Tetracyclines and Mineralized Tissues: Review and Perspectives [1]

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INTRODUCTION

The tetracyclines have proved to be an extremely important group of drugs because of their wide range of antibiotic activity (1–3). (For general background, antibiotic activity, and pharmacology of the tetracyclines, we refer the reader to recent review articles by Weinstein (1), Kucers (3), Weisblum and Davies (4), and Garrod *et al.* (5).) In addition, they have the unique attribute of being incorporated into skeletal and dental tissues at sites of active mineralization (6–7), where they may be straightforwardly detected in both whole specimens and sections. Under ultraviolet irradiation, the tissues exhibit fluorescence—a characteristic intrinsic to the tetracycline molecule (8).

We will first briefly outline the components, mechanics, and metabolism of the several normal human mineralized tissues. The chemistry of tetracyclines will be discussed, followed by the biochemistry, with emphasis on their incorporation into calcified tissues and the information that such a phenomenon provides. We will consider mineral–tetracycline reactions, fluorescent labeling of bone and teeth, and the effects of tetracyclines on mineralized tissues at different levels. Observations made in our laboratory will illustrate and confirm several concepts found in the tetracycline literature. There are, as might be anticipated, further avenues of research to be explored.

MINERALIZED TISSUE DYNAMICS

Mineralized tissues, bones, and teeth, are characterized by an intimate mixture of organic and inorganic constituents (9, 10). The organic fraction of bone and the dentin and cementum portions of the tooth organ are predominantly ($> 90\%$) the asymmetric protein, collagen, plus minor glycoproteins and mucopolysaccharides. The inorganic fraction is a calcium phosphate whose crystal chemistry most closely approximates $Ca_{10}(PO_4)_6(OH)_2$, the mineral known as hydroxyapatite (HA). The proportion of inorganic to organic is predictable for particular mineralized tissues but is dependent on age or maturation level, systemic conditions, and/or any local pathological considerations for a specific sample. Enamel, the most highly mineralized tissue in the body ($> 98\%$ inorganic) is distinctive in having a noncolla-

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genous protein matrix and becoming acellular at maturity. Bone and cementum, 50–80% inorganic, normally contain actively metabolizing cells involved in the formation and remodelling of the tissue in response to growth, nutrition, and homeostasis. Dentine, about 70% inorganic, has minimal, if any, turnover once formed, but cells clustered in the pulp cavity have processes (in tubules) throughout which contribute to tissue viability.

Mineralized tissue formation is known to proceed from specialized tissue cells (osteoblasts, dentinoblasts, cementoblasts, ameloblasts) by elaboration of a protein-mucopolysaccharide matrix, which is extruded from the cell and subsequently mineralizes. The cells may become enveloped by the calcified matrix they produce, but they continue to metabolize, providing nutrients and fluid to the tissue from the blood via a system of anastamosing cell processes within canaliculi. Such cells are one example of the several different types found in calcified tissues and involved as functioning units in skeletal dynamics.

Highly mineralized cortical bone at maturity may contain elaborate super structures called osteons (Haversian systems). Embedded cells are arranged in a concentric pattern around a central canal that contains both arteries and venules. Osteons mature from the circumference toward the canal and may achieve a diameter of 100 μm. The structure may be altered or removed in time through vascular displacement resulting in overlapping (secondary) osteons. Trabecular or spongy bone may exhibit osteons, in the mineralized spicules of lamellar bone, but the predominant cell population and activity is concentrated at the interface between mineralized and unmineralized tissue. Osteoblastic activity or bone formation (increasing the mineralized tissue mass) may take place side by side with resorption by osteoclasts, cells that destroy the entire structure (inorganic plus organic). Bone tissue is dynamic, continually altering structurally both at cellular and intercellular sites. The rates of formation for bone and teeth and resorption for bone, as well as the individual cell activity and mineralization of the organic matrix of all calcified tissues, are influenced by hormones and other circulating factors, such as tetracycline, which will be discussed in this paper.

CHEMISTRY OF TETRACYCLINE

Molecular Structure

Tetracyclines are a family of compounds, basically polycyclic napthacene carboxamides (6). The gross structure of oxytetracycline was first determined by Woodward and collaborators (11) using chemical degradation of the compound and ultraviolet and infrared spectral and analytical techniques. X-ray crystallographic structural analyses on aureomycin hydrochloride (12) (= chlortetracycline) unambiguously demonstrated the concordance with terramycin hydrochloride (oxytetracycline) at the level of the configuration of asymmetric carbon atoms. Similarity in chemical and biological properties presumes this concordance.

The usual chemical presentation of the four-ring system of tetracyclines (Fig. 1A) does not successfully express the fact that the A-ring and its constituents are rotated with respect to the plane of the B-C-D ring. This structural characteristic, more obvious in the stereochemical presentation (Fig. 1B), is probably essential for the stability and activity of the molecule. Epimerization at C_4, which takes place rapidly under acidic conditions, results in almost complete loss (95%) of activity (13). The appropriate configuration of substituents on the A-ring is critical for antibiotic activity (14). The OH on C_6 (C-ring) is highly labile, responding to both acid
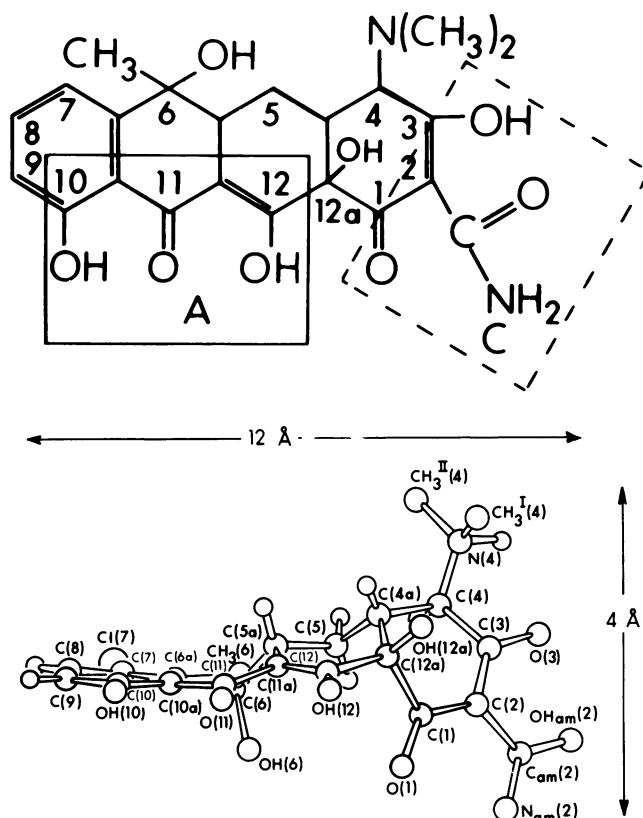
FIG. 1. (A) Tetracycline molecule with areas of potential metal cation chelation in boxes. (B) Stereo configuration of tetracycline after Donahue *et al.* (12).

and base degradations (6); this will lead to a relative loss of activity, as do any substitutions on the C_{11} or C_{12} atoms (6). Investigations into the tetracycline molecule (15) and its biosynthesis (16) have shown that the hydroxyl on C_{12a} is present early in the pathway and required for antibiotic function. In general, it has been demonstrated that structural variations at the C_{11}, C_{12}, C_{12a}, C_6, C_5, and C_4 sites may result in a loss of *in vitro* antibiotic activity of the molecule.

Molecular Reactions

No specific protein binding site has been designated on the molecule, but tetracyclines complex with aliphatic/carboxylic acids or urea derivatives in the molecular ratio of two tetracyclines to one (17). Further, the sites enclosed in boxes in Fig. 1A have been implicated in cation binding, which is of importance to *in vivo* antibiotic activity (18, 19). Electronic interaction is possible between these sites, from which it is inferred that complexation could take place at either or both. Mutual dependence and/or enhancement of cation binding for optimum molecular fit (and activity) *in vivo* is reminiscent of the stereochemical configurations "required" for enzyme catalysis (20). Modification of the basic molecule or of substituents to maintain molecular stability while maximizing antibiotic characteristics is only one aspect of the activity *in vivo* (6). Chemical and structural environment of the reaction site is probably equally important in the efficacy of these compounds as antibiotics (21).

*Fluorescence of the Molecule in the Presence of Cations and/or
Proteins and Its Stability*

Table 1 presents the absorption and emission (fluorescence) spectral maxima we observed on tetracycline hydrochloride and tetracycline hydrochloride in association with $CaCl_2$, $FeCl_2$, and $FeCl_3$ in methanol solutions. The inherent fluorescence of the tetracycline molecule is enhanced in the presence of $CaCl_2$ and decreased in the presence of both $FeCl_2$ and $FeCl_3$. A definite shift in ultraviolet absorption maxima was observed only at high $CaCl_2$ concentrations (10^{-2} M). Low concentrations (10^{-5}) of the cations were used for comparison fluorescence and absorption to minimize interference by Fe, which is obvious at higher concentrations. No significant changes in absorption maxima or fluorescence were noted even after 4 days of cation association with the tetracycline hydrochloride. The solutions were kept in the dark at low temperatures during that time. The ultraviolet absorption maxima determined by us for tetracycline hydrochloride are very similar to those given by Conover *et al.* (22) (see Table 1).

Tetracyclines are amphoteric and soluble in aqueous bases or acids. Acid tetracycline salts readily dissolve in most organic solvents (6) giving high fluorescence. The fluorescence of tetracyclines increases with the quantity of ethanol in solution concomitant with a blue shift of the fluorescent maxima (23). A slight red shift of the fluorescent maxima is found with metal cation–tetracycline complexes (23) (see Table 1). Increased fluorescence directly proportional to the concentration of magnesium (Mg) has been demonstrated (20). The presence of Mg ions enhances the fluorescence of tetracycline bound to ribosomes (24). Complexes of calcium and tetracycline show fluorescence (25, 26), which increases with concentration but not

TABLE 1

Ultraviolet[a] and Fluorescence Data on Tetracycline Hydrochloride and Tetracycline
Hydrochloride + Metal Chlorides

Sample	Ultraviolet				Fluorescence (relative I)
	λ maximum	Σ	λ maximum	Σ	
TC · HCl in MeOH Conover, 1953 (50)	363	13 800[b]	268	18 600[b]	N.D.
TC · HCl	370	13 742	273	15 857	24
after 4 days	370	13 646	273	16 145	
TC · HCl + CaCl₂ 10⁻²	377	15 760	277	14 031	
after 4 days	400	15 760	280	14 799	
TC · HCl + CaCl₂ 10⁻⁵	370	14 031	273	15 760	60
after 4 days	375	13 646	273	14 992	
TC · HCl + FeCl₂ 10⁻⁵	370	14 031	273	16 337	10
after 4 days	370	13 262	273	19 220	
TC · HCl + FeCl₃ 10⁻⁵	373	14 031	273	15 953	10
after 4 days	373	12 493	273	16 145	
TC · HCl + CaCl₂ 10⁻⁵ + FeCl₂ 10⁻⁵	373	14 031	275	16 145	45
after 4 days	375	12 685	274	16 337	
TC · HCl + CaCl₂ 10⁻⁵ + FeCl₃ 10⁻⁵	373	13 646	275	16 145	43
after 4 days	373	11 916	273	15 568	

[a]All analyses by Elaine Yamaguchi, Department of Chemistry, Yale University. Ultraviolet determinations were made using Beckman grating Spectrophotometer Model No. DBG, Σ (extinction coefficient) = A (absorption)/(C × 1) (cell length = 1 cm). Relative intensity of fluorescence was determined on Jarrell Ash Model 82-410 Fluorescence Emission Spectrometer at OD = 0.1 using quinine bisulfate (MW = 548.6) as standard. Values have been rounded to nearest whole number and did not change appreciably over the 4-day period.

[b]Converted from log Σ (50).

in a linear fashion. There is a drop in fluorescence at high calcium concentrations (10^{-2} M) (23). Fe^{3+}-plus-tetracycline complexes are not photochemically active (27). Examination of metal cation (Me)-plus-tetracycline (TC) precipitates are consistent with the metal ion occupying an octahedrally coordinated site (19) in the solid. A general formula for the complex $Me(TC)_2(H_2O)_2$ assumes that tetracycline is a bidentate group (19).

The association of iron plus tetracycline in aqueous solutions, especially Fe^{3+}, shows high stability, several orders of magnitude higher than other metal complexes (28). Heinrich and Oppitz (29) have shown that tetracycline inhibits iron absorption in man. The bacteriostatic activity of the drug in serum fell during ingestion of iron by patients. In view of the requirements of iron to sustain bacterial growth (30), perhaps part of the antibiotic activity of tetracycline is related to the formation of iron–tetracycline complexes, lowering the level of iron available to bacteria. Iron complexation (18) might explain the response of the gastrointestinal flora to tetracycline ingestion and the rapid reversibility of the effects upon cessation of use. Cation complexation and inhibition of protein synthesis could reduce bacterial proliferation. Radioactive tracer compounds might be useful to discriminate between mechanisms of action.

Complexes of tetracyclines and bovine serum albumin also exhibit enhanced fluorescence and a blue shift (23). Ten to 20 times higher fluorescence (quantum yield) has been found for tetracyclines in hydrophobic environments than in any of these solvents (31). Caswell (32) and others (33) have used tetracyclines as fluorescent probes, monitoring changes in divalent cation distribution in mitochondrial membranes, which upon energization, permit transport of ions.

MINERAL–TETRACYCLINE REACTIONS

In vitro studies show that crystals of $CaCO_3$ and HA bind tetracycline and exhibit fluorescence (34), but $CaSO_4 \cdot 2H_2O$ or $CaHPO_4 \cdot 2H_2O$ crystals do not (35). In the latter two cases, calcium is in a different crystal structural configuration which may make it less available to chelation by the tetracycline molecule. The formation of the normal inorganic mineral phase in statoliths and nematocysts, $CaSO_4 \cdot 2H_2O$, is inhibited by the presence of tetracycline, but tetracycline is not incorporated (35). Precipitation of HA is also inhibited by tetracycline, but high concentrations are required (36). At lower tetracycline concentrations, HA crystals, especially those with a Ca/P ratio of 1.61 (less than stoichiometric HA, 1.67), incorporate tetracycline in higher proportions than fully formed HA crystals exposed to tetracycline-containing solutions (36). Tetracycline also has been shown to inhibit the transition of amorphous to crystalline apatite (37).

A possible stereoconfiguration of tetracycline binding to HA has been proposed (38). The regions of the tetracycline molecule implicated in metal cation binding were outlined in Fig. 1A. Perrin (38) oriented the tetracycline molecule to superpose three calcium ions of the HA structure if HA is viewed in the c-axis projection (Fig. 2). Tetracycline replaces two PO_4 ions. The "O" of the C_{10}, C_{12}, and C_2 of tetracycline can "fit" three calcium ion positions of HA with minimal distortion while maintaining electric neutrality of the complex. The complex "presents" an essentially hydrophobic surface to the environment (38) minimizing dissociation in aqueous media. If the suggested orientation of bound tetracycline parallels the "c" axis of HA (the third dimension), the effect may be to substitute for part of the structure as depicted in Fig. 2.
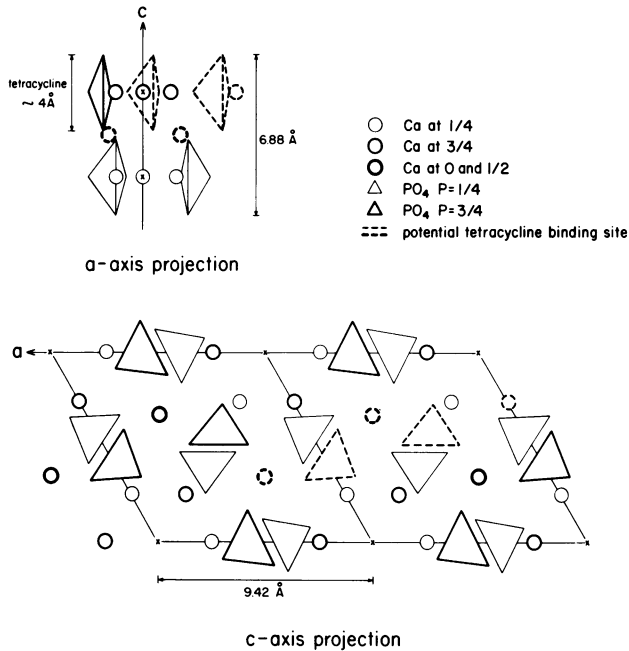
FIG. 2. Suggested association of tetraycline on the lattice of hydroxyapatite, after Perrin (38).

Anorganic bone, alcohol-ether extracted bone (39, 40), or excised dead bone (41) will exhibit fluorescence after soaking in tetracycline solutions, whereas bone treated with EDTA before or after tetracycline soaking will not fluoresce (39). Un-mineralized osteoid does not exhibit fluorescence (42), and *in vitro* studies confirmed that although collagen can bind tetracycline, the complex does not fluoresce (34). However some noncalcifying, fast growing tissues (nails, hair, tumors, and wounds) will fluoresce after long periods of tetracycline ingestion (39, 43).

TETRACYCLINE AS AN *IN VIVO* LABEL

The tetracyclines have been employed as antibiotics for various infections of bony tissues (44) and arthritic joints (45) and as a prophylactic measure preceding or-thopedic surgery (46), where selective localization in bone might aid their clin-ical effectiveness. However, it is the application in studies of skeletal dynamics that makes the tetracyclines a particularly exciting and unique class of drugs. Tetracy-clines have a distinctive nonantibiotic use as a fluorescent probe specific for areas of forming hard tissues (47, 48).

By combining ultraviolet microscopy with autoradiography ([45]Ca, ^{14}C, ^{3}H), mi-croradiography, or dyes such as Alizarin or lead acetate, it has been shown that tetracyclines localize at the site of developing hard tissues, both in the skeleton (48–55) and in the teeth (56–62). Tetracycline localization is demonstrated by the characteristic golden fluorescence of the intact hard tissues irradiated with ul-traviolet light (63). The fluorescence is qualitatively identical to that of the tetracy-cline solutions described above and in Table 1. The tetracycline derivative as well as the specific inorganic species influences the color and intensity of the fluorescence (64).

In the design of *in vivo* studies on hard tissues, careful attention is given to the choice of tetracycline derivative and the dose, duration, and mode of administration,

in order to minimize nonspecific binding and to obtain maximum fluorescence without disrupting mineralization. A lag time (2–3 days) to clear excess drugs is usual before excision of tissues (64, 65).[3] Optimal preparative procedures of the excised tissue (67) to retain distinct fluorescent label with minimal artifact suggests freeze-drying or immersion of the sample in alcohol (48, 68). The golden ultraviolet-induced fluorescence of the tetracycline-labeled areas is easily distinguished from autofluorescence of the tissue (49, 69). Comparison of the section exhibiting fluorescence with its microradiograph unambiguously defines forming mineral areas and is probably cautiously wise methodology (48) in using the label for quantitative measurements of bone formation.[4]

Embedded samples have shown no diminution of fluorescence after 6 yr (42), but there is evidence of some fading in bulk samples stored at room temperature and exposed to light (42). Photochemical oxidation of tetracyclines producing brown or yellow discoloration can be observed in some samples (43). Chemical changes after excision, such as degradation of the organic constituents in a sample, incomplete dehydration, or uptake of water from the atmosphere by the sample may also contribute to fading or loss of the tetracycline label (74).

Histological examination of undecalcified sections has shown that the tetracycline label does not cross osseous lamellae, cement, or other incremental growth lines (48). The width of a tetracycline label, besides depending on the duration of tetracycline ingestion and rate of mineralization of the organ at the site of sampling, is a function of the angle or direction of sectioning (75). Tetracycline marks all areas of active hard tissue formation (76) and, therefore, with appropriate experimental design and control, can be used reliably and accurately to determine the rate of hard tissue formation.

Tetracycline Label in Bone

Figure 3 illustrates tetracycline label in a ground section of bone as seen with ultraviolet microscopy. This specimen is a portion of rib from a normal dog given several doses of tetracycline intravenously. Each dose of tetracycline incorporated into a growing Haversian system produces a fluorescent circle conforming to the osteonal pattern. Calculations of accretion rates for bone have been devised by several investigators (70, 77). Frost and his collaborators have examined tetracycline-labeled rib and iliac-crest biopsies from normal individuals and patients with a variety of skeletal disorders. In a series of papers (77–85), bone dynamics for

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[3] A small proportion of the tetracycline label incorporated into bone may be recycled and redeposited (66) during this short cessation period.

[4] A typical protocol to obtain sharp tetracycline labels on forming mineralized tissues in adult animals is intravenous or intraperitoneal administration of tetracycline at a dose of 20 mg/kg of body wt/day for 3–6 days with cessation before excision of about 1 week. The time course depends on the purpose of the study and the metabolism of the patient or animal. After excision, soak the sample in 100% alcohol, embed, and section. Section thickness, dictated by the topic under investigation, may range from 10 to 12 μm to study high fluorescent single osteons, to 100 μm, for poorly labeled lamellar or woven bone. Ultraviolet, or white light with appropriate filters (Wratten 18a, 47, or 47a, between microscope and light source and Wratten 8, 9, or 2b or Illford 108 in the eye piece) (52, 70), provides the excitation wave length, approximately 360 cm^{-1} necessary for generating tetraycline fluorescence (6, 48, 41). Bone autofluorescence may be blue, white, or green depending on the filter system. Variations in the yellow color of tetracycline fluorescence, golden with chlortetracycline and green-gold with oxytetracycline, have been used to good advantage: one tetracycline to label the control, the other to label the experimental period (70). In other sequential studies more than two tetracycline varieties have been used, together with dyes (72). Some variations in shade of fluorescence may be related to the dose.
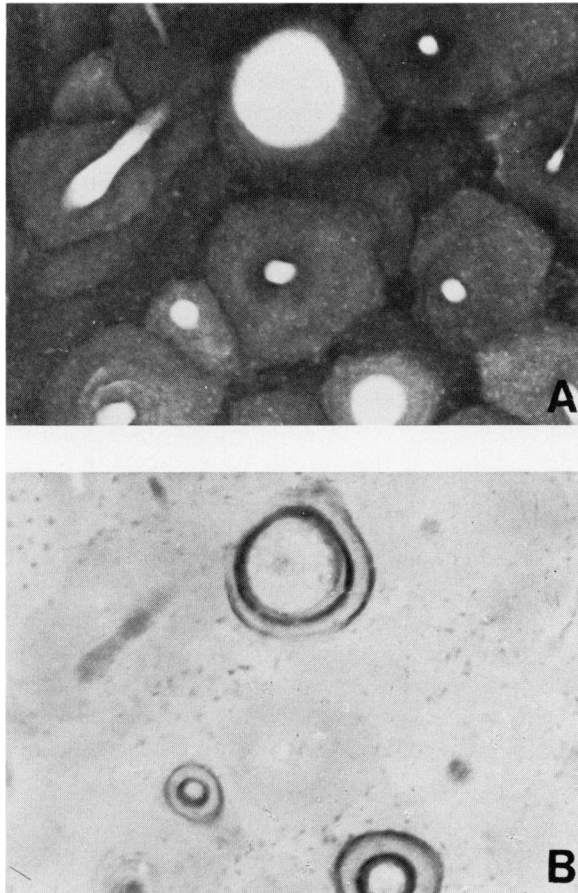
FIG. 3. Ground section' of rib bone from a normal dog given several doses of tetracycline intravenously. (A) Viewed with transmitted white light. (B) The same area under ultraviolet light shows three osteons, each with two fluorescent rings (dark circles). × 175. Photographs courtesy of Dr. J. P. Albright.

different metabolic situations have been discussed. Quantitative histological methods are applied to bone labeled *in vivo* with tetracycline. The rate of formation and quantity of bone within Haversian systems are measured. The cellular activity in several sections (number of cells actively metabolizing) are calculated and the results extrapolated to the organ level. Differences in bone activity at the several levels of resolution were observed and calculated between samples from patients and age-matched normals. These detailed investigations have led to suggestions on the form or type and on the level of the metabolic abnormality in patients. Disease states investigated by this group include: osteoporosis (78), osteomalacia (79), pseudohypoparathyroidism (80), acromegaly (81), primary hypophosphatemic rickets (82), osteogenesis imperfecta (83), osteopetrosis (84), and pycnodysostosis (85). The effects of fluoride (86) and acetyl salicylic acid (aspirin) (87) on bone have been investigated. In an editorial (88) that includes details of the methodology, the systematic errors and the distinction between cellular, tissue, and organ level dynamics, Frost presented averaged appositional rates for Haversian remodelling,

1.5–0.72 μm/day[5] in the normal rib, for periods from birth to age 89. Time for osteon formation over the same age range and grouping was 0.13–0.30 yr/osteon and a bone (rib, cortical) formation rate of 0.85–0.044 mm^2/mm^2/yr.

Tetracycline labeling of bones from normal animals has been used to confirm variable rates of bone formation within a bone at the level of individual osteons (89) and between trabecular and cortical bone (88). The thickness of osteoid seams has been estimated using the usual histologic techniques (90). Differences at the organ level, rib versus femur (34, 91) at the intraorgan level, metaphysis versus diaphysis (92), and the complicated cell-mediated-metabolic control of skeletal dynamics were known but perhaps insufficiently appreciated before the advent of tetracycline as a unique bone-seeking label and quantitative histological examination of bone biopsies (88, 93).

Tetracycline Label in Teeth

Although the use of tetracycline antibiotics has been curtailed, especially for the young, past use and incorporation in dental hard tissues are readily apparent. A recent article (94) graphically illustrates both the visible and the ultraviolet light macroappearance of tetracycline-affected whole teeth. Examination of ground sections of teeth that have incorporated tetracycline show sharp bands, permanent records of the specific time and duration of tetracycline ingestion (61). A voluminous literature exists on tetracyclines and human teeth (95–103), especially describing adverse cosmetic effects of the altered tooth enamel and fluorescent banding observed in ground sections. Most specimens gathered and studied have only one or a few bands. Weyman (95) reported from one to five bands per tooth in a series of 30 patients. Baker (104) reported on fluorescent bands in crowns. In a study of 83 teeth, 14 of the most seriously affected specimens averaged 8.9 lines per tooth.

Observations on Tetracycline in a Tooth Section

We obtained specimens of maxillary and mandibular third molars that must be almost unique.[6] Sixty distinct bands of tetracycline were observed within the dentin of a longitudinal ground section of the maxillary molar (Fig. 4). A regimen of oral tetracycline (Sumycin) in the usual dose range, repeated at intervals by this adolescent female virtually throughout the entire period of tooth gestation, resulted in serial incorporation expressed as individual bands. The tooth was anatomically well formed and virtually normal in color, lacking gross hypoplastic effects and having no obvious reduction in size. Under ultraviolet excitation, the yellow fluorescent bands in the section are striking. The two bands in the cuspal dentin (d) of the specimen (Fig. 5A) can be traced and shown to correspond developmentally to two bands in the enamel (e) by confluence at the dentino-enamel junction (DEJ) (Fig. 5A, arrow). In the dentin, the rootward bending of bands at the outer edge of the tooth crown, faithfully following the DEJ, as well as band curvatures in the more central part of the tooth, depicts the normal incremental pattern of dentin formation and mineralization (105). A few ultraviolet bands correspond to lines noted on the section when viewed with ordinary white transmitted light, indicating some local variations in mineralization. The width of most bands at the site of least sectioning

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[5] For details of calculations and rationale of the extrapolation from sequentially labeled Haversian systems to whole organ dynamics, the reader should consult the Frost articles, in particular (88).

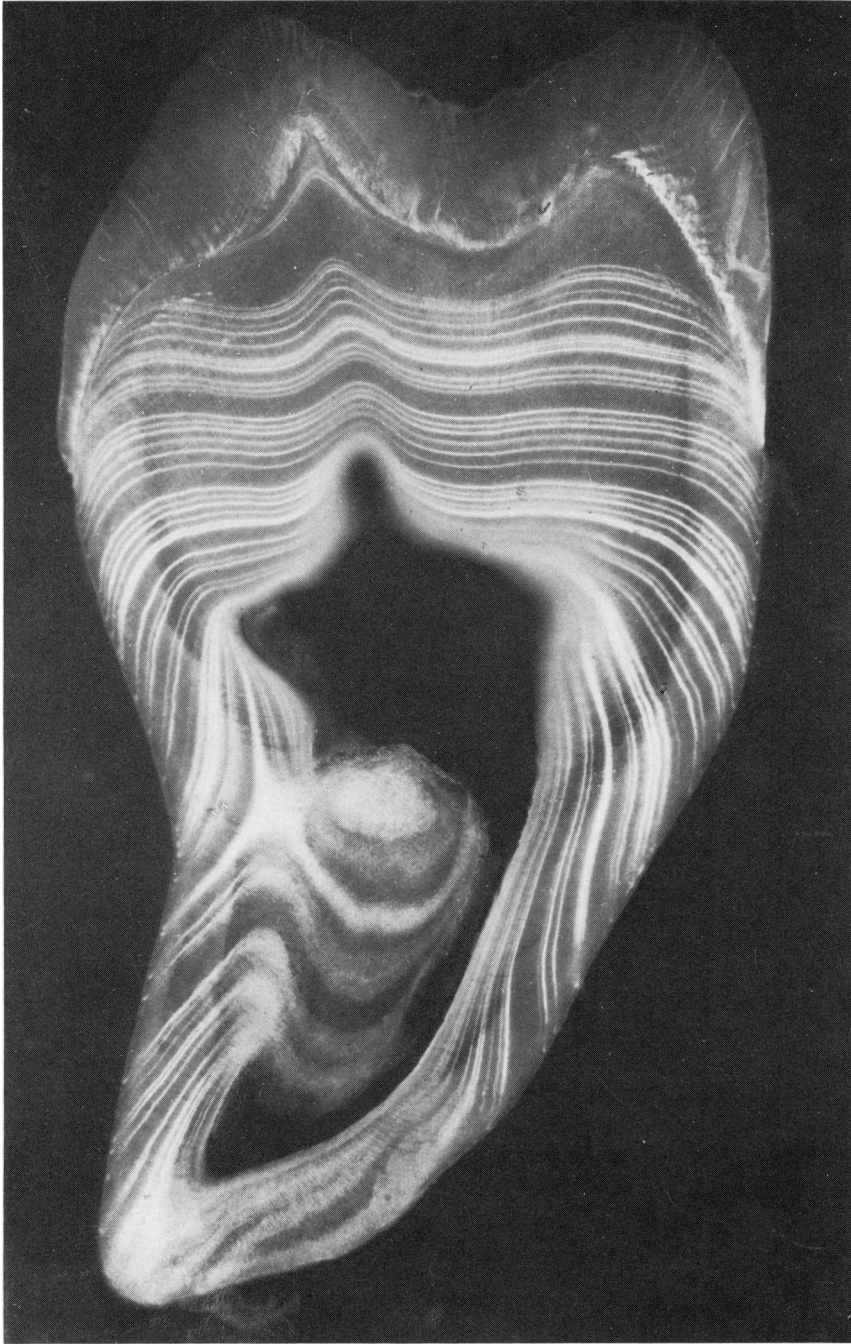[6] Courtesy of Dr. Stanton Fater, D.D.S., New Haven.

Figure 4A

FIG. 4. A ground section of a maxillary third molar from a 19-yr-old female known to have taken repeated oral doses of tetracycline. (A) Photographed with ultraviolet light. Bands of tetracycline fluorescence are prominent in the dentin. Enamel shows marked fluorescence near the DEJ. × 10. (B) Photographed with transmitted white light. Incremental lines are visible in the dentin and enamel, corresponding to tetracycline bands. × 4

distortion measured approximately 35 μm, which we infer corresponds to the period of ingestion of tetracycline (12 days). The 35μm value is exceedingly constant in the sample (except for a few narrower bands) and corroborates the rate of growth of dentin in molars postulated by Schour and Poncher (106), approximately 3 μm/day.[7]

Microscopic observations of the tooth section shown in Figs. 4 and 5 show several prominent lines of Retzius in the enamel and Von Ebener's lines in the dentin and some, but not an unusual amount of, interglobular dentin. These incremental lines corresponded to fluorescent bands seen with ultraviolet light. The following more detailed observations were made: (a) The pulpal aspect of the fluorescent bands was less sharply demarcated as compared to the peripheral side (Fig. 5). This and many of the following observations are expected, based on our knowledge of tooth embryology and the pharmacology of tetracyclines, and have been reported by others (45, 100, 107). (b) On close inspection, the bands often resolved into scalloped configurations (Fig. 5B) as if tetracycline incorporation occurred at the proximal surfaces of globules of calcification. In some cases the entire periphery of a globule was fluorescent. By first locating a globule under ultraviolet light, it was possible to revert to ordinary transmitted light and pinpoint the same globule, recognizing a subtle difference in the optical property of the dentin not readily distinguishable. (c) Some interglobular dentin revealed fluorescence even when not situated along the fluorescent bands. (d) The dentinal tubules showed a constant degree of fluorescence along their entire length. No disturbance, distortion, bifurcation, or blockage of any tubule was seen anywhere in the section examined by us. Observations to the

[7]The actual data given by Schour and Poncher (105) are for deciduous teeth; enamel, 3.9 μm/day; dentin, 3.8 μm/day; and permanent tooth enamel, 2.7 μm/day. We extrapolate that permanent dentin accretion should be somewhat less than 3 μm/day.
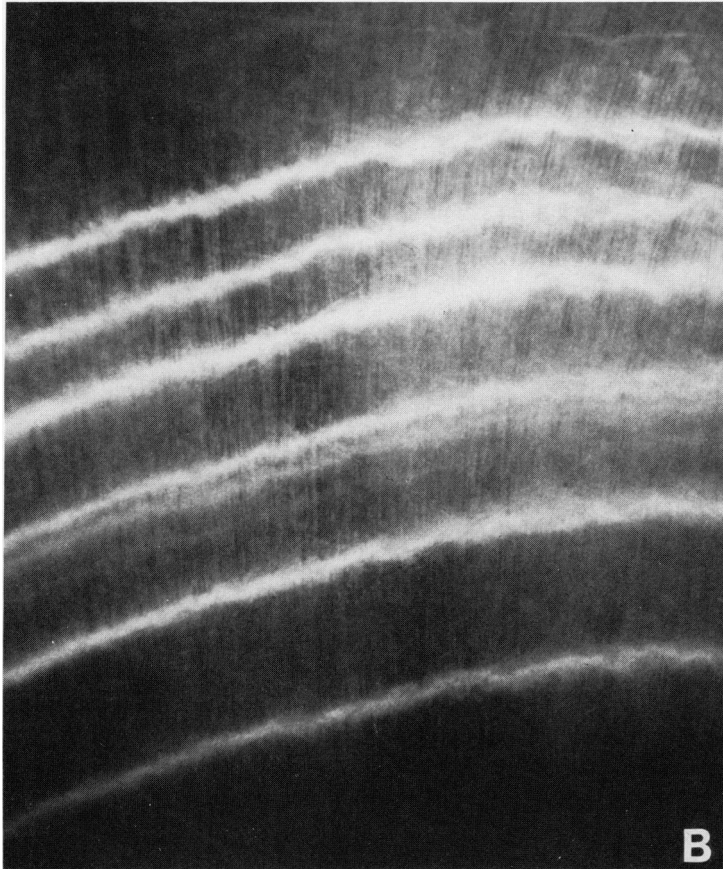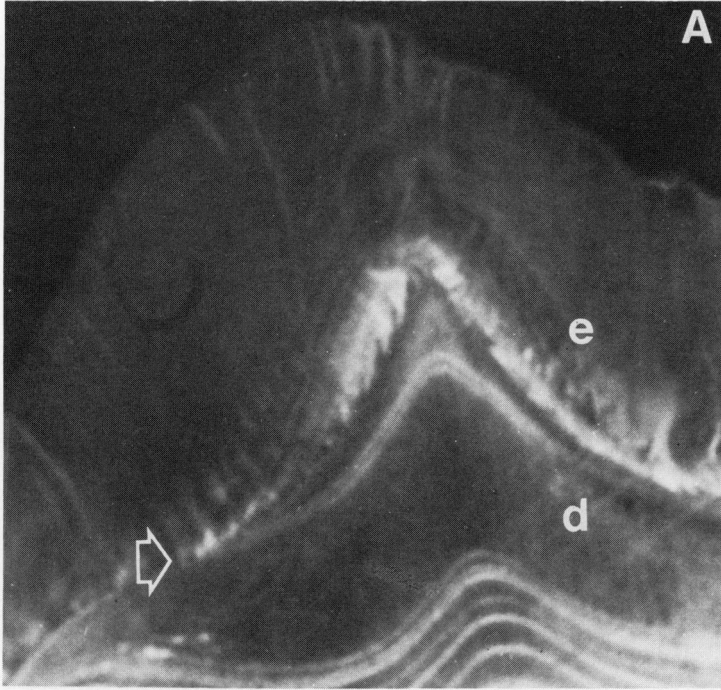
contrary have been recorded (108) and may very well depend on the individual dose level, total quantity ingested, and type of tetracycline. (e) The cementum was highly fluorescent throughout, with several bands distinguishable. (f) The two bands in the inner enamel had considerably reduced intensity relative to their corresponding dentin bands (Fig. 5A). The same was true for several short, faint bands seen in the cervical enamel, corresponding to a later series of dentin bands. Possible reasons for this difference are discussed below. (g) The most prominent fluorescence in the enamel was located along the DEJ with extensions outward in a pattern corresponding to Hunter–Schreger bands. The fluorescence was rather disseminated and diffuse. There was no fluorescence in the dentin that could be unambigously defined as corresponding to this zone in the enamel. (h) Enamel lamellae, tufts, and spindles also revealed some degree of fluorescence, with the spindles generally brighter than their dentinal counterparts.

Tetracycline Fluorescence in the Several Mineralized Tissue Systems

As confirmed in our tooth specimen (Figs. 4 and 5), tetracycline bands in the enamel exhibit less fluorescence than those in the dentin, or else they tend to be more diffuse (96, 104, 109). This may be related to differences in the development of the two tissues. With dentin, a small amount of matrix (predentin) is formed and then calcifies quite abruptly. In enamel, the initial influx of mineral into the matrix represents only a fraction of its ultimate complement, and there is a prolonged process of maturation as crystallites increase in size. Enamel crystals at maturity are several times those found in dentin. The smaller-size HA crystals in dentin imply greater surface area and presumably a larger number of potential tetracycline binding sites (25). There are other possible differences. The enamel mineral phase is distinct from that of dentin and bone not only in size and rate of formation but in composition (110, 111). Enamel not only has a higher proportion of mineral to organic matrix but the composition of the organic matrix is different from that in dentin and bone.

Examination of adult rat teeth and bone after tetracycline injection (109) showed that the incisors were more highly fluorescent than rat femurs. This is a demonstration of basic differences in response to tetracycline of two normal mineralized tissues and organs. Over a period of time, fluorescence in bone will diminish and virtually disappear, since bone tissue is a dynamic cellular system that not only deposits but may resorb and redeposit the extracellular material over a period of time within the organ. In fact, evidence has been presented to suggest that tetracycline deposited in the skeleton may be released in the course of remodelling, reenter the circulation, and be incorporated in other hard tissues, dentin, for example (66). Enamel and dentin, on the other hand, are not normally reworked (at the tissue level) once formed; hence, the initial tetracycline label is effectively permanent. The mechanisms of localization and concentration of tetracycline are probably distinctive for each of the calcified tissues.

THE NONFLUORESCENT EFFECTS OF TETRACYCLINE IN MINERALIZED TISSUES

The administration of tetracycline for antibiosis always presents a risk of disruption of the mineralizing skeletal tissues, although small for most instances. There is probably little way of distinguishing between the disruption of calcified tissues

FIG. 5. Higher magnification of portions of the tooth in Fig. 4A. (A) Two adjacent fluorescent lines in the dentin (d) converging (arrow) with less intense enamel (e) lines. × 20. (B) Wavy or saclloped tetracycline lines and tetracycline outlining dentinal tubules (perpendicular to tetracycline lines). ×80.

caused by tetracycline and that due to concomitant fever or other problems related
to the disease for which the antibiotic has been prescribed. At the cellular level, espe-
cially in bone where the structural evidence is not sequentially laid down, the disrup-
tions may be destroyed (reworked) in time. It is estimated that the average lifetime
of human bone tissue is 10 yr (112), but as mentioned above, the period of formation
of specific structures (Haversian systems or osteons) is of the order of days to
months (88, 111). The effects of tetracycline as well as traumatic systemic events
can be examined long after their incorporation in the mineralized tissue (114). For
example, the event of birth will be recorded in the developing teeth of the fetus (115).
Examination of the deciduous teeth years later, after exfoliation, may permit ob-
servation of this event. The ingestion of tetracyclines by an expectant mother, or by
any individual, is permanently recorded in teeth undergoing active formation at the
time of ingestion. There is a distinct advantage in studying teeth to determine the
nonfluorescent aspect of tetracycline incorporation in mineralized tissues.

Adverse side effects of the human use of the drug, such as tooth discoloration and
hypoplasia, are the result of tetracycline administered during the period of dental
development (47, 103). The relationship of tetracycline to incidence of caries (101–
103) and the devastation of the whole tooth organ with continual high doses of
tetracycline, such as those required in treatment of systemic diseases, for example,
cystic fibrosis (116) (see Fig. 6), have prompted in-depth studies of the effects on
teeth. Ultraviolet light, and electron microscopy, microradiography, and other
more subtle techniques (104, 117) have been utilized.

Gross examination of tetracycline-affected human enamel (104) showed (a)
hypoplasia associated with fluorescence, (b) tooth discoloration related to the
intensity of the fluorescence and the number of microscopically demonstrated
tetracycline episodes (bands) in the dentin, and (c) a lower specific gravity and mi-
crohardness for the severly discolored areas of enamel. X-ray microradiographs on
tissue sections confirm the correlation of hypoplasia and the location of tetracycline
fluorescence.

Ultrastructural studies on rat enamel by Omnell et al. (109) and Nylen et al. (117)
have shown that hypomineralized increments can be produced with single dose (30–
130 mg/kg body wt) and gross hypoplastic lesions with multiple (intraperitoneal) in-

FIG. 6. Permanent tooth discoloration and hypoplasia in a patient who was treated with tetracycline
for cystic fibrosis during the dental development period.

jections of tetracycline. There were disturbances in packing and organization of enamel prisms sometimes persistent beyond the period of tetracycline ingestion. Reduction in number, size, shape, and location of crystals, absence of mineral relative to organic, or the inverse, a hypermineralized layer, were the more drastic effects directly related to the size of the dose and/or multiple injections. Occasional areas of dense crystals were found, and within tetracycline bands, crystallites of thicker size were observed, relative to those outside the bands at the same developmental stage. Dense crystalline aggregates were also found along the DEJ, an otherwise normal area, adjacent to tetracycline bands. All are examples of a generalized disruption of mineralization. It is obvious that tetracycline ingestion, especially in high doses, interferes with normal enamel formation.

INTERPRETATION AND INTEGRATION OF THE OBSERVATIONS— A TETRACYCLINE "EFFECT"

The fact that enamel possesses a high degree of structure that is known in detail (118–121) enables differences in development to be recognized. Examination of dentin has not shown such striking morphological discrepancies between unaffected and affected teeth (96) except that at high doses dentinal tubules may be disrupted (108). Fluorescence is usually more prominent in dentin (not in predentin) than corresponding bands in the enamel (109). Enamel fluorescence may be weak or absent and observed only with multiple injections of tetracycline (109) or a high initial dose (117). The wavy structure of tetracycline bands in enamel and globular outlines in dentin support the suggestion that tetracycline incorporation is contemporaneous with ingestion and contiguous for the entire formative zone in these tissues. Further, since interband areas can be structurally normal and nonfluorescent, some of the tetracycline localization in mineralizing tissues is probably directly related to specific cellular activity.

The stage in the cycle of a cell, elaboration of matrix or maturation of that matrix, may be critical to the degree or level of tetracycline interference. Perhaps there are times when cells are more susceptible to tetracycline or perhaps the effects observed are more striking. Major morphological disruptions within the tooth organ, of dentinal tubules or enamel prisms, suggest interference at the time of matrix formation. The effect may be in the mode described for antibiotic action: "interruption of the elaboration of protein." The effect appears reversible for most tetracycline derivatives, provided the (adult human) dose is less than 1 g/day (approximately 10 mg/kg body wt) and of a duration less than 2 weeks. The dose threshold for morphologic disturbance may be distinctive from the dose affecting crystalline maturation. Maturation interference may be expressed as a hypoplastic or hyperplastic layer. A hypoplastic layer will be observed only if the sample is excised and examined a short time after the tetracycline insult or if the cell does not complete maturation in the area. The hypoplastic layer may be reworked, especially in enamel (117), and a hypermineralized zone may be formed following tetracycline insult.

The metabolism of tetracycline by the cell is influenced not only by the amount but by the variety and the method of administration (122–124). Subcutaneous and certainly oral doses of the antibiotic may circulate longer than an intravenous injection, although the latter mode would achieve higher blood concentrations most rapidly. The severity of the effects of tetracycline, intensity of fluorescence, and width of fluorescent bands are related to the variety of tetracycline, size of dose,

mode of delivery, duration of ingestion, and specific metabolic stage and physical and chemical environment of the cells. The effects are unique for each mineralizing tissue.

CONCLUSIONS AND DISCUSSION

The proposed mechanisms of antibiotic action of tetracyclines are at least twofold (bifaceted) and related to the stereochemistry of the molecule (125, 131): (a) Tetracycline sterically inhibits the elaboration of normal bacterial protein by binding to the 30 S ribosome. (b) Other sites on the tetracycline molecule may chelate metal cations essential for bacterial cell metabolism, thereby depressing growth. Both "activities" are potentially reversible for a bacterial population, and the sensitivity of a specific bacterial stain within the population may partially depend on the stage of the bacterial cell cycle as well as the titer and transport characteristics of the particular tetracycline derivative.

Partition, concentration, and fixation of tetracycline at the site of forming mineralized tissues may also be a combination of these two molecular level options. The "blast" activity of the several mineralizing cell systems rapidly synthesizes protein in the early stages, elaborating matrix. Matrix can become "deranged" morphologically in the presence of tetracyclines (although derangement can also be caused by other factors). The degree of derangement is related to the level, timing, and duration of the tetracycline dose. Mineralization of the matrix of vertebrate hard tissue is inhibited in the presence of tetracycline (132) and is thought to be due to the tetracycline chelation of calcium. The "incorporation" of tetracycline with specific mineral species, $CaCO_3$ and HA, has been documented and may be essential to the mechanism by which tetracycline is "fixed" in mineralized tissues.

Tetracycline presence in tissues can be detected by the characteristic golden fluorescence, an intrinsic property of the molecule and certain metal–tetracycline complexes, on ultraviolet excitation. It has been shown that tetracycline is a specific label for actively mineralizing sites in hard tissues. Collagen can bind tetracycline *in vitro,* but the fact that unmineralized osteoid (*in vivo*) fluoresces only immediately after the dose, not following a period of cessation, begs the question of whether tetracycline is complexed to osteoid in a minimally or nonfluorescent form (Fe^{3+} + TC, perhaps) or not at all. Excised bone and dead bone soaked in tetracycline solutions exhibit the fluorescent label on exposed surfaces.

We can speculate that the rapid collagen synthesis stage (which has Fe^{2+} as cofactor) (132) of "blast" cells in mineralizing systems may be effective in concentrating tetracycline from the serum. The presence of tetracycline may interfere with the elaboration of protein either producing an "abnormal" collagen or by altering the rate. In the formation of the extracellular matrix, some tetracycline may be carried along or additional extracellular tetracycline may become associated with the matrix. Calcium ions at the site of mineralization may be chelated by the extracellular tetracycline, and any crystallites forming on or within the collagenous matrix may be the foci for tetracycline incorporation. A Ca-plus-tetracycline complex is stable and would remain until the entire structure of the tissue, organic and inorganic, disintegrates or is reworked.

The concentration of tetracycline taken up by the different mineralizing cell systems—enamel, bone, or dentin—may be a reflection of the distinctive protein produced by these systems (and cofactors required) and/or the amount of calcium or other ions available for chelating tetracycline. The permanence of the tetracycline is dictated by the biology of the cell system (i.e., absence of remodeling). Differential

fluorescence in tissues implies differences in the particular hard tissue system and in the amount of tetracycline available. However, lack of fluorescence should not be taken as assured absence of tetracycline, i.e., fluorescence is an inadequate base on which to establish a quantitative assay for tetracycline (134–136).

Future work elucidating the precise metabolism of tetracycline, as well as concentration and localization (immobilization) mechanisms, must be undertaken. Further, the effects of tetracycline on different mineralizing cell systems so precisely documented morphologically on enamel should be extended to dentin and bone. Integrating the tetracycline biochemistry with morphological effects will add insight to our understanding of the chemical reactions of mineralizing systems at the cellular level. This knowledge is basic to, and will complement and extend, the elegant use of tetracycline as a "tree ring" marker in interpreting the kinetics of bone metabolism.

ACKNOWLEDGMENTS

REFERENCES

1. Weinstein, L., IV the Tetracyclines: Chlortetracycline, Oxytetracycline, Tetracycline, and Demethylchlortetracycline. *In* "The Pharmacological Basis of Therapeutics" (L. G. Goodman and A. Gilman, Eds.), 3rd ed., p. 1242. Macmillan, New York, 1968.

2. Dowling, H. F., "Tetracycline." Medical Encyclopedia, New York, 1955.

3. Kucers, A., Tetracycline. *In* "The Use of Antibiotics," p. 271. Lippincott, Philadelphia, 1972.

4. Weisblum, B., and Davies, J., Antibiotic inhibitors of the bacterial ribosome. *Bacteriol. Rev.* 32; 493, 1968.

5. Garrod, L. P., Lambert, H. P., and O'Grady, F., "Antibiotic and Chemotherapy," 4th ed. Churchill–Livingstone, Edinburgh, 1973.

6. Boyne, P. J., The use of tetracycline in studies of bone healing. *Advan. Oral Biol.* 3; 121, 1968.

7. Storey, E., Tetracycline antibiotics and their effects on calcified and non-calcified tissues. *Australas. Ann. Med.* 12; 325, 1963.

8. Blackwood, R. K., Tetracyclines, Encycl. Chem. Tech. 20; 325, 1963

9. Bourne, Geoffrey H. (Ed.) "The Biochemistry and Physiology of Bone," Vols. I–III, 2nd ed. Academic Press, New York, 1972.

10. Miles, A. E. W., (Ed.) "Structural and Chemical Organization of Teeth," Vols. I, II. Academic Press, New York, 1967.

11. Hochstein, F. A., Stephens, C. R., Conover, L. H., Regna, P. P., Pasternack, R., Brunings, K. J., and Woodward, R., Terramycin VII. The structure of terramycin. *J. Amer. Chem. Soc.* 74; 3708, 1952.

12. Donahue, J., Dunitz, J. D., Trueblood, K. N., and Webster, M. S., The crystal structure of aureomycin (chlortetracycline) hydrochloride. Configuration, bond distances and conformation. *J. Amer. Chem. Soc.* 85; 851, 1963.

13. Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M., and Waring, H., "The Molecular Basis of Antibiotic Action," p. 315. Wiley, London, 1972.

14. Stephens, C. R., Conover, L. H., Gordon, P. N., Pennington, F. C., Wagner, R. L., Brunings, K. J., and Pilgrim, F. J., Epitetracycline—the chemical relationship between tetracycline and "Quatrimycin." *J. Amer. Chem. Soc.* 78; 1515, 1956.

15. Von Dreele, R. B. and Hughes R. E. Crystal and molecular structure of S,12a diacetyloxytetracycline. *J. Amer. Chem. Soc.* 93; 7290, 1971.

16. Money, T., and Scott, A., Recent advances in chemistry and biochemistry of tetracyclines. *Progr. Org. Chem.* 7; 1, 1968.

17. Inouye, S., and Iitaka, Y., Crystallographic data on the molecular complexes of tetracycline salts. *Acta Crystallogr.* **17**; 207, 1964.

18. Weinberg, E., The mutual effects of antimicrobial compounds and metallic cations. *Bacteriol. Rev.* **21**; 46, 1957.

19. Baker, W. A., Jr., and Brown, P. M., Metal binding in tetracyclines. Cobalt (II) and nickel (II) complexes. *J. Amer. Chem. Soc.* **88**; 1314, 1966.

20. Sompolinsky, D., and Samra, Z., Influence of magnesium and manganese on some biological and physical properties of tetracycline. *J. Bacteriol.* **110**; 468, 1972.

21. Hlavka, J. L., and Boothe, J. H., The tetracyclines. *Progr. Drug Res.* **17**; 211, 1973.

22. Conover, L. H., Moreland, W. T., English, A. R., Stephens, C. R., and Pilgrim, F. J., Terramycin, XI. Tetracycline. *J. Amer. Chem. Soc.* **75**; 4622, 1953.

23. Popov, P. G., Vaptzarova, K. I., Kossekova, G. P., and Nikolov, T. K., Fluorometric study of tetracycline–bovine serum albumin interaction. The tetracyclines—a new class of fluorescent probes. *Biochem. Pharmacol.* **21**; 2363, 1972.

24. Hash, J. H., Antibiotics mechanisms. *Annu. Rev. Pharmacol.* **12**; 35, 1972.

25. Ibsen, K. H., and Urist, M. R., Complexes of calcium magnesium with oxytetracycline. *Proc. Soc. Exp. Biol. Med.* **109**; 797, 1962.

26. Finerman, G. A., and Milch, R. A., *In vitro* binding of tetracycline to calcium. *Nature London* **198**; 486, 1963.

27. Calvert, J., and Pitts, J., "Photochemistry." Wiley, New York, 1966.

28. Albert, A., and Rees, C. W., Avidity of the tetracyclines for the cations of metals. *Nature London* **177**; 433, 1956.

29. Heinrich, H. C., and Oppitz, K. H., Tetracycline inhibits iron absorption in man. *Naturwissenschaften* **60**; 524, 1973.

30. Weinberg, E. D., Iron and susceptibility to infectious disease. *Science* **184**; 952, 1974.

31. Caswell, A. H., and Hutchinson, J. D., Selectivity of cation chelation to tetracyclines: Evidence for special conformation of calcium chelate. *Biochem. Biophys. Res. Commun.* **43**; 625, 1971.

32. Caswell, A. H., The migration of divalent cations in mitochondria visualized by a fluorescent chelate probe. *J. Membr. Biol.* **7**; 345, 1972

33. Brewer, G., Chlorotetracycline as a fluorescent probe for membrane events in the action of Colicin K on Escherichia Coli. *Biochem.* **24**; (13) 5038, 1974.

34. Ibsen, K. H., and Urist, M. R., Competition between oxytetracycline and citrate for binding sites on hydroxyapatite crystals. *Calcif. Tissue Res.* **1**; 243, 1967.

35. Spangenberg, D. B., and Beck, C. W., Tetracycline effects on statolith and nematocyst differentiation in *Aurelia. Calcif. Tissue Res.* **9**; 122, 1972.

36. Urist, M. R., and Ibsen, K. H., Chemical reactivity of mineralized tissue with oxytetracycline. *Arch. Pathol.* **76**; 484, 1963.

37. West, V. C., and Storey, E., The *in vitro* effect of fluoride and tetracycline on phase transformation of calcium phosphate. *Calcif. Tissue Res.* **9**; 207, 1972.

38. Perrin, D. D., Binding of tetracyclines to bone. *Nature London* **208**; 787, 1965.

39. Milch, R. A., Tobie, J. E., and Robinson, R. A., A microscopic study of tetracycline localization in skeletal neoplasms. *J. Histochem. Cytochem.* **9**; 261, 1961.

40. Milch, R. A., Rall, D. P., and Tobie, J. E., Fluorescence of tetracycline antibiotics in bone. *J. Bone Joint Surg.* **40A**; 897, 1958.

41. Deleau, J., and Bohr, H., Uptake of tetracycline by human bone *in vitro. Nature London* **204**; 1103, 1964.

42. Frost, H. M., Villanueva, A. R., and Roth, H., Measurement of bone formation in a 57 year old man by means of tetracyclines. *Henry Ford Hosp. Med. Bull.* **8**; 239, 1960.

43. Douglas, A. C., The deposition of tetracycline in human nails and teeth: A complication of long term treatment. *Brit. J. Dis. Chest* **57**; 44, 1963.

44. MacAusland, W. R., Jr., and Eaton, R. G., The management of sepsis following intramedullary fixation for fractures of the femur. *J. Bone Joint Surg.* **45A**; 1643, 1963.

45. Cobbs, C. G., and Kaye, D., Arthritis in infectious diseases. *Clin. Orthop.* **57**; 57, 1968.

46. Bingham, R., Fleenor, W. H., and Church, S., The local use of antibiotics to prevent wound infection. *Clin. Orthop.* **99**; 194, 1974.

47. Wallman, I. S., and Hilton, H. B., Teeth pigmented by tetracycline. *Lancet* **I**; 827, 1962.

48. Harris, W. H., Jackson, R. H., and Jowsey, J., The *in vivo* distribution of tetracyclines in canine bone. *J. Bone Joint Surg.* **44A**; 1308, 1962.

49. Frost, H. M., and Villaneuva, A. R., Tetracycline staining of newly forming bone and mineralizing cartilage *in vivo*. *Stain Technol.* **35;** 135, 1960.

50. Yen, P. K., and Shaw, J. H., Effects of tetracycline on membranous bone growth and dentin apposition in young Rhesus monkeys. *J. Dent. Res.* **53;** 897, 1974.

51. Frost, H. M., Tetracycline labelling of bone and the zone of demarcation of osteoid seams. *Can. J. Biochem. Physiol.* **40;** 485, 1962.

52. Frost, H. M., An economical microfluorescence set-up for detection of tetracyclines in bone. *Henry Ford Hosp. Med. Bull.* **8;** 197, 1960.

53. Frost, H. M., Human osteoblastic activity—part II. Measurement of the biological half-life of bones with the aid of tetracyclines. *Henry Ford Hosp. Med. Bull.* **9;** 87, 1961.

54. Saxen, L., Tetracycline: Effect on osteogenesis *in vitro*. *Science* **149;** 870, 1965.

55. Kaitila, I., Effect of tetracycline on mineralization in cycloheximide treated bones *in vitro*. *Calcif. Tissue Res.* **7;** 46, 1971.

56. Bevelander, G., Rolle, G. K., and Cohlan, S. Q., The effect of the administration of tetracycline on the development of teeth. *J. Dent. Res.* **40;** 1020, 1961.

57. Antalovska, Z., Jirkalova, V., Lonska, V., and Strnad, L., Uber die Beziehungen zwischen Fluoreszenz, Menge und biologischer Aktivitat der in Zahnhartgeweben fixierten Tetrazyklinantibiotiko. *Deutsch. Stomat.* **17;** 184, 1967.

58. Antalovska, Z., Laws of tetracycline antibiotic deposition in rat incisors. *J. Dent. Res.* **45;** 1430, 1966.

59. Hennon, D. K., Dental aspects of tetracycline therapy: Literature review and results of a prevalence survey. *J. Ind. Dent. Ass.* **44;** 484, 1965.

60. Ibsen, K. H., Urist, M. R., and Sognnaes, R. F., Differences among tetracyclines with respect to the staining of teeth. *J. Pediatr.* **67;** 459, 1965.

61. Tetracyclines and the teeth. *Lancet* **I;** 917, 1966. (Lead article.)

62. Owen, L. N., The effects of administering tetracyclines to young dogs with particular reference to localization of the drugs in the teeth. *Arch. Oral Biol.* **8;** 715, 1963.

63. Bevelander, G., The effect of tetracycline on mineralization and growth. *Advan. Oral Biol.* **1;** 205, 1964.

64. Harris, W. H., A microscopic method of determining rates of bone growth. *London Nature* **188;** 1038, 1960.

65. Epker, B. N., Tetracycline in human dentin: Deposition in the zone of mineralization. *J. Dent. Res.* **45;** 1825, 1966.

66. Stewart, D. J., The re-incorporation in calcified tissues of tetracycline released following its deposition in the bones of rats. *Arch. Oral Biol.* **18;** 759, 1973.

67. Frost, H. M., Preparation of thin undecalcified bone sections by rapid manual method. *Stain Technol.* **33;** 273, 1958.

68. Hulth, A., and Olerud, S., Tetracycline labelling of growing bone. Acta *Soc. Med. Upsal.* **67;** 219, 1962.

69. Milch, R. A., Rall, D. P., and Tobie, J. E., Bone localization of the tetracyclines. *J. Nat. Cancer Inst.* **19;** 87, 1957.

70. Harris, W. H., and Weinberg, E. H., Microscopic method of measuring increases in cortical bone volume and mass. *Calcif. Tissue Res.* **8;** 190, 1972.

71. Kuper, S. W., and May, J. R., Detection of acid-fast organisms in tissue sections by fluorescence microscopy. *J. Pathol. Bacteriol.* **79;** 59, 1960.

72. Olerud, S., and Lorenzi, G. L., Triple fluoroschrome labeling in bone formation and bone resorption. *J. Bone Joint Surg.* **52A;** 274, 1970.

73. Harcourt, J. K., Johnson, N. W., and Storey, E., *In vivo* incorporation of tetracycline in the teeth of man. *Arch. Oral Biol.* **7;** 431, 1962.

74. Skinner, H. C. W., Unpublished observations.

75. Harris, W. H., Haywood, E. A., Lavorgna, J., and Hamblen, D. L., Spatial and temporal variations in cortical bone formation in dogs. *J. Bone Joint Surg.* **50A;** 1118, 1968.

76. Bohr, H., Ravn, H. O., and Werner, H., The osteogenic effect of bone transplants in rabbits, *J. Bone Joint Surg.* **50B;** 866, 1968.

77. Frost, H. M., Relation between bone tissue and cell population dynamics. Histology and tetracycline labeling. *Clin. Orthop.* **49;** 65, 1966.

78. Villanueva, A. R., Ilnicki, L., Duncan, H., and Frost, H. M., Bone and cell dynamics in the osteoporoses: A review of measurements by tetracycline bone labelling. *Clin. Orthop.* **49;** 135, 1966.

79. Ramser, J. R., Villanueva, A. R., and Frost, H. M., Cortical bone dynamics in osteomalacia measured by tetracycline bone-labeling. *Clin. Orthop.* **49;** 89, 1966.

80. Takahashi, H., Frost, H. M., and Kuhn, T., Bone tissue and cell dynamics determined by tetracycline labeling in a case of pseudohypoparathyroidism (and in the patient's mother). *Clin. Orthop.* **49;** 163, 1966.

81. Ramser, J. R., Frost, H. M., and Smith, R., Tetracycline-based measurement of the tissue and cell dynamics in rib of a 25-year-old man with active acromegaly. *Clin. Orthop.* **49;** 169, 1966.

82. Arnstein, A. R., and Frame, B., Primary hypophosphatemic rickets and osteomalacia: A review. *Clin. Orthop.* **49;** 109, 1966.

83. Ramser, J. R., Villanueva, A. R., Pirok, D., and Frost, H. M., Tetracycline-based measurement of bone dynamics in 3 women with osteogenesis imperfecta. Clin. Orthop. **49;** 151, 1966.

84. Frost, H. M., Villanueva, A. R., Jett, S., and Eyring, E., Tetracycline-based analysis of bone remodelling in osteoporosis, *Clin. Orthop.* **65;** 203, 1969.

85. Sarnsethsiri, P., Hitt, O. K., Eyring, E. J., and Frost, H. M., Tetracycline-based study of bone dynamics in pycnodysostosis. *Clin. Orthop.* **74;** 301, 1971.

86. Epker, B. N., A quantitative histologic study of the effects of fluoride on resorption and formation in animal and human bone. *Clin. Orthop.* **49;** 77, 1966.

87. Ramser, J. R., Duncan, H., Landeros, O., and Frost, H. M., Measurements of bone dynamics in seven patients with salicylate-treated rheumatoid arthritis. *Arthritis Rheum.* **9;** 424, 1966.

88. Frost, H. M., Tetracycline-based histological analysis of bone remodelling. *Calcif. Tissue Res.* **3;** 211, 1969.

89. Löe, H., Bone tissue formation. A morphological and histochemical study. *Acta Odontol. Scand.* **17;** 325, 1959.

90. Wilson, R. V., Ramser, J. R., and Frost, H. M., Thickness of osteoid seams in some diseases of man. *Clin. Orthop.* **49;** 119, 1966.

91. Amprino, R., and Marotti, G., A topographic quantitative study of bone formation and reconstruction. *In* "European Bone and Tooth Symposium," (H. J. J. Blackwood, Ed.), p. 21. Macmillan, New York, 1964.

92. Hansson, L. I., Daily growth in length of diaphysis measured by oxytetracycline in rabbit normally and after medullary plugging. *Acta Orthop. Scand. Suppl.* **101;** 1967.

93. Marshall, J. H., Rowland, R. E., and Jowsey, J., Microscopic metabolism of calcium in bone. V. The paradox of diffuse activity and long-term exchange. *Radiat. Res.* **10;** 258, 1959.

94. Moffitt, J. M., Cooley, R. O., Olsen, N. H., and Hefferren, J. J., Prediction of tetracycline-induced tooth discoloration. *J. Amer. Dent. Ass.* **88;** 547, 1974.

95. Weyman, J., The clinical appearances of tetracycline staining of the teeth. *Brit. Dent. J.* **118;** 289, 1965.

96. Weyman, J., Microscopic appearances of tetracycline deposition in human dentin. *J. Dent. Res.* **47;** 742, 1968.

97. Weyman, J., and Porteous, J. R., Tetracycline staining of teeth: A report of post mortem material. *J. Dent. Res.* **42;** 1112, 1963.

98. Grossman, E. R., Walchek, A., and Freedman, H., Tetracyclines and permanent teeth: The relation between dose and tooth color. *Pediatrics* **47;** 567, 1971.

99. Johnson, N. W., Distribution and stability of tetracycline in dental tissues. *In* "Antibiotic Advances in Research, Production, Clinical Use," (M. Herold and Z. Gabriel, Eds.). Butterworth, London, 1966.

100. Witkop, C. J., Jr., and Wolf, R. O., Hypoplasia and intrinsic staining of enamel following tetracycline therapy. *J. Amer. Med. Ass.* **186;** 1008, 1963.

101. Zipkin, I., Larson, R. H., and Rall, D. P., Reduced caries in off-spring of rats receiving tetracycline during various prenatal and post-partum periods. *Proc. Soc. Exp. Biol. Med.* **104;** 158, 1960.

102. Brearly, L. J., and Porteous, J. R., Characteristics and caries experience of tetracycline-affected dentitions. *J. Dent. Res.* **52;** 508, 1973.

103. Porter, P. J., Sweeney, E. A., Golan, H., and Kass, E., Controlled study of the effect of prenatal tetracycline on primary dentition. *Antimicrob. Agents Chemother.* **1965;** 668, 1965.

104. Baker, K. L., The fluorescent, microradiographic, microhardness and specific gravity properties of tetracycline-affected human enamel and dentine. *Arch. Oral Biol.* **17;** 525, 1972.

105. Schour, I., and Massler, M., Studies in tooth development: The growth pattern of human teeth. Part I, II. *J. Amer. Dent. Ass.* **27;** 1778, 1940.

106. Schour, I., Poncher, H. G., Rate of apposition of enamel and dentin, measured by the effect of acute fluorosis. *Amer. J. Dis. Child.* **54;** 757, 1937.

107. Löfgren, C. G., Omnell, K. A., and Hylen, M. U., Effect of intraperitoneal injections of tetracycline hydrochloride and oxytetracycline on forming enamel of rat incisors. *Calcif. Tissue Res.* **2**; 145, 1968.

108. Antalovska, Z., Melkova, V., and Smutna, V., Electron microscopy of the calciotraumatic response in rat incisor dentin and bone to parenteral and oral administration of tetracycline. *J. Dent. Res.* **48**; 521, 1969.

109. Omnell, K. A., Löfgren, C. G., and Nylen, M. U., Tetracycline-induced enamel defects in the rat incisors. *Arch. Oral Biol.* **15**; 645, 1970.

110. Rowles, S. L., Chemistry of the mineral phase of dentin. *In* "Structural and Chemical Organization of Teeth" (A. E. W. Miles, Ed.), Vol. II, Chap. 17, p. 201. Academic Press, New York (1967).

111. Brudenold, F., and Soremark, R., Chemistry of the mineral phase of enamel. *In* "Structural and Chemical Organization of Teeth" (A. E. W. Miles, Ed.), Vol. II, Chap. 18, p. 247. Academic Press, New York (1967).

112. Frost, H. M., "Bone Dynamics in Osteoporosis and Osteomalacia." Thomas, Springfield, Ill., 1966.

113. Taylor, T. C., Epker, B. N., and Frost, H. M., The appositional rate of Haversian and endosteal bone formation measured in tetracycline labeled human ribs. *J. Lab. Clin. Med.* **67**; 633, 1966.

114. Porter, P. J., Controlled study of the effect of prenatal tetracycline on primary dentition. *Antimicrob. Agents Chemother.* **5**; 668–671, 1965.

115. Massler, M., Schour, I., and Poncher, H. G., Developmental pattern of the child as reflected in the calcification pattern of the teeth. *Amer. J. Dis. Child.* **62**; 33, 1941.

116. Schwachman, H., Fekete, E., Kulczychi, L. L., and Foley, G. E., The effect of long-term antibiotic therapy in patients with cystic fibrosis of the pancreas. *Antibiot. Annu.* **1958–1959**, 692, 1959.

117. Nylen, M. U., Omnell, K. A., and Löfgren, C. G., An electron microscopic study of tetracycline-induced enamel defects in rat incisor enamel. *Scand J. Dent. Res.* **80**; 384, 1972.

118. Boyde, A., The development of enamel structure. *Proc. Roy. Soc. Med.* **60**; 923, 1967.

119. Frank, R. M., and Nalbandian, J., Ultrastructure of amelogenesis. *In* "Structural and Chemical Organization of Teeth" (A. E. W. Miles, Ed.), Vol. I, p. 399. Academic Press, New York (1967).

120. Nylen, M. U., Eanes, E. D., and Omnell, K. A., Crystal growth in rat enamel. *J. Cell Biol.* **18**; 109, 1963.

121. Travis, D. F., and Glimcher, M. J., The structure and organization of and the relationship between the organic matrix and the inorganic crystals of embryonic bovine enamel. *J. Cell Biol.* **23**; 477, 1964.

122. Saxen, L., Drug-induced teratogenesis *in vitro:* Inhibition of calcification by different tetracyclines. *Science* **153**; 1384, 1966.

123. Buyske, D. A., Eisner, H. J., and Kelly, R. G., Concentration and persistence of tetracycline and chlortetracycline in bone. *J. Pharmacol. Exp. Ther.* **130**; 150, 1960.

124. Kunin, C. M., Dornbush, A. C., and Finland, M., Distribution and excretion of four tetracycline analogues in normal young men. *J. Clin. Invest.* **38**; 1950, 1959.

125. Kunin, C. M., and Finland, M., Clinical pharmacology of the tetracycline antibiotics. *Clin. Pharmacol. Ther.* **2**; 51, 1961.

126. Steigbigel, N. H., Reed, C. W., and Finland, M., Susceptibility of common pathogenic bacteria to seven tetracycline antibiotics *in vitro. Amer. J. Med. Sci.* **255**; 179, 1968.

127. Hill, A., Susceptibility of seven common pathogenic bacteria to doxycycline (Vibramycin), tetracycline HCl and ampicillin *in vitro. Med. J. Aust.* **2**; 1295, 1972.

128. Hammarström, L., Different localization of tetracycline and simultaneously injected radiocalcium in developing enamel. *Calcif. Tissue Res.* **1**; 229, 1967.

129. Olson, C. A., and Riley, H. O., Jr., Complications of tetracycline therapy. *J. Pediatr.* **68**; 783, 1966.

130. Neuvonen, P. J., Gothoni, G., Hackman, R., and Björksten, K., Interference of iron with the absorption of tetracyclines in man. *Brit. Med. J.* **4**; 532, 1970.

131. Council on Drugs. *J. Amer. Med. Ass.* **209**; 549, 1969.

132. Harris, W. H., Lavorgna, J., Hamblen, D. L., and Haywood, E. A., The inhibition of ossification *in vivo. Clin. Orthop.* **61**; 52, 1968.

133. Miller, E. J., and Martin, G. R., The collagen of bone. *Clin. Orthop.* **59**; 195, 1968.

134. Klastersky, J., and Daneau, D., Bacteriological evaluation of minocycline, a new tetracycline. *Chemotherapy* **17**; 51, 1972.

135. Lever, M., Improved fluorometric determination of tetracyclines. *Biochem. Med.* **6**; 216, 1972.

136. Jackman, K., Klein, L., and Lacey, S. H., Recovery and determination of ^{3}H-tetracycline from whole bones. *Biochem. Med.* **8**; 114, 1973.