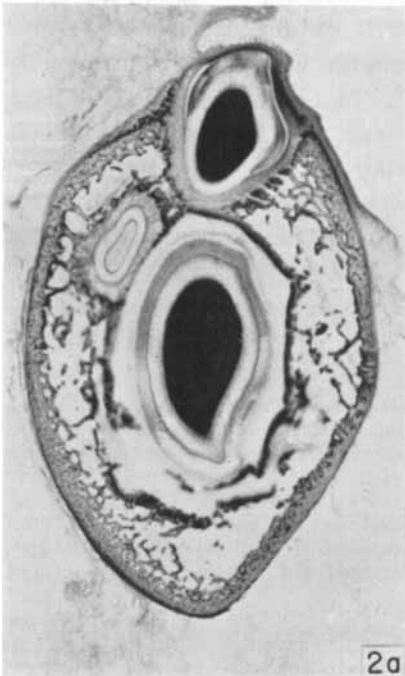
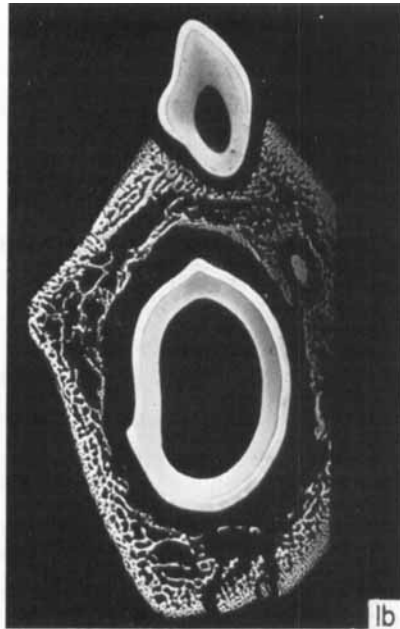
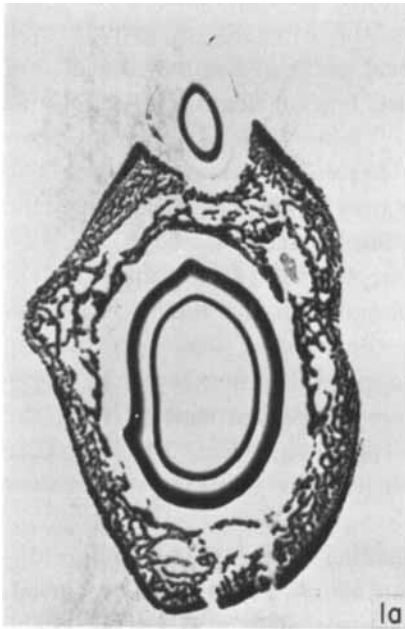
In the microradiographic examination of the ground sections from the jaws of the dogs on the diet deficient in vitamin D various pictures of the disturbances in mineralization were

Fig. 1 a. Autoradiogram of 100 μ ground section from the mandible of a control dog injected with $\text{Ca}^{45}\text{Cl}_2$. The first premolar, deciduous and permanent canines. $\times 4.5$.

Fig. 1b. Microradiogram of the same section as 1a.

Fig. 2 a. Autoradiogram of 100 μ ground section from the mandible of a control dog injected with $\text{Na}_2\text{S}^{35}\text{O}_4$. First premolar, deciduous and permanent canines. $\times 4.5$.

Fig. 2b. Microradiogram of same section as 2a. $\times 4.5$.



found in the teeth that had developed during the course of the experiment. In the regions where the intensity of growth was greatest — for example, the cervical parts of the carnassial, and the permanent canines — there was a great deal of interglobular dentine (Figs. 3 b, 5 b, 10). The globuli were of different sizes but appeared to have the same degree of mineralization. The latter was not thought to be poorer than that of the dentine formed prior to the beginning of the experiment (Figs. 5 b, 10). The inferiorly mineralized contour lines of Owen appeared in the more slowly growing occlusal parts of the teeth (Figs. 3 b, 7 b, 9). In the lower carnassial the dentine developed during the experiment over the pulp tip appeared to be normal in structure but to have a considerably lower mineral content than the dentine formed prior to the experiment (Fig. 7 b). Further cervically in the same tooth interglobular dentine was formed (Fig. 8 b).

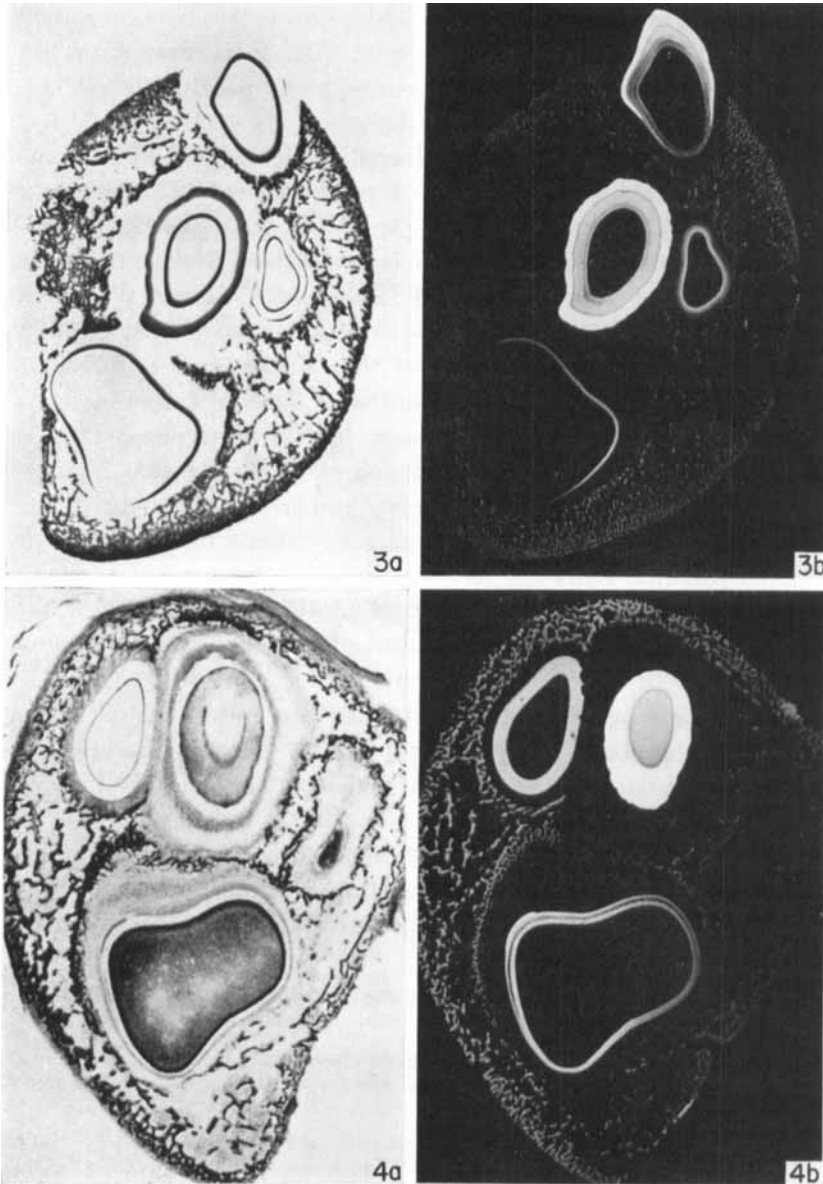
The enamel defects varied according to the stage of development of the enamel at the beginning of the deficiency period. The least evidence of change was the poorly mineralized striae of Retzius (Fig. 10). In some teeth the surface of the enamel was uneven and pitted, but the enamel was well and uniformly mineralized (Figs. 3 b, 4 b, 7 b). In other teeth there was a poorly mineralized superficial zone with hypoplastic defects (Fig. 5 b). Circular defects following the striae of Retzius showing little, if any, mineral content were observed in the teeth of the animals later given vitamin D with the experimental diet (Fig.

Fig. 3a. Autoradiogram of 100 μ ground section from the mandible, of a "deficient" animal injected with $Ca^{45}Cl_2$. First premolar, permanent and deciduous canines, permanent third incisor. $\times 4.5$.

Fig. 3b. Microradiogram of the same section as in Fig. 3a. The lines of Owen in the crown dentine and the interglobular dentine in the root of the first premolar. The lines of Owen in the permanent canine the enamel of which has an uneven surface. $\times 4.5$.

Fig. 4a. Autoradiogram of a 100 μ ground section from a dog, which first had a deficiency of vitamin D and then received the vitamin in the diet. Intravenous injection of $Na_2S^{35}O_4$. Permanent and deciduous canines, first and third permanent incisors. $\times 4.5$.

Fig. 4b. Microradiogram of the same section as Fig. 4a. Resorption cavities in the deciduous canine. Interglobular dentine in the third permanent incisor. $\times 4.5$.



9). Evidence of healing was observed in both the enamel and the dentine in the regions which developed when the animals received milk with the experimental diet. It is seen from the microradiograms that the jaw bones were poorly mineralized in the animals fed the deficient diet.

The microradiograms of the ground sections through the jaws of the control animals revealed no disturbed mineralization (Figs. 1 b, 2 b), the pictures being in accord with those of normal animals (*Engfeldt, Bergman and Hammarlund-Essler, 1954*). In the dentine of the control and "deficient" animals a further observation was made. The microradiograms of transverse sections from the canines showed that the dentine adjacent to the enamel had a peculiar distribution of mineral salts (Fig. 10). In this area of the dentine are seen highly mineralized streaks alternating with areas of low degree of mineralization forming a comblike structure. These highly mineralized structures are about 5μ in width and are running perpendicular to the dentino-enamel junction reaching 20–30 μ into the dentine. This region contains many branching and anastomotic dentinal tubules and has been said to resemble the granular layer of Tomes (*Mellanby, 1929*).

The autoradiographic examination of the uptake in the dental tissues of the control dogs of Ca^{45} and S^{35} supplied in vivo has given results similar to those obtained in an earlier study of normal dog teeth. The Ca^{45} was taken up at the dentine — pre-dentine junction, while the rest of the dentine showed no evidence of the isotope. In the tooth germs the Ca^{45} was found either in the superficial enamel or throughout its thickness, depending on the stage of development (Fig. 1 a).

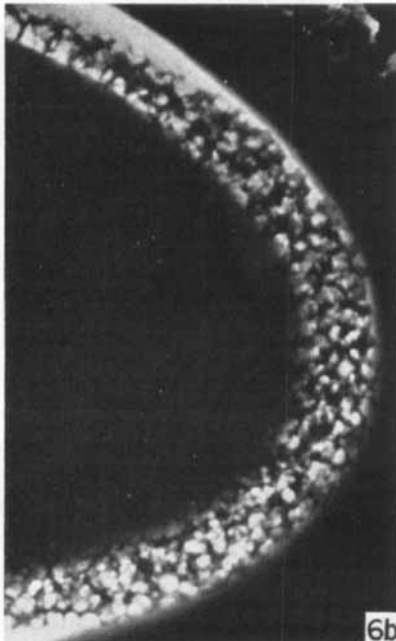
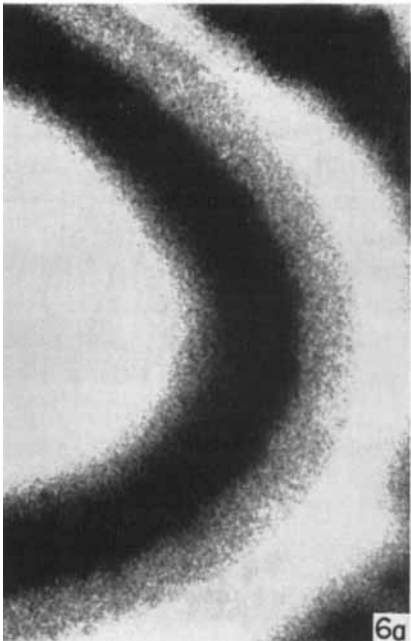
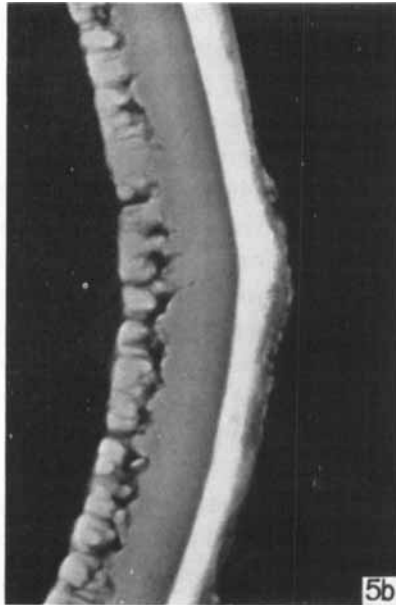
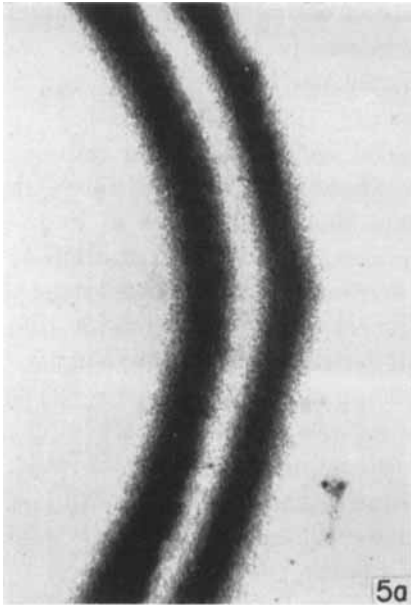
The uptake of S^{35} was pronounced in the pre-dentine zone.

Fig. 5a. Autoradiogram from a permanent lower canine of a dog with vitamin D deficiency. Uptake of Ca^{45} in a wide zone with irregular boundary with the normal dentine. $\times 50$.

Fig. 5b. Microradiogram of the same area as in Fig. 5a. Low and irregular mineralization of the outer enamel and interglobular dentine. $\times 50$.

Fig. 6a. Autoradiogram from the root of the first permanent molar from the same dog as Fig. 3. Uptake of Ca^{45} in the areas of interglobular dentine. $\times 50$.

Fig. 6b. Microradiogram of the same regions as in Fig. 6a, showing a great deal of interglobular dentine. $\times 50$.



There was also evidence of the radiosulphur in the pulpal tissue of the unerupted teeth. The uptake of S^{35} in the dentino-enamel junction — mentioned in the previous paper — was also revealed, and in the rest of the enamel the isotope was found at the same sites as the Ca^{45} (Fig. 2 a).

The autoradiograms of the ground sections from the animals fed the deficient diet show on the whole a marked concentration of radioactive isotopes at the same sites in the jaws as in the control animals. There are exceptions, however. In addition to the normal uptake of Ca^{45} at the dentine-pretentine border there was also some reaction in the adjacent parts of the dentine that were impaired through the vitamin deficiency (Figs. 3 a, 5 a, 6 a). The boundary between the zone of uptake of the defective dentine and of the normal, older dentine was an irregular wavy line.

The impaired dentine shows also an uptake of S^{35} . A weak reaction is visible within the dentine region of low mineral salt content (Fig. 7 a), and the regions between the calcium salt globuli have a small but definite uptake (Figs. 4 a, 8 a).

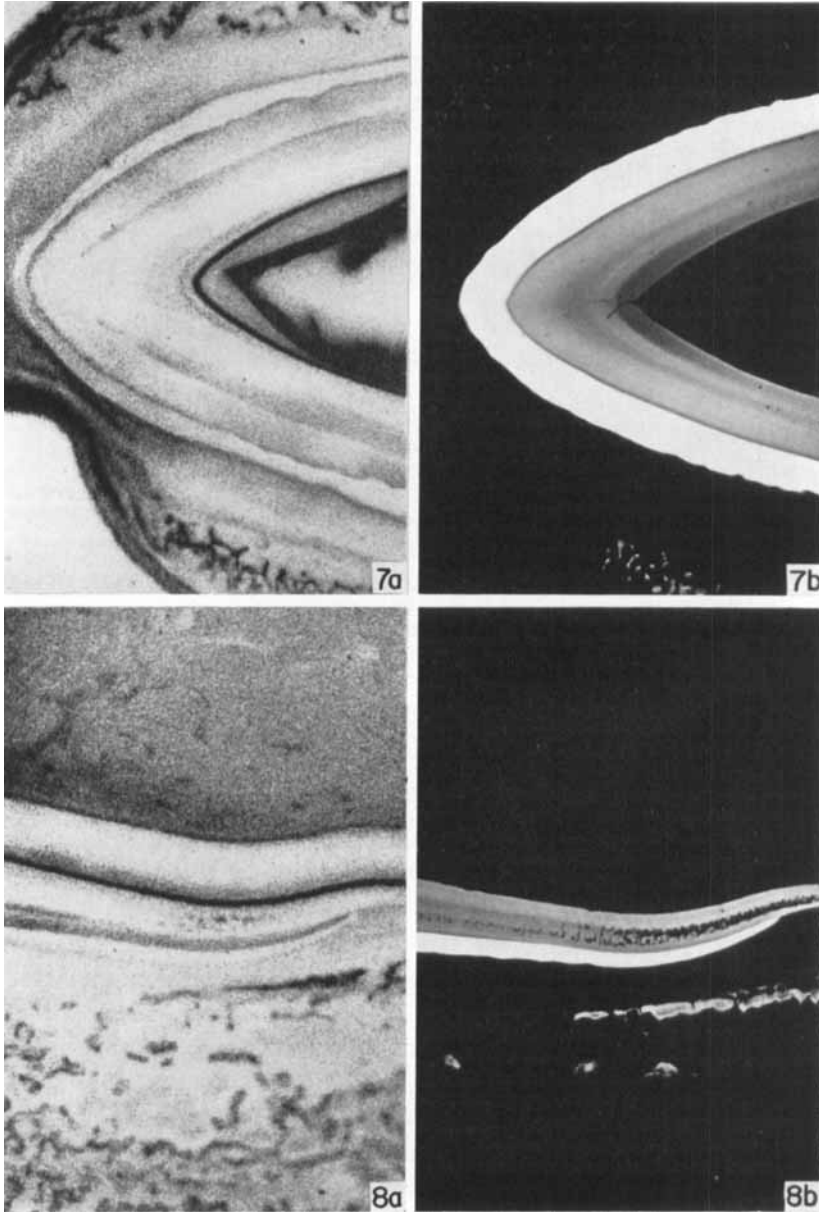
In the histologic examination of sections from decalcified material and of ground sections, it was found that for the pretentine the PAS reaction was positive both in control and "deficient" dogs. Staining with toluidin blue and thionin showed, moreover, that the pretentine was metachromatic (Figs. 11 a, 11 b, 12). Sometimes a narrow metachromatic zone was also observed at the dentino-enamel junction (Fig. 11 a). Metachromasia was also distinguished in the dentinal tubules but not in the normal ground substance of the dentine. In the interglobular dentine, however, there was clear evidence of metachromasia (Fig. 12). The pulp tissue of all teeth under development were clearly metachromatic.

Fig. 7a. Autoradiogram from a lower carnassial of the dog, that first had deficiency of vitamin D and then was given the vitamin in the diet. Uptake of S^{35} of different intensity in the dentine over the pulp tip. $\times 17$.

Fig. 7b. Microradiogram of the same region as Fig. 7a.

Fig. 8a. Autoradiogram from the cervical part of the same tooth as Figs. 7a and 7b. Uptake of S^{35} in the interglobular dentine. $\times 17$.

Fig. 8b. Microradiogram of the same area as Fig. 8a. Interglobular dentine between older normal dentine and normal "healing" dentine. $\times 17$.



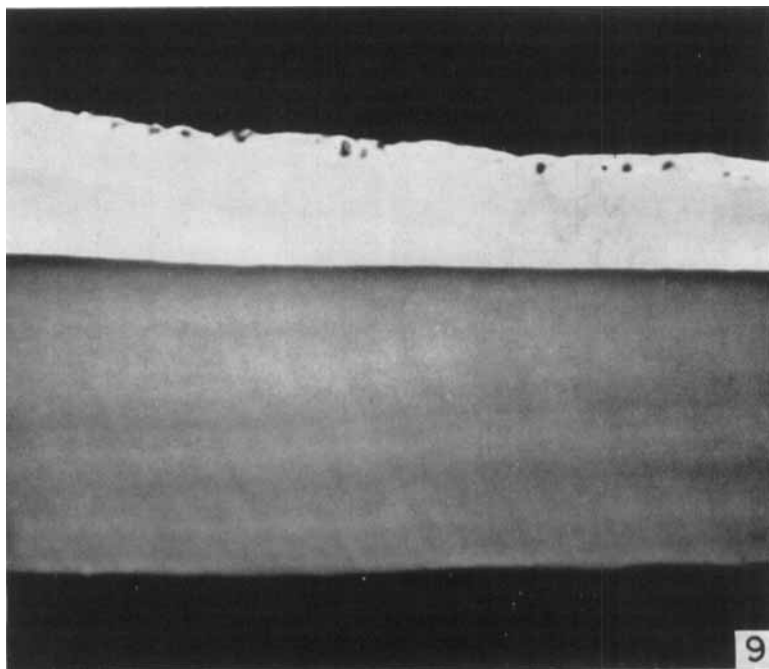


Fig. 9. Microradiogram from a lower carnassial of a dog with vitamin D deficiency. Enamel defects are seen along a striae of Retzius. In the dentine the lines of Owen are visible. $\times 80$.

DISCUSSION

When deficient amounts of vitamin D are provided to growing animals disturbances occur in those parts of the hard tissues of the teeth which are under development. Such changes in the mineralization of these tissues can easily be observed and studied in the microradiograms. Thus it could be demonstrated in the tooth germs where the roots were under development that the defects in the development of the dentine were most pronounced in the apical parts — that is those parts that were formed by the last odontoblasts to be differentiated. In the dentine over the tip of the pulp a lower mineral content than normal was found. On the other hand, interglobular dentine was found in the apical parts. This is in accord with the earlier postulate that the youngest parts of the odontoblastema reacts first and most strongly to the restricted supply of the vitamin.

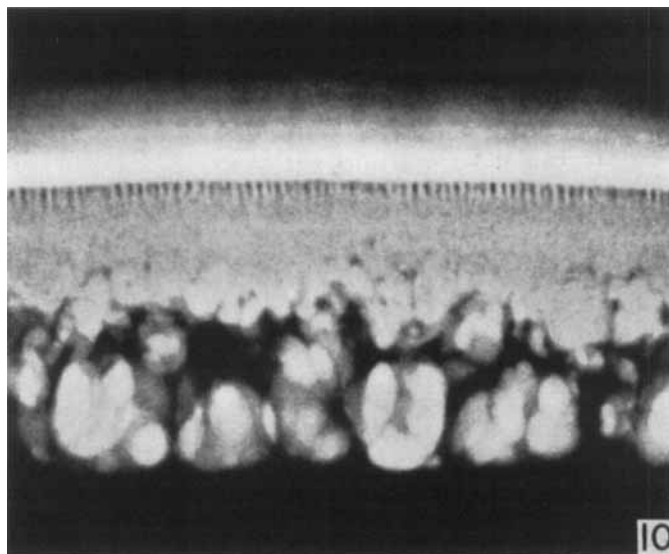


Fig. 10. Microradiogram from a permanent lower canine of a dog with vitamin D deficiency. Striae of Retzius and interglobular dentine. Calcium salt globuli with high and uniform mineralization. Note the distribution of the mineral salts in the dentine adjacent to the enamel. $\times 80$.

The microradiograms reveal, moreover, that the calcium salt globuli of varying size formed in the dentine have a high mineral salt content and are uniformly mineralized. No marked difference in the degree of mineralization could be established for these and normal dentine. Taken as a whole, the dentine formed during the period of deficiency of vitamin D, however, had a low mineral content, as the regions between the calcium salt globuli were not mineralized.

The autoradiograms show that compared with the normal narrow zone of uptake of Ca^{45} at the predentine-dentine border this zone is considerably wider in the corresponding region of teeth developed during the vitamin deficiency period. This is due to the fact that the uptake occurs in the whole of the defective dentine up to the normally developed dentine formed prior to the deficiency, against which the defective region has an irregular boundary. This boundary corresponds to the demarcation of the mineralization defects against the normally developed dentine which may be studied on the microradiograms (Figs. 5 a, 5 b).

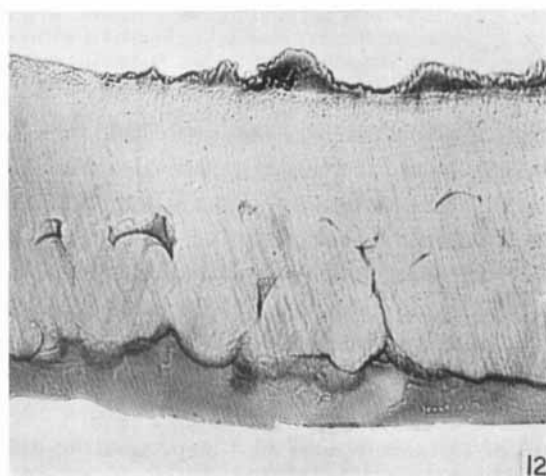
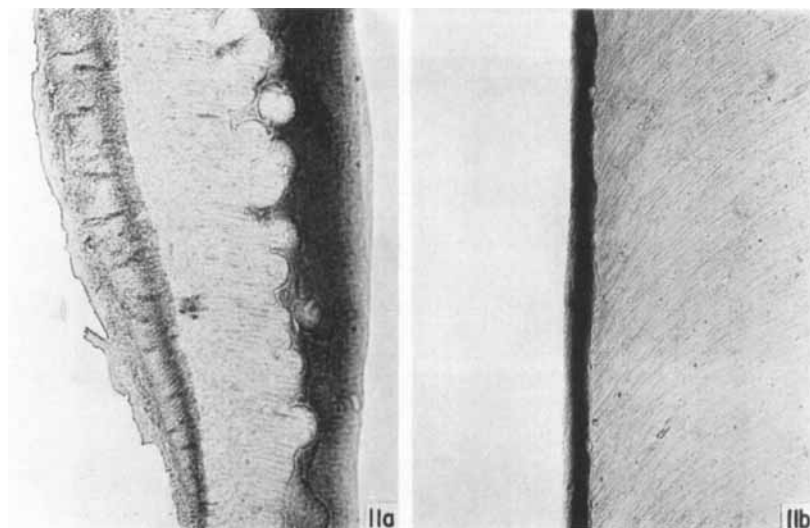


Fig. 11 a. Photomicrograph of a ground section of an upper carnassial of a dog with vitamin D deficiency. Staining with toluidin blue. Metachromatic regions are dark. Note the wide metachromatic predentine zone (right), and the metachromasia of the dentino-enamel junction. $\times 110$.

Fig. 11b. Photomicrograph of a ground section of an upper carnassial of a control dog. Staining with toluidin blue. Note the metachromasia of the predentine. $\times 110$.

Fig. 12. Photomicrograph of a ground section of an upper carnassial of a dog with vitamin D deficiency. Staining with thionin. Metachromasia of the interglobular dentine and predentine. $\times 110$.

The wide predentine zone in the "deficient" animals injected with radio-sulphate demonstrates the uptake of radioactive sulphate throughout the width of the predentine zone. There is thus a comparatively marked increase in the width of the zone of uptake. Interglobular dentine isolated from the predentine by an area of healing dentine shows definite radio-activity due to the sulphate. (Fig. 8 a). No uptake of Ca^{45} by interglobular dentine of similar location could be observed. The uptake of Ca^{45} and S^{35} in the enamel showed no real difference in the teeth of the control and "deficient" dogs. Upon comparison between the pictures of the distribution of mineral salts on the microradiograms and the radioactive reaction in the enamel on the autoradiograms it was found that badly developed enamel in the final mineralization has an uptake of Ca^{45} and S^{35} similar to that occurring in the normally developed enamel.

The mechanism of the uptake of Ca^{45} in the hard tissues of the teeth may be either a deposition of mineral salts or an exchange of ions in the body fluids with ions in the already deposited mineral salts. The situation is more complex for radioactive sulphate as it can be deposited together with the mineral salt fraction and moreover can be incorporated in the organic component of the hard tissues (*Engfeldt and Hjertquist, 1954, 1955*). It has been demonstrated that the predentine zone shows a positive PAS reaction and stains metachromatically with toluidin blue and thionin. Similar reactions have been demonstrated for the interglobular dentine. It is thus conceivable that these regions contain acid mucopolysaccharides and since there is a pronounced uptake of sulphate, a part of the S^{35} deposit in these regions is probably due to the incorporation of sulphate in such substances. These findings suggest the formation of acid mucopolysaccharides in these regions. The same observation as made in the predentine applies to the pulp tissues of teeth in the course of development, so that here too a formation of acid mucopolysaccharides may be in progress.

SUMMARY

The disturbances occurring in the dental hard tissues through experimentally induced vitamin D deficiency in young dogs have

been studied by means of microradiography, autoradiography and various histologic staining methods.

The microradiograms show that the non-fusing calcium salt globuli formed in the dentine in vitamin D deficiency were uniformly mineralized. The degree of mineralization was apparently not less than that of the dentine developing prior to the beginning of the experiment. The lines of Owen had a low mineral content. The microradiograms also reveal various kinds of defects and disturbances in enamel mineralization of the teeth that had developed during the course of the experiment.

The autoradiograms indicate that, as a rule, Ca^{45} and S^{35} are taken up in the dental tissues at the same sites in vitamin D deficiency as under normal conditions. In deficiency, however, the zone of Ca^{45} uptake in the predentine-dentinal border is wider than normal. The predentine is wider than usual and so is the band of uptake of sulphate in this zone. S^{35} was also taken up by the interglobular dentine of animals with deficiency of the vitamin. There was no corresponding uptake of Ca^{45} .

The mechanism of the uptake of Ca^{45} may be a deposition of calcium salts or an ionic exchange between mineral salts already present in the tissues. S^{35} may be included both in mineral salts and in the organic component.

In the animals with vitamin D deficiency the interglobular dentine and the predentine presented metachromasia, and gave a positive PAS reaction. This suggests that acid mucopolysaccharides occur in these regions. The S^{35} uptake in the same areas may be due to a formation of these acid mucopolysaccharides. The same applies to the pulpal tissue of the developing teeth.

RÉSUMÉ

ÉTUDES SUR LES TISSUS DENTAIRES MINÉRALISÉS

X. Observations microradiographiques, autoradiographiques et histochimiques sur les tissus durs de chiens atteints expérimentalement d'avitaminose D.

Les perturbations observées dans les tissus durs à la suite d'une avitaminose D provoquée chez de jeunes chiens ont été étudiées par les méthodes microradiographique et autoradiographique, ainsi que par différentes techniques histologiques de coloration.

Les microradiographies attestent que les globules non fusionnés de sel de calcium formés dans la dentine dans les cas d'avitaminose D étaient minéralisés uniformément. Le degré de minéralisation ne paraissait pas moins avancé que dans la dentine de développement antérieur à l'expérience. Dans les lignes de contour d'Owen, le contenu minéral était peu élevé. Les microradiographies révélaient également divers défauts et perturbations dans la minéralisation de l'émail formé en cours d'expérience.

Les autoradiographies indiquent qu'en général le Ca^{45} et le S^{35} ont été absorbés par les tissus dentaires aux mêmes points dans les cas d'avitaminose que dans les cas normaux. Mais la zone d'absorption du Ca^{45} à la limite de la dentine et de la pré-dentine était plus large que la normale. Etant donné que, dans les cas d'avitaminose D, la pré-dentine est plus large que la normale, la zone d'absorption de sulfate y était plus large également. Le S^{35} était absorbé aussi par la dentine interglobulaire chez les animaux atteints d'avitaminose, tandis que le Ca^{45} était absent dans cette région.

Le mécanisme d'absorption du Ca^{45} peut être soit un dépôt de sels de calcium, soit un échange ionique entre les sels minéraux déjà présents dans les tissus. Le S^{35} peut faire partie à la fois des sels minéraux et du composant organique.

Chez les animaux atteints d'avitaminose, la dentine interglobulaire et la pré-dentine présentaient des métachromasies, la pré-dentine ayant en outre une réaction positive au PAS. Ceci tend à prouver que des mucopolysaccharides acides se trouvent dans ces régions; il est probable que l'absorption du S^{35} y est due à la formation de ces mucopolysaccharides acides. Il en va de même des tissus pulpaire des dents en cours de développement.

ZUSAMMENFASSUNG

STUDIEN AN MINERALISIERTEN ZAHNGEWEBEN

X. Eine mikroradiographische, autoradiographische und histochemische Untersuchung der Zahnhartgewebe von Hunden mit experimentell hervorgerufenem D-Vitaminmangel.

Mit Hilfe der Mikroradiographie und verschiedener histologischer Färbemethoden wurden die Störungen, die in den Hartgeweben des Zahnes von Hunden mit experimentell hervorgerufenem D-Vitaminmangel entstehen, untersucht.

Die Mikroradiogramme zeigen, dass die nicht zusammenfließenden Kalkglobuli, die bei D-Vitaminmangel im Dentin gebildet werden, gleichmäßig mineralisiert sind. Ihr Mineralisierungsgrad ist nicht sichtbar kleiner als der, des vor Versuchsbeginn gebildeten Dentines. Die vorhandenen Owenschen Linien haben einen geringeren Mineralgehalt. Verschiedene Formen von Defekten und Mineralisierungsstörungen im Schmelz an den Zähnen, die sich während der Versuchszeit entwickelten, sind auch im Mikroradiogramm zu sehen.

Die Autoradiogramme zeigen, dass bei D-Vitaminmangel, Ca^{45} und S^{35} an den gleichen Stellen in die Zahngewebe aufgenommen werden wie unter normalen Verhältnissen. Was Ca^{45} betrifft, ist jedoch das Aufnahmegebiet in der Prädentin-Dentingrenze breiter als normal. Dadurch, dass das Prädentin bei D-Vitaminmangel breiter ist als normal, ist auch die Sulfataufnahme in dieser Zone breiter als normal. S^{35} wird auch in das Interglobulärdentin von Tieren mit D-Vitaminmangel aufgenommen. Eine entsprechende Aufnahme von Ca^{45} findet nicht statt.

Die Aufnahme von Ca^{45} kann eine Deposition von Kalksalzen, oder aber ein Ionenaustausch zwischen bereits vorhandenen Mineralsalzen sein. S^{35} kann sowohl in Mineralsalzen als auch in der organischen Komponente vorhanden sein.

Bei Tieren mit D-Vitaminmangel zeigen sowohl Interglobulärdentin wie auch Prädentin Metakromasie. Das Prädentin zeigt auch eine positive PAS Reaktion. Das deutet auf das Vorkommen von sauren Mukopolysacchariden in diesen Gebieten. Die S^{35} Aufnahme in diesen Gebieten beruht wahrscheinlich darauf, dass hier eine Bildung von sauren Mukopolysacchariden stattfindet.

Für das Pulpagewebe von in der Entwicklung begriffenen Zähnen treffen die gleichen Verhältnisse zu.

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