The inhibitory effect of tetracycline on osteogenesis in organ culture

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Two characteristics of the tetracycline-group antibiotics have recently focused the special attention of teratologists: these drugs are known to pass readily across the placental ‘barrier’ (Charles, 1954; Frost & Villanueva, 1960; Gibbons & Reichelderfer, 1960; Hughes, Lee & Flood, 1965; Simpson, Burnette & Bawden, 1967), and they are incorporated into the foetal skeleton, where they may remain for long periods and can be detected owing to their brilliant fluorescence (Rall, Loo, Lane & Kelly, 1957; Milch, Rall & Tobie, 1957, 1958; Bevelander, Rolle & Cohlan, 1961; Urist & Ibsen, 1963; Michelson, 1964). The question of whether they exert a harmful effect on bone formation and mineralization in mammals, however, is still a matter of controversy. There is no doubt that tetracycline interferes with mineralization of the skeletal tissue in certain lower vertebrates (Bevelander, Goldberg & Nakahara, 1960; Bevelander & Cohlan, 1962), and prevents calcification of mammalian bones in organ culture (Saxén, 1966a, b). Reports of in vivo studies in mammalian species have yielded more confusing results.

In 1957, Filippi & Mela reported minor skeletal malformations in mouse foetuses after maternal tetracycline administration, and later Cohlan, Bevelander & Tiamsic (1963) observed depression of the growth rate of the long bones of premature children receiving high doses of tetracycline. Correspondingly, a reduction of the foetal weight has been reported to result from administration of tetracycline to the mother (Bevelander & Cohlan, 1962; Cohlan et al. 1963). On the other hand, neither experiments on rats nor observations on children have confirmed all these reports, as oxytetracycline appears to have no adverse effects on mineralization (Chu, O’Hara & Keitel, 1963; Likins & Pakis, 1965). In addition to differences in the dose and mode of administration, these conflicting results may also be due to differences between the various members of the
tetracycline group, as suggested by comparative studies both in vivo and in vitro (Owen, 1963; Hughes et al. 1965; Saxén, 1966b).

Consequently, the exact site of action of tetracycline and the mechanism by which it inhibits osteogenesis are not known. High concentrations of the drug in the culture medium of embryonic bones seem to interfere with the uptake of labelled thymidine and proline, suggesting that the synthesis of DNA and protein are affected (Bennet, Proffit & Norton, 1967), but the conclusion is based on experiments employing concentrations much higher than those needed to prevent cumulative calcium uptake and the elongation of the mineralized zone (Saxén, 1966b). In a recent study, the results suggested that tetracycline injected into pregnant rats inhibits the biosynthesis of collagen in the foetal bone and skin (Halme & Aer, 1968). The antibiotic action of tetracyclines has been attributed to several possible mechanisms. Interference with protein synthesis, inhibition of phosphorylation and of oxidation of glutamate, or inhibition of bacterial oxygen consumption have been suggested (see Goodman & Gilman, 1965). As a potent chelate, tetracycline may act directly by binding divalent cations and thus either interfering with the formation of bone mineral crystals or removing cations participating in enzymic processes (Ibsen & Urist, 1962; Finerman & Milch, 1963; Urist & Ibsen, 1963; Sternberg, 1966). At the moment, it has not been established which of these many alternatives are operative in the inhibitory action of mineralization and whether we are dealing with a multitude of different effects on the chain reaction, starting from the matrix synthesis and ending in the formation of bone minerals.

The mechanism of bone calcification process has been, until now, only partially elucidated. It includes, however, the formation of the osteoid matrix, where the protein-polysaccharides and collagen are the most important components. The sulphated mucopolysaccharides and collagen are both synthesized by the same cells in the embryonic cartilage (Godman & Porter, 1960; Prockop, Pettengill & Holtzer, 1964). The evidence indicates that gradual depolymerization of chondroitic mucopolysaccharides precedes mineralization (Gersh, 1952; Dziewiatkowski, 1966) and that there is a close parallel relation between collagen biosynthesis and mineralization in osteogenesis (cf. Glimcher, 1959; Laitinen, 1967; Bauer & Shtacher, 1968) and between collagen and mineral turnover in bone (Firschein, 1967). Collagen, which comprises about 95% of the organic bone matrix, contains about 14% of the hydroxyproline found almost exclusively in collagen and formed from proline by hydroxylation only (cf. Prockop & Kivirikko, 1967).

As previous experiments performed in organ cultures of embryonic bones have suggested the usefulness of this method, some of the unsolved problems related to the inhibitory action of tetracycline on osteogenesis were re-analysed in these conditions. It was hoped especially that these highly standardized conditions would allow comparative studies with different concentrations of the drug, and thus enable a distinction to be made between its different toxic and/or
more specific actions. To give a more detailed impression of the effects of organ culture on osteogenesis, a brief comparison of growth \textit{\textit{in vitro}} and \textit{\textit{in vivo}} will also be presented.

\textbf{MATERIAL AND METHODS}

\textit{Organ culture}

Random-bred Swiss mice were used throughout the work. The day 0 of pregnancy was determined by the presence of a vaginal plug. Bone rudiments from 16- to 17-day embryos were isolated under a dissecting microscope without enzyme treatment. The rudiments were carefully freed from as much of the surrounding tissue as possible without damage to the periosteum. The bones were cultivated in a chemically defined medium, BGJb (Biggers, Gwatkin & Heyner, 1961) supplemented with ascorbic acid, which has been shown to maintain good viability and growth of the skeletal tissues and to allow mineralization to take place (Reynolds, 1966; Saxén, 1966a, b). The culture dishes, each with ten rudiments and 10 ml of medium, were incubated in a humidified 5\% CO$_2$ atmosphere at 37 °C.

\textit{Tetracycline}

The purified tetracycline hydrochloride was acquired through the courtesy of the Pfizer Corporation. A stock solution was prepared by diluting dry powder in sterile distilled water. The pH remained constant in the buffered culture medium.

\textit{Measurements of length and weight}

The length of the mineralized zone was measured from drawings of individual bone rudiments made with a camera lucida, with which the dense calcified zone was clearly distinguishable from the translucent, non-mineralized epiphyseal parts of the bone. The actual lengths were calculated from these measurements. The dry weights of the rudiments were determined with a ‘Cahn’ electric micro-balance by a method described by Biggers (1960).

\textit{Histology}

The undecalcified rudiments were fixed in 10\% formalin or in Zenker solution. Histological preparations stained with H.E., toluidine blue, PAS and von Kossa stain were made by conventional methods.

\textit{Electron microscopy}

After cultivation, the bones were rinsed with phosphate-buffered saline, fixed for 30 min at 4 °C with 4\% glutaraldehyde in phosphate buffer at pH 7.2 and postfixed for 30 min with 1\% OsO$_4$ at the same temperature and in the same buffer (Millonig, 1962; Sabatini, Bensch & Barrnett, 1963). The fixed bones were
dehydrated in graded alcohol and embedded in Epon 812. Thin sections were cut with glass knives on a Porter–Plum MT-2 microtome, stained with lead citrate (Reynolds, 1963), and examined and photographed in a Philips 200 electron microscope.

Radioactive labelling

Sodium $[^{35}S]$sulphate (spec. act. 35-0 mCi/mm), $[^{45}Ca]$calcium chloride (spec. act. 8-25 and 11-8 mCi/mm), $[^{3}H]$thymidine (spec. act. 20600 mCi/mm) and $[^{3}H]$tetracycline hydrochloride (spec. act. 80-0 mCi/mm) were obtained from the New England Nuclear Corporation, Boston, Mass., and $[^{1-3}H]$proline (spec. act. 100 mCi/mm) from the Radiochemical Centre, Amersham, England. The label was either added to the original culture medium or in pulse experiments the unlabelled medium was replaced with fresh medium containing the label. The isotope concentrations used are indicated in the legends to the figures.

After labelling, the bone rudiments were harvested, washed for 15 min in cold distilled water and placed for 1 h in the counting vials to dry in an incubator at 60 °C. Hyamine hydroxide (Nuclear Enterprises, Sighthill, Edinburgh) was added for 10 min. Scintillation liquid (Nuclear Enterprises, Sighthill, Edinburgh) or PPO, 5 g/l, and dimethyl-POPOP, 0·3 g/l, in toluene was used as scintillation fluid. The radioactivity was determined by an ‘I.D.L., Tritomat 6020’ or ‘Packard Tricarb 3375’ liquid scintillation counter. For the determination of $^{46}Ca$- and $^{3}H$-activities in the double-labelling experiment, the bones were carefully washed after culturing with distilled water and hydrolysed in 6 N-HCl at 138 °C for 8 h. The hydrolysates were then evaporated to dryness and dissolved in water. $^{46}Ca$ content was then measured from the aliquots with a gas-flow counter (Tracerlab) after previous precipitation of calcium with ammonium oxalate. The supernatants obtained were further evaporated to dryness at 110 °C for 5 h and dissolved again in water. Samples of the aliquots were taken for assay of total $^{3}H$ content in Bray's solution and of the specific activity of hydroxyproline by the method of Peterkofsky & Prockop (1962) after previous separation of $[^{3}H]$proline from hydroxy$[^{3}H]$proline with a Dowex 50-X8 column (hydrogen form).

Figure 1

Comparison of mouse embryonic bones in vivo and in vitro and the effect of tetracycline on the morphology of bone. PAS stain. × 100.

(A) 16-day ulna.
(B) 17-day ulna.
(C) 16-day ulna cultivated for 12 days in vitro.
(D) 16-day ulna cultivated for 12 days in the presence of 1 μg/ml of tetracycline in vitro.
(E) As D, but tetracycline, 10·0 μg/ml.
Autoradiography

Autoradiographs were prepared from tissue sections, using the stripping film technique (Kodak AR-10). After 4 days exposure, the autoradiographs were developed and the tissue sections stained with haematoxylin.

Measurement of total calcium

The total calcium of the bones was determined by dry combustion in a maximal temperature of 600 °C. The ash was dissolved in concentrated HCl subsequently diluted with distilled water. The calcium was measured in a Beckman DU flame photometer at a wavelength of 422.7 nm. The determinations were performed from pooled samples of ten bones.

RESULTS

Comparison of development in vivo and in vitro

It was of interest to compare the extent of depression of development resulting from the culture conditions. In histological preparations made after different

Fig. 2. Comparison of the elongation of the mineralized zone of embryonic ulnae *in vivo* and *in vitro*. Cultivation was begun on the 16th day. (Each dot is based on measurements of 10–20 bones, mean ± S.D.)
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periods of cultivation (Fig. 1 A, B, C) it was clearly seen that the mitotic activity of the chondroblasts at the epiphyseal plate decreased during cultivation; the proliferating zone, which is 20–30 cell layers thick in vivo, diminished continually, until after 14–16 days in vitro only a few layers remained. The hypertrophy of the chondroblasts, which normally occurs towards the metaphysis, was also retarded; beyond the proliferating zone there existed a resting zone 10–30 cell layers thick, which was not found in vivo and where the cells were morphologically uniform. In the next zone the cells were flattened in the longitudinal plane, another phenomenon that could not be found in vivo. After this zone, the chondroblasts hypertrophied in a few layers as in vivo and the intercellular matrix lost the metachromatic stainability typical of chondroitic tissue. The periosteal cells and the matrix, as well as the osteoid tissue in the diaphysis, were like in vivo rudiments. In the cultivated bones no traces of haematopoietic tissue could be found. The length of the mineralized zone increased steadily during cultivation, although at a greatly depressed rate (Fig. 2). The length achieved in vivo during 1 1/2 days was reached after 12 days of cultivation. During a 12-day cultivation period the total calcium content of a tibia was about doubled, whereas in vivo it increased eightfold.

Localization of tetracycline in the bone

Tetracycline was localized in the bones by two methods: either by making use of the typical bright yellow fluorescence of the drug or by employing labelled tetracycline in autoradiography. When examined in ultraviolet light, the tetracycline fluorescence was found to be localized exclusively in the mineral of the diaphysis of bones grown in the presence of the drug. When subsequent counterstaining of the bones was performed by von Kossa’s method for calcium, a very good correlation was found between the tetracycline fluorescence and the stained calcium (Fig. 3 A, B, B'). Bones were also cultured in the presence of radioactive tetracycline for different periods of time. Autoradiographs prepared from them indicated that the drug is not incorporated into the epiphyseal cartilage. The first grains were noted in the zone of hypertrophied chondroblasts, where the first calcium incorporation likewise takes place, as judged by the distribution of the von Kossa stain. The bulk of the radioactive tetracycline was found in the mineralized diaphysis on the mineral crystals (Fig. 3C, D). The comparison of the U.V.-fluorescence and autoradiographic 45Ca grains in the same section showed an exact correspondence; the drug was localized on the mineralizing trabeculae (Fig. 4A, B).

Inhibition of calcification by tetracycline

The localization of the tetracycline fluorescence and the radioactivity to the calcifying areas of the bone matrix is accompanied by inhibition of mineralization, as has previously been reported (Saxén, 1965, 1966a, b). The inhibitory effect of tetracycline was further confirmed here by the use of randomized pairs
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of bones, consisting of the right and left long bones of the same embryos, one of each pair being chosen, according to random numbers, to be treated with tetracycline, while the other served as the control. With these pairs, the effect of the drug was assessed by the elongation of the mineralized zone and by the uptake of $^{45}$Ca. Fig. 5 shows the effect of different concentrations of tetracycline on the mineralization of 16-day mouse embryo ulnae cultivated for 11 days in the presence of 0.1 $\mu$Ci/ml $^{45}$Ca in the medium. The results indicate that in all bone pairs tetracycline causes inhibition in the elongation of the calcified zone and a decrease in the uptake of radiocalcium. A dose response is also presented, in which as little as 1 $\mu$g/ml tetracycline decreases the incorporation of $^{45}$Ca by 40 % while an effect of 70–75 % inhibition is reached with 10 $\mu$g/ml tetracycline. The depressive effect on the elongation of the calcified zone is even more pronounced (Fig. 6).

The effect of tetracycline on the synthesis of DNA

The effect of tetracycline on the synthesis of DNA was studied to establish the growth-depressing concentration of the drug. It can be seen (Fig. 7) that tetracycline up to 10 $\mu$g/ml had no effect on the uptake of labelled thymidine, whereas 100 $\mu$g/ml greatly depressed the synthesis of DNA. Recently, it has been found in a similar organ culture system, but with a different medium, that the same kind of reduction in the synthesis of DNA occurs in concentrations of tetracycline exceeding 50 $\mu$g/ml (Bennet et al. 1967).

The effect of tetracycline on the dry weight of the bone

To obtain further knowledge of the effect of tetracycline on the overall growth of bone rudiments in vitro, ulnar bones were cultivated in numbers of 5 to 10 for different periods of time in the presence of 100 $\mu$g/ml of tetracycline (Fig. 8). The results are expressed as the dry weight for an individual bone. The amount

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**FIGURE 3**

Localization and effect of tetracycline on bone.

(A) 16-day embryonic ulna grown for 5 days in culture, stained by von Kossa's method for calcium. × 100.

(B) Ultraviolet photograph of a similar bone to that in A, but grown in a medium containing 5 $\mu$g/ml tetracycline.

(B') The same section as in B after staining for calcium by von Kossa's method. The localization of the U.V.-fluorescence in the calcified area is clear.

(C) Autoradiograph of the epiphyseo-diaphyseal transition zone where calcium can be seen in B'. 16-day embryonic ulna grown for 6 days in the presence of 5 $\mu$g/ml of $[^{3}H]$tetracycline. The drug is seen to be localized in the calcifying matrix between the hypertrophied chondroblasts. × 1500.

(D) Autoradiograph of a periosteal section. The $[^{3}H]$tetracycline grains are localized on the foci of calcifying matrix. Insert shows source of material for C and D.
of tissue increased about 40% in the controls during the first 4 days, after which the rate of increase slowed down. The dry weight of the bones cultivated in tetracycline-containing medium increased only 10% during the first 4 days. Thereafter, a slow degradation of the already formed tissue seemed to take place and after 11 days of cultivation the weight was about the same as at the beginning. On the 11th day there was a difference of about 45% between the dry weights of the control and the experimental bones.

The effect of tetracycline on the morphology of the bone

The morphology of the cells in the various zones of ulnar bone rudiments was relatively little changed by different concentrations of tetracycline in the medium (Fig. 1C, D, E). The two additional zones of chondrocytes that were not seen in vivo and which were described above in the control cultures could still be found at the epiphysial plate after 12 days cultivation of 16-day embryonic bones in a medium containing 1·0 or 10·0 μg/ml of tetracycline. No intensely stained matrix was found between the hypertrophied chondrocytes after PAS staining, as reported by Rolle (1967). On the other hand, the von Kossa stain revealed slight amounts of calcium in these regions, as was also noticed by her. A poorly formed diaphyseal matrix of reduced density and a short mineralized zone were clearly seen in bones treated with 10·0 μg/ml of tetracycline. In sections stained with toluidine blue a gradual loss of metachromasia at the metaphysis with advancing age could be seen in the control and in the tetracycline-treated bones.

Electron microscopy of calcification under the influence of tetracycline

For the electron microscopic studies of the inhibition of calcification by tetracycline, ulnae of 16-day mouse embryos were cultured in the presence of 5 μg/ml tetracycline or as controls for varying lengths of time up to 13 days in vitro. Special attention was paid to the calcified parts where tetracycline was localized by its fluorescence and by autoradiographic methods as described above.

Tetracycline treatment did not seem to have any apparent effect on the ultrastructure of the osteoblasts in the area of calcification (Fig. 9A). The nuclei

**FIGURE 4**

Comparison of the distribution of tetracycline fluorescence and radiocalcium. A 16-day embryonic ulna was grown for 3 days in the presence of 10 μg/ml tetracycline and 0·02 μCi/ml ⁴⁶Ca. × 1500.

(A) Ultraviolet photograph of the bone, showing the localization of the typical tetracycline fluorescence.

(B) Autoradiograph of the same section as in A, showing the close correspondence of the localization of the newly deposited ⁴⁶Ca and tetracycline on the trabeculae of bone matrix.
Fig. 5. Inhibitory effect of different concentrations of tetracycline on the elongation of the mineralized zone and on the uptake of $^{45}{\text{Ca}}$ into bones cultured for 11 days. The choice of the experimental bone from the same embryo was randomized. The results are presented pair by pair. The percentage of inhibition is calculated from the bone pairs and expressed as pooled samples.
Fig. 6. Effect of tetracycline on the elongation of the calcified zone. 16-day embryonic ulnae were cultivated for different periods of time. (Each dot is based on determinations from 10–20 bones, mean ± S.D.)

Fig. 7. Effect of tetracycline on [³H]thymidine uptake. 16-day embryonic ulnae were cultivated for 5 and 11 days in the presence of 1.0, 10.0 and 100.0 μg/ml of tetracycline. At the end of the cultivation a 2 h pulse with [³H]thymidine, 5 μCi/ml, was given. (Each column is based on determinations of 10 bones, mean ± S.D.)
were without signs of damage, and the cytoplasm of the cells was rich in mitochondria. An especially noticeable feature was the large amount of rough-surfaced endoplasmic reticulum which was frequently distended and filled with homogeneous electron-dense material. Between the cells was a network of unoriented fibres, mostly lacking clear periodicity (Fig. 9B). Numerous circular sites of calcification of various sizes were in close relation to the matrix network. At higher magnification a very elaborate network of thicker 350–500 Å fibres and thinner 200 Å fibrils was revealed, with dense particles or granules of 200–500 Å in close association with the latter especially (Fig. 9B, Fig. 10A, B). Some granules contained radially arranged needles and appeared to be possible sites of onset of calcification. The granules often seemed to contain a lighter core and a more electron-dense ring in the periphery (Fig. 10B).

Electron micrographs of larger calcified sites showed a meshwork of partly aligned needles of high electron density. The needles were 20–40 Å in diameter.

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**Figure 9**

(A) Electron micrograph of embryonic ulna grown in organ culture for 13 days in the presence of 5 μg/ml tetracycline. The osteoblasts show no sign of a toxic effect of the drug. They have an elaborated distended endoplasmic reticulum. Abundant fibres are present in the matrix. Smaller and larger electron-dense calcified areas are seen adjacent to the fibres. × 7200.

(B) Higher magnification of an electron micrograph of the same specimen in which a network of thick fibres is visible with an adjoining calcified area consisting of small needle-type structures in a more heterogeneous substance. × 34000.
and embedded in a less electron-dense and more amorphous structure (Fig. 11 A). Compared with the tetracycline-treated bones, the electron microscopy of the calcified diaphysis in the control bones revealed a similar picture. The cells and the matrix components did not indicate any differences. The areas of calcification were more extensive and at higher magnification also had a more electron-dense appearance (Fig. 11 B).

The effect of tetracycline on the proteinpolysaccharides of the bone

The proteinpolysaccharides in the matrix of bone, which are mainly chondroitin sulphates, were labelled with radioactive sulphate. The $^{35}$SO$_4^-$ autoradiography showed grains distributed all over the embryonic bone, but most abundantly in the epiphyseal portions of the long bone after 6-day cultivation and a 24 h pulse. The type of distribution was to be expected, because of the great abundance of mucoproteins in the chondroitic intercellular matrix. No relation to the localization of tetracycline was seen. In order to find out whether tetracycline in concentrations effectively inhibiting calcification would have any influence on the chondroitin sulphate, which has been reported to undergo degradation during the mineralization process (Dziewiatkowski, 1966), the rudiments were cultured for 9 days in the presence of $^{35}$SO$_4^-$ and $^{45}$Ca. The length of the mineralized zone and the total calcium were determined from seven rudiments at the beginning and end of cultivation as well as the uptake of $^{35}$SO$_4^-$ and $^{45}$Ca (Fig. 12). Tetracycline, in concentrations of 1.0 and 10.0 $\mu$g/ml, had a markedly depressing effect on the growth of the mineralized zone, on the total calcium and on the uptake of $^{45}$Ca of the bone. It did not seem to have a significant influence on the uptake of $^{35}$SO$_4^-$. In an experiment elucidating the cumulative incorporation of $^{35}$SO$_4^-$, the bone rudiments were cultured for 1, 3, 6, 9, and 12 days with different concentrations of tetracycline (Fig. 13). The $^{35}$SO$_4^-$ radioactivity of the rudiments increased continuously until the 9th day of cultivation, and tetracycline seemed to have no effect on the amounts of acid mucopolysaccharides in the rudiments.

Figure 10

(A) Electron micrograph of an ulna cultivated for 13 days in the presence of 5 $\mu$g/ml tetracycline. Adjacent to the osteoblast an interlacing network of thin fibrils can be seen. Seemingly attached to the fibrils are irregularly shaped granules, which are also of varying size and electron density. $\times$ 34000.

(B) At a higher magnification a variety of different-sized particles are seen to be attached to the thin fibrils. The arrow marks a radial arrangement of electron-dense projections on one of the particles. $\times$ 130000.
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![Graph showing the effect of tetracycline on bone calcification](image)

**Fig. 12.** Effect of tetracycline on the uptake of $^{35}$SO$_4$ in concentrations inhibiting calcification. The results of three parallel experiments are included. The length of the mineralized zones of 7 ulnae were measured and the pooled sample then used for determination of total calcium. The uptakes of $^{45}$Ca and $^{35}$SO$_4$ were determined from 10 bones after a 24-h pulse of 0.02 μCi/ml or 0.2 μCi/ml respectively in different cultures (mean ± S.D.).

**The effect of tetracycline on the synthesis of collagen in the bone**

*In vitro* experiments in our laboratory concerning the influence of tetracycline on collagen biosynthesis suggested that the drug inhibits both the incorporation of proline into protocollagen and the hydroxylation of proline into hydroxyproline (Halme, Kivirikko, Kaitila & Saxén, 1969). The results also suggested that the tetracycline concentrations affecting collagen synthesis were higher than the lowest concentrations still inhibiting mineralization, and that, therefore, the final inhibition of osteogenesis can be obtained through two different pathways.

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**Figure 11**

(A) Calcified site in an ulna grown for 8 days in the presence of 5 μg/ml tetracycline. Longitudinal and cross sections of needle-type structures are seen embedded in a more heterogeneous material. ×335000.

(B) Extensive calcification in an ulna grown for 8 days *in vitro*. Thinner needles and thicker fibrils extend from dense areas of calcification. ×130000.
To analyse further this bimodal effect, a comparative study was made in which both collagen biosynthesis and calcification were measured in the same bones. Despite our recent finding, which showed the favourable effect of a daily change of the medium on collagen biosynthesis, this experiment was performed under conditions which were earlier tested and found suitable for mineralization, i.e. without the daily replacement (Saxen, 1966a). The bones were cultured for 4 and 8 days in the presence of different concentrations of tetracycline and double-labelled during the last 24 h with radioactive calcium and proline. The incorporation of $^{45}$Ca into bone rudiments was taken as a criterion for the rate of mineralization and the incorporation of $[^3H]$proline as hydroxy$[^3H]$-proline for the rate of collagen synthesis. The results showed (Table 1) that none of the tetracycline concentrations used caused significant changes in the rate of hydroxy$[^3H]$proline synthesis or in the total uptake of $^3$H. The incorporation of $^{45}$Ca was slightly depressed in rudiments cultured in the medium containing only 0.1 $\mu$g/ml of tetracycline. Compared with the control rudiments, the percentage incorporations of $^{45}$Ca on days 4 and 8 in dishes containing 1.0 $\mu$g/ml of tetracycline were 72 and 77 % and in the ones containing 10.0 $\mu$g/ml 40 and 31 %, respectively. Slight retardation in the growth of the rudiments was also observed in the dishes containing 10.0 $\mu$g/ml of tetracycline.

Table 1. Effect of tetracycline on the dry weight, mineralization, and collagen biosynthesis of bone after 4 and 8 days of culture

<table>
<thead>
<tr>
<th>Tetracycline $^{(\mu g/ml)}$</th>
<th>Dry weight $^{(cpm/mg bone)}$</th>
<th>$^{45}$Ca incorporation $^{(cpm x 10^2/mg)}$</th>
<th>$^3$H total incorporation $^{(cpm x 10^2/mg)}$</th>
<th>$[^3H]$hypro. spec. activ. $^{(cpm/\mu g)}$</th>
</tr>
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<tr>
<td>None</td>
<td>87</td>
<td>2000</td>
<td>404</td>
<td>529</td>
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<tr>
<td>0.1</td>
<td>90</td>
<td>1880</td>
<td>387</td>
<td>522</td>
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<td>1.0</td>
<td>78</td>
<td>1440</td>
<td>412</td>
<td>553</td>
</tr>
<tr>
<td>10.0</td>
<td>66</td>
<td>800</td>
<td>399</td>
<td>421</td>
</tr>
</tbody>
</table>

Cultures of 10 mouse embryonic ulnae were incubated under conditions described in the text. 24 h before harvesting the bones were transferred to a medium containing 1 $\mu$Ci of $[^3H]$proline and 0.1 $\mu$Ci of $^{45}$Ca per ml. The dry weight is expressed per bone.

**DISCUSSION**

Comparison between the development of the bones in our culture conditions and *in vivo* indicated a definite retardation, but suggested that developmental events followed a normal course during the first 8–10 days of organ culture. The experimental model-system thus appeared to be suitable for studies on the effect of certain exogenous factors and their modes of action, and was used to analyse the effect of tetracycline antibiotics. These have earlier been shown to have a specific affinity for calcifying tissues (Milch *et al.* 1957; Bevelander *et al.* 1960), and our results, obtained in fluorescence microscopy and in autoradio-
graphs of bones treated with [\textsuperscript{3}H]tetracycline, confirmed these observations. It has previously been shown that tetracycline may interfere with the calcification process both \textit{in vivo} and \textit{in vitro} (Introduction), and in the present study a significant inhibitory effect was observed with tetracycline concentrations as low as 1 \( \mu \text{g/ml} \).

**Fig. 13.** Effect of tetracycline on the cumulative uptake of \textsuperscript{35}S\textsubscript{4}. 16-day embryonic ulnae were cultivated for different periods of time in the presence of 0.1, 1.0 and 10.0 \( \mu \text{g/ml} \) tetracycline, and 1 \( \mu \text{Ci/ml} \textsuperscript{35}S\textsubscript{4} \). (Each column is based on 5 bones, mean \( \pm \) S.D.).

The above observations in light and electron microscopy, as well as the determination of thymidine uptake, suggested that the effect on mineralization of low tetracycline concentrations could not be attributed to an action affecting cell viability or proliferation. Hence attention was focused on the formation of the bone matrix, and the synthesis of its main organic components, protein-polysaccharides and collagen fibres.

The role of protein-polysaccharides in the calcification process is as yet obscure. It has been suggested that the ground substance polysaccharides might...
act as compatible polymer diluents or plasticizers for collagen structures (Milch, 1966). A remarkable loss of proteinpolysaccharides in the process of endochondral ossification has been noticed, probably due to a degrading protease-like enzyme (Dziewiatkowski, 1966). If it is assumed that calcification is preceded by this kind of degradation of acid mucopolysaccharides, it might be proposed that tetracyclines would prevent the diminution of the protein-polysaccharide content in bone rudiments when inhibiting calcification. As chondroitin sulphate contains abundant sulphur, radiosulphate might serve as a marker for the proteinpolysaccharides in the bone matrix. Under the conditions used, the concentration of tetracycline that was found to inhibit mineralization seemed to have no effect on the cumulative or short-term incorporation of radiosulphate. The results have thus given no proof of a relation between the inhibitory effect of tetracycline on osteogenesis and a specific influence on the proteinpolysaccharide degradation in the calcifying bone.

Our earlier quantitative evaluations on the effect of tetracycline on collagen biosynthesis have demonstrated a clear inhibition at high concentrations (Halme et al. 1969). Present results, on the other hand, indicate that inhibition of mineralization can be obtained with lower drug concentrations. Our double-labelling experiment recording the rate of mineralization and the synthesis of collagen in the same bone rudiments shows a clear inhibition of mineralization in concentrations of 1.0 and 10.0 μg/ml, whereas the synthesis of collagen seems to be unaffected.

Electron microscopy of cartilage calcification has been studied in detail recently (Anderson, 1967; Bonucci, 1967). Two structural components of the calcifying cartilage have attracted special interest: matrix fibres, presumably collagen, in amorphous background material (Martin, 1954; Robinson & Cameron, 1956; Scott & Pease, 1956; Godman & Porter, 1960), and dense matrix granules (Robinson & Cameron, 1956; Godman & Porter, 1960; Takuma, 1960; Revel & Hay, 1963). The latter have been shown by Matukas, Panner & Orbison (1967) preferentially to deposit colloidal iron, indicating that they contain acid mucopolysaccharide. No differences in these structures between tetracycline-treated and untreated bones were detected in this investigation. A close relationship between proteinpolysaccharide and collagen has been detected by chemical methods (Gross, Mathews & Dorfman, 1960; Campo & Dziewiatkowski, 1962), and in electron microscopy (Revel & Hay, 1963; Anderson, 1967; Matukas et al. 1967) fibrillar projections are seen to be attached to the granules. The tetracycline treatment in the present study did not cause any apparent changes in the relationship between the fibrils and granules.

The process of calcification has been reported to be associated with enlargement of the mucopolysaccharide granules in the zone of hypertrophic cells in the cartilage (Matukas et al. 1967), and calcification starts with the appearance of small needle-shaped apatite crystallites in these granules (Bonucci, 1967). The localization of the crystallites to the granules could also be established in the
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present case. Decalcification of ultrathin sections leaves an organic framework of strands within the previously calcified granule, which has the appearance of a 'ghost', showing the sites of the apatite needles (Bonucci, 1967).

The tetracycline-treated bones and their controls in this study revealed an apparently similar ultrastructure, with thicker (350–500 Å) fibres and thinner (200 Å) fibrils, amorphous ground substance and homogeneous (200–500 Å) granules, some of which contained electron-dense needlelike structures of 20–40 Å diameter within them or were more heavily calcified. In essence, the picture was like that in other cartilages or calcifying bones (Anderson, 1967; Bonucci, 1967; Matukas et al. 1967). The inhibition of calcification by tetracycline in bone rudiments, demonstrated by the experiment with randomized pairs of bones, was reflected in the ultrastructure only as a decrease in the number of foci of calcification present.

It has been shown that tetracyclines are bound to the calcium rather than to the organic matrix of bone (Finerman & Milch, 1963). Tetracycline has an affinity for collagen in vitro but only in the presence of calcium and possibly only by means of it (Jacobs, Harris, Katz & Glimcher, 1964). Constructed models of unit cells of hydroxyapatite and tetracycline molecules have given strong support for the view that tetracycline binds without steric strains to calcium atoms of bone apatite (Perrin, 1965). The inhibition of calcification by tetracycline could therefore well be a direct blocking effect of the further growth of a mineral crystal, like that suggested for inorganic pyrophosphate in regulating the initiation of calcification (Fleisch et al. 1966).

Our present observations on the inhibitory effect of low tetracycline concentrations on mineralization in bones, where the synthesis of the organic matrix was apparently unaffected, give support to the hypothesis of depressed growth of mineral crystals. In high concentrations impairment of osteogenesis seems to be due both to depressed matrix formation and to inhibited mineralization.

SUMMARY

1. The effect of tetracycline hydrochloride on osteogenesis was tested on mouse embryonic long bones cultivated in vitro in a chemically defined medium. In these conditions development was retarded, but morphological and electron microscopic studies suggested a normal course of differentiative events.

2. Tetracycline at concentrations exceeding 1 µg/ml had an inhibitory effect on bone mineralization as judged from measurements on the elongation of mineralized zone, the incorporation of radiocalcium and the increase in the total amount of calcium in the rudiments.

3. No specific morphological changes were detected by light or electron microscopy, apart from the decreased amount of histochemically stainable calcium and of electron-dense bone mineral crystals.

4. Incorporation of labelled thymidine was not affected by tetracycline
concentrations not exceeding 10 μg/ml, but was greatly inhibited at a concentration of 100 μg/ml.

5. Electron microscopy, histochemical stainings and determination of $^{35}$SO$_4$ incorporation suggested bone proteinpolysaccharides were unaffected in the presence of tetracycline at concentrations inhibiting calcification.

6. Negative results were also obtained as regards the inhibition of collagen synthesis by drug concentrations of 10 μg/ml or less.

7. The results suggested that low concentrations of tetracycline antibiotics prevent mineralization by exerting a direct action on the formation of bone mineral crystals. An increased concentration may lead to a similar end-result by affecting collagen biosynthesis, while still larger amounts of the drug interfere with DNA synthesis and cell proliferation as well as with general protein synthesis.

**RÉSUMÉ**

**Effet inhibiteur de la tétracycline sur l'ostéogenèse en culture d'organes**

1. L'action du chlorhydrate de tétracycline sur l'ostéogenèse a été éprouvé sur des os longs embryonnaires de souris cultivés in vitro dans un milieu chimiquement défini. Dans ces conditions, leur développement a été retardé, mais les recherches morphologiques et au microscope électronique suggèrent que les processus de différenciation se déroulent normalement.

2. À des concentrations supérieures à 1 μg/ml, la tétracycline a eu un effet inhibiteur sur la minéralisation de l'os, si on en juge d'après des mesures de l'élongation de la zone minéralisée, l'incorporation de radiocalcium et l'accroissement de la teneur globale en calcium des ébauches.

3. On n'a pas décelé de modifications morphologiques spécifiques au microscope ordinaire ou électronique, en dehors de la diminution de la teneur en calcium colorable histochimiquement et en cristaux minéraux osseux denses aux électrons.

4. L'incorporation de thymidine marquée n'a pas été affectée par des concentrations de tétracycline n'excédant pas 10 μg/ml, mais a été fortement inhibée à une concentration de 100 μg/ml.

5. La microscopie électronique, les colorations histochemiques et la détermination de l'incorporation de $^{35}$SO$_4$ permettent de penser que les polysaccharides protéiques de l'os ne sont pas affectés en présence de tétracycline à des concentrations inhibant la calcification.

6. On a également obtenu des résultats négatifs en ce qui concerne l'inhibition de la synthèse du collagène par des concentrations égales ou inférieures à 10 μg/ml.

7. Les résultats obtenus permettent de penser que de faibles concentrations de tétracycline empêchent la minéralisation en exerçant une action directe sur la formation des cristaux minéraux osseux. Une concentration accrue peut conduire à un résultat final similaire en affectant la biosynthèse du collagène, tandis
que des quantités encore plus élevées d’antibiotique interfèrent avec la synthèse du DNA et la prolifération cellulaire ainsi qu’avec les synthèses protéiques générales.

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