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TETRACYCLINE IN DEVELOPING RAT ENAMEL IN RELATION TO PROTEIN SYNTHESIS AND MATURATION

by

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INTRODUCTION

Ever since the first reports by *Schwachman & Schuster* (1956) and *Wallman & Hilton* (1962) of a probable association between tetracycline medication and discolorations and hypoplasias of the teeth a great number of investigations have been carried out on this problem. The mechanisms behind these effects are still incompletely known. The tetracyclines are strongly accumulated in the skeletal tissues mainly in the mineralizing parts. The affinity for these tissues is considered to be due to binding to divalent metal ions, mainly calcium, but some organic components seem also to be implicated (for review see *Storey*, 1963; *Johnson*, 1964; *Bevelander*, 1965). In a recent study it was found that the distribution patterns of simultaneously injected ^{45}Ca and tetracycline were different and the initial uptake of tetracycline seemed to occur only where new matrix was formed (*Hammarström*, 1967). Moreover the distribution of tetracycline was changed with time in a way which was very similar to that of a number of amino acids, as described by several investigators (*Hwang & al.*, 1963; *Young & Greulich*, 1963; *Greulich & Slavkin*, 1965). This might indicate an association between tetracycline and some protein component of the developing enamel. The purpose of the present investigation was to correlate the uptake of tetracycline in the enamel, as shown by fluorescence microscopy, with the formation of enamel proteins, as indicated by the autoradiographic pattern of

simultaneously injected ^{14}C-proline, and also to correlate this uptake with the maturation process in the developing enamel, as shown by microradiography of the same sections.

Proline, which is the most abundant amino acid in the proteins of the developing enamel (*Eàstoe*, 1963; *Burgess & Maclaren*, 1965) has previously been used in the study of protein synthesis of dental tissues (*Greulich & Slavkin*, 1965). In order to see if the distribution of tetracycline was dose dependent, two different doses were given. A preliminary histochemical study with Millon's reagent for tyrosine was also performed on the same sections, which had previously been used in the fluorescence microscopic, autoradiographic and microradiographic studies, in order to be able to correlate the various patterns obtained with that of the endogenous proteins of the enamel. With regard to maturation the concentration of most amino acids seem to be reduced at the same time as shown both by histochemistry (*Suga & Gustafson*, 1963) and by chemical analysis (*Burgess & Maclaren*, 1965). The occurrance of tyrosine may therefore illustrate the main protein component of the developing enamel. As experimental animals 10-day-old rats were used. At this age a secondary mineralization is going on in the molar teeth of the rat (see e.g. *Orban et al.*, 1943; *Marsland*, 1952; *Belanger*, 1957; *Suga & Gustafson*, 1963).

MATERIALS AND METHODS

Injected substances

Two aqueous solutions of tetracycline-HCl (Tetradecin, Astra) were prepared, one containing 5 mg/ml and the other containing 25 mg/ml. 50 μci (microcuries) of uniformly labelled ^{14}C-proline (The Radiochemical Centre, Amersham) with a specific activity of 125 mci/mM in 2 ml of a 1 % ethanol solution was also used.

Animals and doses

Nine 10-day-old rats of the Sprague-Dawley strain were used. The animals weighing about 20 g were divided into three series with three animals in each. In the first series each animal was given 1 mg (50 mg/kg body weight) tetracycline in 0.2 ml solution and 5 μci (0.23 mg/kg body weight) ^{14}C-proline in 0.2 ml solution. In the second series each animal was given 5 mg (250 mg/kg body weight) tetracycline in 0.2 ml solution and 5 μci (0.23 mg/kg body weight) ^{14}C-proline in 0.2 ml solution. In the third series each animal was given 5 μci (0.23 mg/kg body weight) ^{14}C-proline in 0.2 ml solution and no tetracycline.

Experimental procedure

The tetracycline was given intraperitoneally and the ^{14}C-proline was given subcutaneously in the back of the neck at the same time. The injections were given directly after each other. After the injections one animal from each series was killed at the end of each time interval of 60 min., 24 hours and 4 days. One of the animals given 5 mg tetracycline died before the planned survival period of 4 days and was discarded. At the end of the predetermined survival periods, the animals were anesthetized with ether and then killed by freezing in hexane mixed with solid CO_2 (—75°C).

Immediately before the freezing the animals were embedded in an aqueous solution of carboxymethyl cellulose applied on a large microtome stage. After freezing in hexane-CO_2 the specimens were thus ready for sectioning, which was performed in a freezebox (—10°C). To obtain whole sections through the non-decalcified tissues of various hardness a piece of a Scotch tape (No. 810, Minnesota Mining & Manufacturing Co.) was attached to the exposed surface of the frozen specimen before cutting. The sections then came off adhering to the tape. Sections, 10 μ thick, were taken and freeze-dried in the box for two days and then allowed to reach room temperature in an air-tight box.

The sections were then examined under ultraviolet light in a Zeiss fluorescence microscope (HBO 200-watt mercury lamp, 4 mm BG 12 filter). Microphotographs were taken in Scopix (Gevaert).

After the examination of the fluorescence autoradiograms of the same sections were obtained by pressing the sections against Structurix X-ray film (Gevaert). After 5 weeks' exposure the sections were separated from the films and the films were developed in G 230 and fixed in G 305 (Gevaert). The freeze-sectioning and the autoradiographic technique have previously been described by *Ullberg* (1954, 1958).

After the autoradiographic exposure microradiographs of the same sections were made with a Philips X-ray diffraction apparatus type PW 1009 (copper target) using a target-film distance of 25 cm. The voltage was 12 kV and the amperage was 20 mA. Kodak Maximum Resolution Plates were used and the sections were still sitting on the tape when they were put on the photographic plates.

Finally some of the sections were stained with hematoxylin and eosin and some others were fixed with buffered formalin and used for the preliminary histochemical study with Millon's reaction (Bensley & Gersh modification as described by *Pearse* 1961). In the last case the sections were fixed for 30 min. in 10 % buffered formaline before they were put into the nitric

acid solution of mercuric nitrate. After 30 min. at +60°C the sections were washed with 2 % nitric acid and then dehydrated with ethanol and embedded in Euparale (Flatters and Garnett Ltd., Manchester, England).

RESULTS

The distribution of ^{14}C-proline in the teeth of those animals which had also received tetracycline was the same as in those which had got only the labelled amino acid and there was no observable difference in its concentration. No yellow fluorescence resembling that of tetracycline was seen in the tissues of the animals injected with only ^{14}C-proline. The distribution of tetracycline after the different doses injected was the same, but the fluorescence was more intense after the high dose. The injections of tetracycline caused no observable changes in the microradiographic pictures or in the histochemical reactions used.

Both the tetracycline fluorescence and the ^{14}C-proline radioactivity in the enamel one hour after the injections were seen in a superficial zone under the tall ameloblasts and in addition tetracycline fluorescence was seen under the ameloblasts just being reduced in length. The distribution of tetracycline could thus be correlated with the functional stage of the ameloblasts, but no correlation could be made with the radiopacity or with the histochemical reactions for tyrosine. After 24 hours and 4 days the distribution of ^{14}C-proline was mainly the same as after 1 hour. The tetracycline fluorescence after one day, however, was spread through the entire thickness of the enamel and covered also more occlusally located parts than those showing fluorescence after 1 hour. There was an inverse relation between the radiopacity and the fluorescence intensity. The fluorescent areas also seemed to be the same as those containing histochemically demonstrable amounts of tyrosine as indicated by Millon's reaction. After four days the fluorescent areas were reduced in the occlusal parts compared with that seen after one day and fluorescence could now be seen in the cervical third of the first molar and in about half the crown in the second molar in the cervico-occlusal direction. In those areas, however, the enamel still showed fluorescence in its entire width. The zone of increased radiopacity showed a corresponding expansion towards the cervical parts and seemed to border to the fluorescent area. The patterns obtained with the various methods will be described more in detail below.

One hour after administration

At this time after injection both tetracycline fluorescence and ^{14}C-proline radioactivity were found in the odontoblasts and ameloblasts. The fluores-

cence intensity in these cells was lower than that of the hard tissues but it was higher than in the surrounding soft tissues. The fluorescence intensity seemed to be a little higher in the ameloblasts, which were just being reduced in length than in the tall and short ameloblasts (Figs. 1 a and 2 a). The highest intracellular concentration of ^{14}C-proline in the developing teeth was found in the odontoblasts and in the tall ameloblasts, whereas the postsecretory ameloblasts had a lower concentration (Figs. 1 b, 2 b).

In the developing enamel a moderate tetracycline fluorescence was found in a narrow superficial zone adjacent to the tall ameloblasts. There was also a deposition of radioactivity of ^{14}C-proline in this zone. The stages of development were different between the first and second molar and also between the different cusps of the teeth. On the distal surface of the first molar the tall ameloblasts covered about the cervical third of the crown. On the mesial surface of the second molar about half the crown was covered by tall ameloblasts (Figs. 1 and 2). Under the ameloblasts just being reduced, the fluorescence was more intense and the fluorescent zone seemed to be wider, but there was no deposition of ^{14}C-proline. Under the short ameloblasts neither fluorescence nor radioactivity was observable in the enamel. The localization of fluorescence and radioactivity in the enamel at this time after injection could not be correlated with any special structure observed in the microradiographic and histochemical studies. The microradiograms showed an increase in radiopacity in the tip of the cusps of the first molar. The remaining part of the enamel of the first molar and of the whole second molar had a lower radiodensity than that of the dentine (Fig. 2 c). These areas also showed a strong positive histochemical reaction for tyrosine.

In the dentine ^{14}C-proline was found in a zone close to the pulp and tetracycline fluorescence was found at the dentine — predentine junction (Figs. 1 a, 1 b, 2 a, 2 b).

Twenty-four hours after administration

At this time after injection no fluorescence could be seen in the ameloblasts and odontoblasts. In the enamel and dentine the concentration had increased and the distribution of tetracycline in the enamel was markedly changed. Fluorescence could now be seen in the whole enamel of the first and second molars except in the most highly mineralized parts in the tips of some cusps (Figs. 3 a and 3 c). The fluorescent parts of the enamel also showed a positive reaction for tyrosine with Millon's reaction. Like the tetracycline fluorescence the staining with this reaction decreased towards the more mineralized parts in the cusps, and there seemed to be a rather good conformity between

Fig. 1. Maxillary first and second molar teeth 1 hour after injection of 1 mg tetracycline and 5 μci ^{14}C-proline. a. Fluorescence microphotograph of tetracycline. (Light areas indicate tetracycline fluorescence.) b. Autoradiogram of ^{14}C-proline. (Dark areas indicate localization of radioactivity.) Tetracycline and ^{14}C-proline have accumulated in the same superficial zone of the enamel. Occlusally of this zone there is a wider zone of increased fluorescence with no corresponding uptake of ^{14}C-proline. The fluorescence of the soft tissues is low, but that of the ameloblasts and odontoblasts seem to be more intense than the background. The odontoblasts and the tall ameloblasts have a high concentration of ^{14}C-proline. In the dentine a narrow fluorescent and radioactive zone can be seen adjacent to the pulp. In the alveolar bone a pronounced uptake of tetracycline and ^{14}C-proline can be seen on the distal surface of the septum between the teeth. ($\times$30).

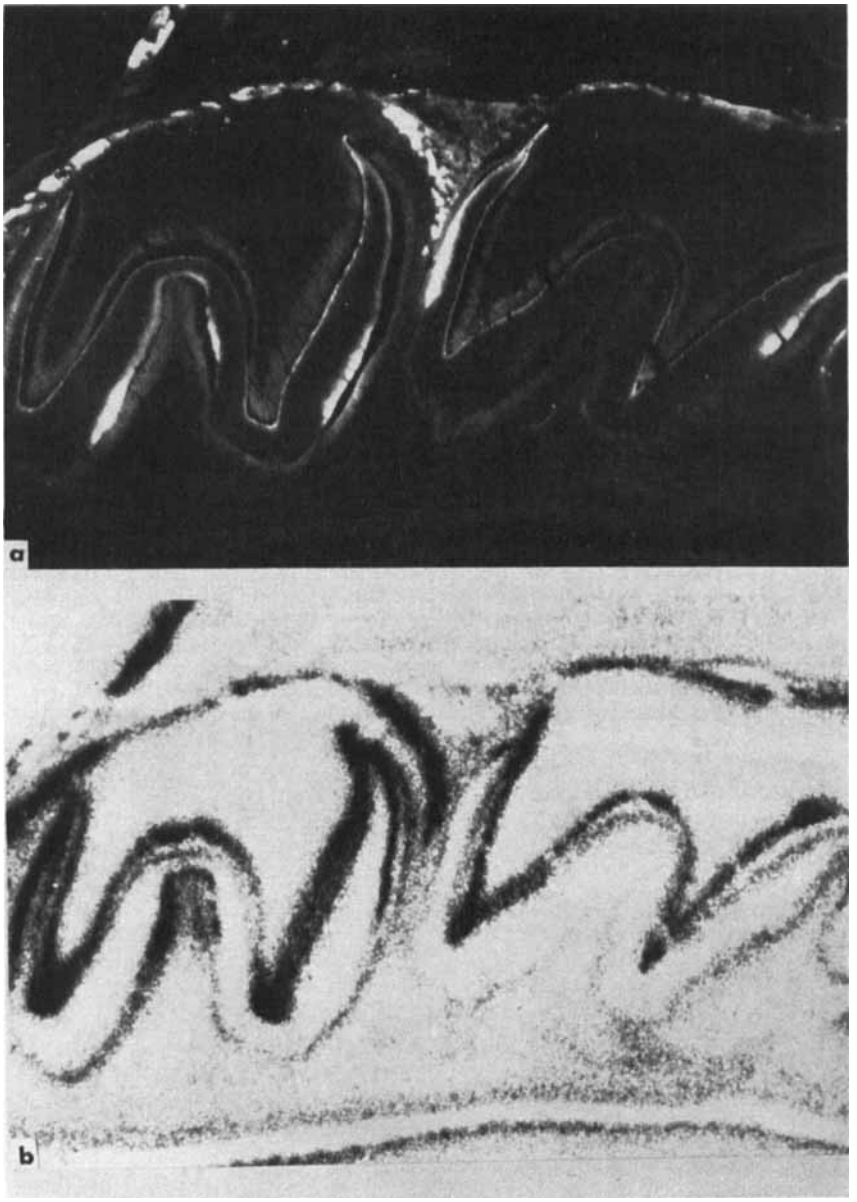

a
b

Fig. 2. Maxillary first and second molar teeth 1 hour after injection of 5 mg tetracycline and 5 μci ^{14}-C-proline. a. Fluorescence microphotograph showing the distribution of tetracycline. (Light areas indicate tetracycline fluorescence.) b. Microradiogram of the same section. (Light areas indicate a high mineral content.) c. Autoradiogram of ^{14}C-proline. (Dark areas indicate localization of radioactivity.) d. Microphotograph of the same section stained with hematoxylin and eosin. The distribution patterns of the tetracycline and ^{14}C-proline are the same as seen in Fig. 1. But note the increased fluorescence in the ameloblasts and odontoblasts. Note also the increased fluorescence under the ameloblasts which are being reduced in height (arrows). The microautoradiograms show an increase in mineral content in the cusps of the first molar, where the radiopacity is about the same as that of the dentine. The remaining parts of the enamel have a lower radiodensity than the dentine. ($\times$30).

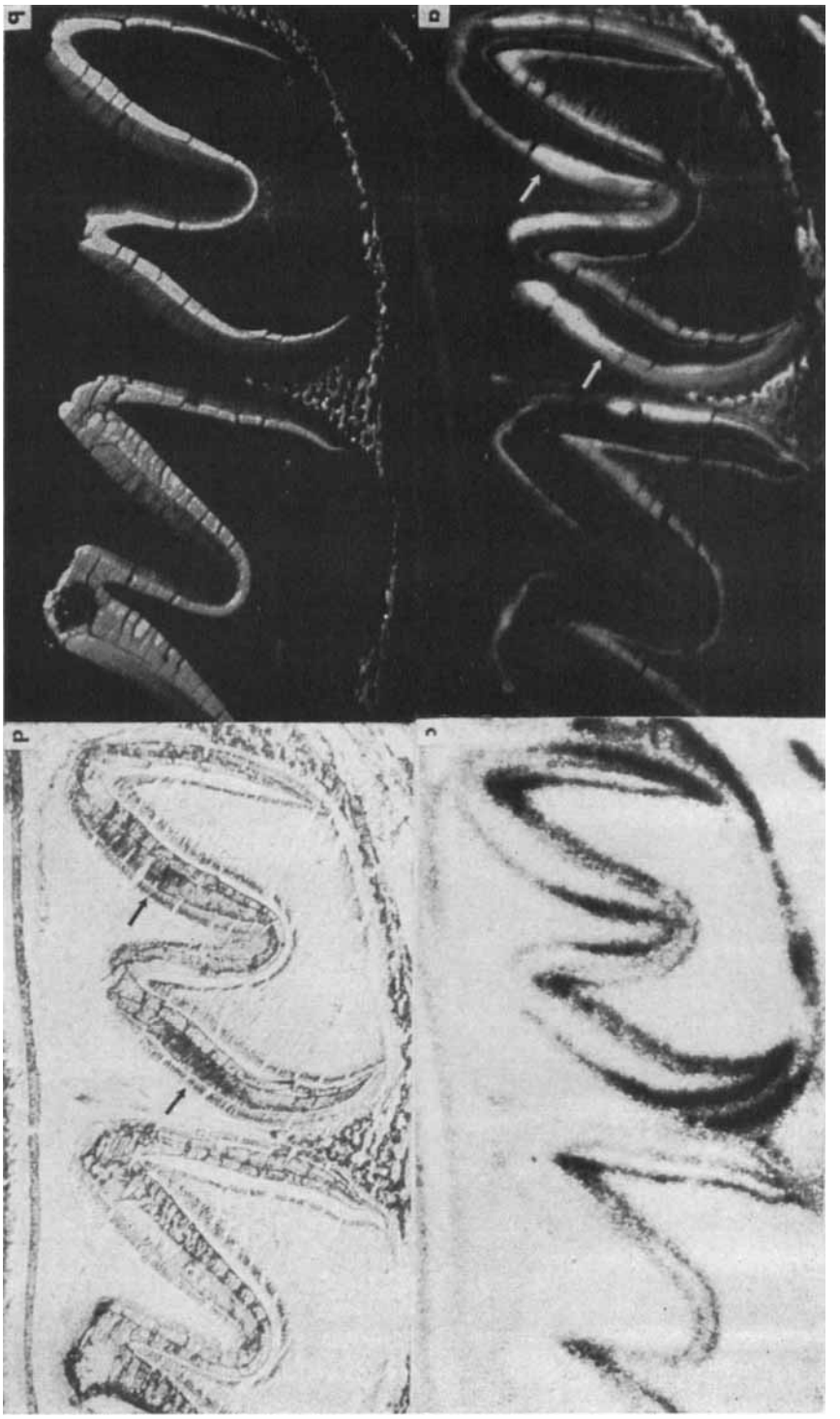
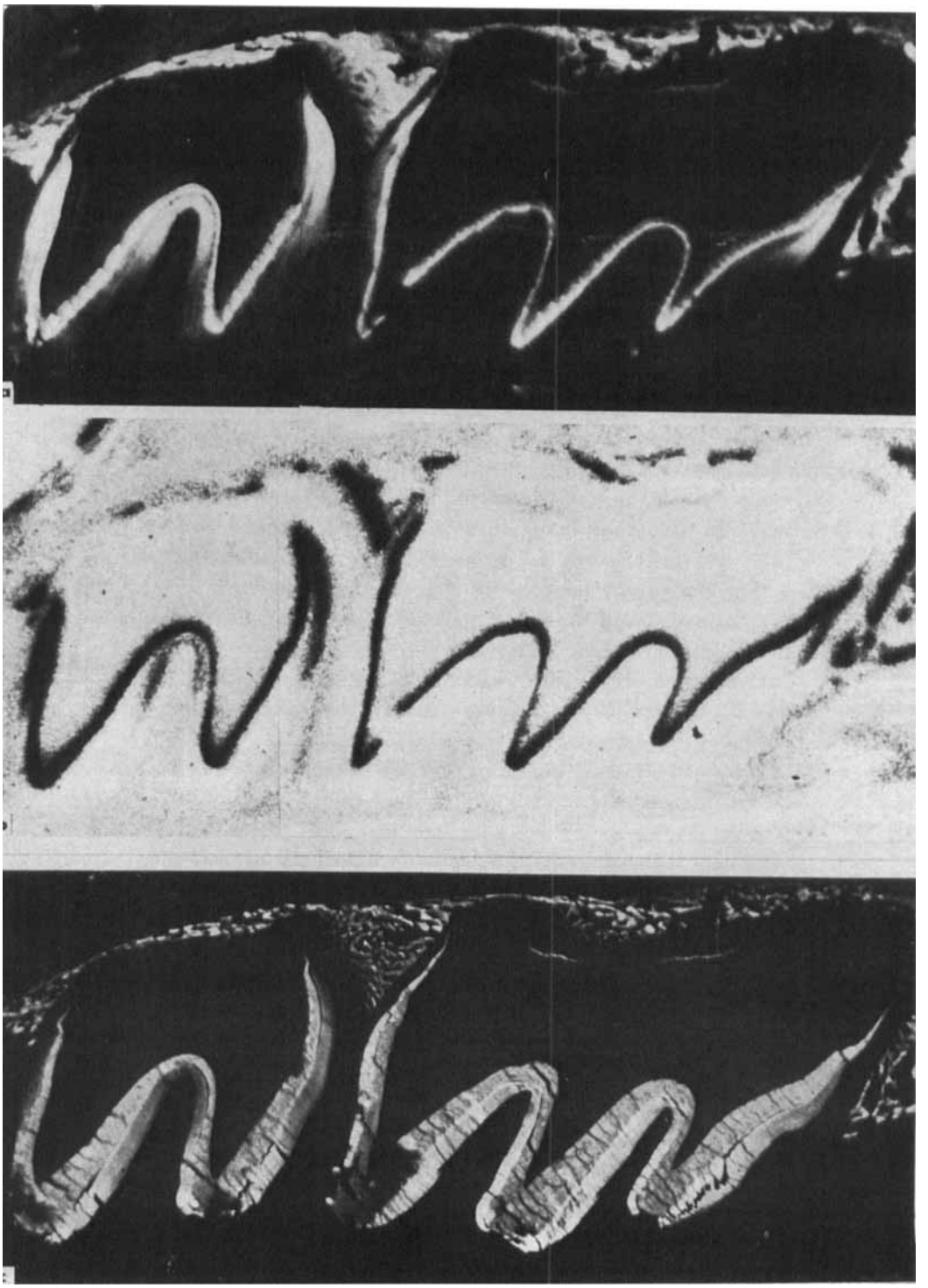

q
p
p
c

Fig. 3. Maxillary first and second molar teeth 24 hours after injection of 1 mg tetracycline and 5 μci ^{14}C-proline. a. Fluorescence microphotograph of tetracycline. (Light areas indicate tetracycline fluorescence.) b. Autoradiogram of ^{14}C-proline. (Dark areas indicate localization of radioactivity.) c. Microradiogram of the same section. (Light areas indicate a high mineral content.) The distribution of tetracycline has markedly changed in comparison with the localization 1 hour after injection. Note the inverse relation between the fluorescence and the radiopacity. The distribution of ^{14}C-proline is mainly the same as that seen after 1 hour. ($\times$ 25).


a
b
c

Fig. 4. Maxillary first and second molar teeth 4 days after injection of 1 mg tetracycline and 5 μci ^{14}C-proline. a. Fluorescence microphotograph of tetracycline. (Light areas indicate tetracycline fluorescence.) b. Autoradiogram of ^{14}C-proline. (Dark areas indicate localization of radioactivity.) c. Microradiogram of the same section. (Light areas indicate high mineral content.) The tetracycline fluorescence has been eliminated from the cusps and can now be seen mainly in the cervical areas of the teeth. The fluorescence of tetracycline reaches about the same level as the radioactivity of ^{14}C-proline. The mineral content of the enamel has increased and is higher than that of the dentine in the non-fluorescent parts, but lower in the fluorescent parts. In the dentine the fluorescent and radioactive zones are localized deep in the tissue and some fluorescence and radioactivity may also be seen in the dentine formed after the injection. In the interdental septum bone has been deposited mainly on the distal side during the time after the injections, as shown by the localization of the fluorescent and radioactive zones. (×30).



these two patterns. However, with maturation the enamel becomes more acid soluble and due to the dissolution of this part of the enamel in the nitric acid used for Millon's reaction it was difficult to determine the »border line» of positive histochemical reaction in the occlusal parts of the teeth. The acid solubility seemed to start in the cusps at the enamel — dentine junction. The main part of the enamel stained orange but sometimes the youngest parts of the enamel showed a more brownish appearance. The enamel and the hairs seemed to have the strongest positive reaction for tyrosine in the whole body of the rats.

Four days after administration

After this survival period fluorescence was observable in the cervical third of the first molar and in half the crown of the second molar (Fig. 4 a). The border line between fluorescent and non-fluorescent areas was rather marked and had a varying configuration. In several cases it was curved with the convexity towards the cervical margin. There seemed to be a corresponding convexity in the border line between the more mineralized occlusal parts and the less mature cervical parts (Fig. 4 c). The radioactive zone of ^{14}C-proline was mainly the same as after one hour but some radioactivity seemed to have spread through the enamel especially in the cervical areas of the second molar. The extension of the radioactive zone in the cervical-occlusal direction was slightly reduced and ended occlusally at the same level as the tetracycline fluorescence. Also the area with a positive staining with Millon's reagent seemed to reach this level.

The radioactivity and fluorescence in the dentine were still in narrow zones, which due to the continuous deposition of new matrix were localized deep in the dentine at this time after injection. Some radioactivity and fluorescence were also seen in the dentine on the pulpal side of these zones.

DISCUSSION

The initial distribution of tetracycline in the developing enamel, which could be correlated to the appearance of the ameloblasts, seems to differ both from the deposition of new protein matrix as shown by the autoradiograms of ^{14}C-proline and from the uptake of minerals as previously shown (*Hammarström*, 1967). The different patterns in the developing enamel of simultaneously injected tetracycline, ^{14}C-proline and ^{45}Ca a short time after the administration may be summarized as follows. Underneath the tall ameloblasts all the three substances are accumulated in a narrow superficial zone

of the enamel; underneath the short, postsecretory ameloblasts there was a pronounced accumulation of ^{45}Ca through the whole thickness of the enamel but no uptake of tetracycline and ^{14}C-proline; underneath the ameloblasts, which are located at the junction between the tall ameloblasts and the short ameloblasts, there was a marked accumulation of tetracycline and ^{45}Ca but no deposition of ^{14}C-proline. This latter observation of a special appearance of enamel in the zone where the function of the ameloblasts is transformed from protein synthesis to some function in the enamel maturation process may indicate a beginning change in the composition of the surface of the developing enamel as a first step in the maturation process, or it may suggest a special function of these cells possibly in the synthesis of the outer surface of the enamel. Of special interest when regarding the increased uptake of tetracycline in the outer surface at the end of enamel matrix formation is the finding by *Nylen et al.* (1964) that a tetracycline induced reduction in crystal size was more widespread in the surface than in the deeper parts of the injured enamel.

The distribution of ^{14}C-proline 60 min. after injection seemed to be in good agreement with previous studies (*Greulich & Slavkin*, 1965). In the present investigation, however, this pattern remained rather unchanged also 1 and 4 days after administration, which is not in agreement with those previous studies, where a diffuse spreading with time has been observed of the radioactivity of labelled amino acids. This disagreement may be due to different stages of development of the enamel studied. In the present investigation the ^{14}C-proline was more spread in the cervical regions of the second molar than elsewhere. This indicates that the »diffusive pattern» of labelled amino acids in the enamel is more marked at the earlier stages of the development, which have previously been studied.

No correlation could be made with the initial uptake of tetracycline and the composition of the enamel as shown by the microradiographic and histochemical methods used. After one and four days, however, there seemed to be good conformity between the fluorescence intensity and positive histochemical reaction for tyrosine and an inverse relation to the degree of mineralization. The curved border line sometimes observed between the cervical fluorescent part and the occlusal part has previously been described by *Johnson* (1965). It is of special interest since there seemed to be a corresponding central zone of high mineral content in the central part of the maturing enamel. *Suga & Gustafson* (1963) have described a similar appearance of the front of increasing mineral content in developing rat enamel.

According to previous histochemical studies the pattern of disappearance at the maturation of the enamel is similar for several amino acids (*Suga &*

Gustafson, 1963). A conformity between the distribution of tetracycline fluorescence and the protein fraction is interesting when regarding the findings by *Loo et al.* (1957), *Milch et al.* (1961) and others, of a binding tetracycline to peptides and may indicate a similar binding of tetracycline to some peptide fraction also in the enamel.

There seem to be a remarkable latency between the reduction in length of the ameloblasts and the appearance of a demonstrable increase in mineral content of the enamel. The present investigation showed that when the ameloblasts were reduced in length at the middle or the lower third of the crown of the tooth germ an increase in radiopacity was demonstrable in the tip of the cusp. Four days later the increased radiopacity had expanded to a region which was slightly cervically of the localization of the most occlusal tall ameloblasts in the 10-day-old rats. An influx of minerals throughout the enamel thickness has been shown to occur as soon as the ameloblasts are reduced in length (*Belanger*, 1957; *Hammarström*, 1967). But apparently there was a latency of almost 4 days until an increased mineral content was microradiographically demonstrable. Four days after the administration the tetracycline fluorescence and autoradiographic image of ^{14}C-proline had the same extension in the cervico-occlusal direction which may further support the idea of an association between the tetracycline and some protein fraction of the enamel.

When given in therapeutic and larger doses the tetracyclines may be toxic to the skeletal tissues affecting both the mineralization and growth (*Cohlan et al.*, 1963; *Nylen et al.*, 1964; *Hansson*, 1967). In the present studies, the period of investigation probably was too short to make these changes demonstrable. *In vitro* has been shown that high concentration of tetracycline may impair the uptake of ^{14}C-proline in growing bone (*Bennet et al.*, 1967). Either did such high concentration not occur in the present experiment or were the quantitative changes too small to be observable with the present method. The mechanism behind the toxic effects of tetracycline is not known. The finding of tetracycline fluorescence in the ameloblasts and odontoblasts may indicate a direct action on the cells elaborating the hard tissues. Tetracycline has been demonstrated in the mitochondria of the cells of a number of tissues and also of bacteria (*Du Boy & Showacre*, 1961). In the present study the resolution did not permit any precise intracellular observation and the fluorescence seemed to cover the whole cells. Also some radioactivity of ^{14}C-proline was localized in the ameloblasts and odontoblasts 60 min. after injection. The accumulation in the tall ameloblasts is in agreement with previous studies (*Greulich & Slavkin*, 1965). It is, however, interesting to note that both ^{14}C-proline and tetracycline were also accu-

mulated in the short, postsecretory, ameloblasts although there was no demonstrable deposition of them in the surface of the underlying enamel at this time of injection.

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SUMMARY

The uptake of tetracycline in the developing enamel of rats, as seen by fluorescence microscopy, has been correlated with the protein synthesis, as visualized by autoradiography of ^{14}C-proline. Correlation with the maturation of the enamel has been made with the aid of microradiography of the same sections, which had been utilized for fluorescence microscopy and autoradiography. The sections were finally stained with hematoxylin and eosin or histochemically stained for tyrosine with Millon's reagent.

The distribution patterns of tetracycline and simultaneously injected ^{14}C-proline were different. After one hour both were found in a superficial zone in the enamel underneath the tall ameloblasts but in addition an increased uptake of tetracycline was found under the ameloblasts which were just being reduced in length. After one and four days the tetracycline fluorescence had been spread through the whole thickness of the non-mature enamel. There was an inverse relation between the fluorescence intensity and the mineral content. There seemed to be a rather good conformity between the enamel with strong fluorescence intensity and the areas with a positive Millon reaction. The distribution of ^{14}C-proline remained rather unchanged during the four days of investigation.

RÉSUMÉ

LA TETRACYCLINE DANS L'ÉMAIL EN VOIE DE FORMATION CHEZ LE RAT, ET SES RAPPORTS AVEC LA SYNTHESE DES PROTEINES ET LA MATURATION

Chez des rats, l'absorption de tétracycline, observée par microfluoroscopie dans l'émail en voie de formation, a été mise en relation avec la synthese des protéines telle qu'elle ressort de l'observation par autoradiographie de la proline C^{14}. Une mise en relation avec la maturation de l'émail a été effectuée en pratiquant des microradiographies des coupes ayant déjà servi

pour la microfluoroscopie et l'autoradiographie. Les coupes ont enfin été colorées à l'hématoxyline et à l'éosine, ou par coloration histochimique pour la tyrosine avec le réactif de Millon.

Les modes de répartition de la tétracycline et de la proline C^{14} injectée simulanément étaient différents. Au bout d'une heure, on les trouvait toutes deux dans une région superficielle de l'émail, au dessous des grands améloblastes, mais, de plus, une absorption plus intense de tétracycline était observée sous les améloblastes dont la longueur était en train de diminuer. Au bout d'un jour et au bout de quatre jours, la fluorescence de la tétracycline s'était étendue à travers à travers toute l'épaisseur de l'émail en voie de maturation. Il existait un rapport inverse entre l'intensité de la fluorescence et la teneur en substances minérales. Mais les régions ayant une réaction de Millon positive semblaient coincider assez bien avec l'émail dont la fluorescence était intense. La répartition de la proline C^{14} est restée à peu pres inchangée pendant les quatre jours qu'a duré l'expérience.

ZUSAMMENFASSUNG

TETRACYCLIN IM WACHSENDEN ZAHNSCHMELZ DER RATTE IM VERHÄLTNIS ZU DER PROTEIN-SYNTHESE UND REIFUNG

Mittels Fluoreszenzmikroskopie und Autoradiographie wurde die Verteilung von Tetracyclin und gleichzeitig injiziertem ^{14}C-Prolin bei im Wachstum stehenden Rattenzähnen studiert. Mittels Mikroradiographie von denselben Schnitten wurde die Verteilung von Tetracyclin und ^{14}C-Prolin mit dem Mineralinhalt des Zahnschmelzes vergleicht. Schliesslich wurden die Schnitte mit Hematoxylin und Eosin oder mit Millons Reagens für Tyrosin gefarbt.

Sowohl Tetracyclin als ^{14}C-Prolin wurden im gebildeten Zahnschmelz angesammelt, zeigten aber grosse Unterschiede in ihrer Verteilung. Nach einer Stunde waren beide in der Oberfläche des Zahnschmelzes unter den hohen Ameloblasten angereichert. Ausserdem war eine erhöhte Fluoreszenz von Tetracyclin unter den sich vermindernden Ameloblasten gefunden. Einen und vier Tage nach der Injektion gab es eine starke Fluoreszenzzunahme in dem ganzen unreifen Zahnschmelz. Ein umgekehrtes Verhältnis zwischen der Intensität der Fluoreszenz und der Mineralinhalt vorlag. Es schien aber eine ziemlich gute Übereinstimmung zwischen dem Zahnschmelz mit Fluoreszenz und den Gebieten mit einer positiven Millon Reaktion zu sein. Die Verteilung von ^{14}C-Prolin verblieb ziemlich unverändert während der vier Tage des Versuches.

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