Tetracycline: Effect on Osteogenesis in vitro

Abstract. *Tetracycline hydrochloride added to cultures of rudiments of embryonic bone in a concentration of 1 microgram per milliliter prevents mineralization of the bones and is found throughout the calcified zones. The inhibition is reversible; upon transfer of a treated culture to a normal medium, mineralization proceeds normally but maldeveloped bones result.*

The difficulties and limitations that are encountered in testing the teratogenic effect of drugs on animals have prompted many similar attempts with tissue cultures. The pioneering work of Fell and Mellanby (*1*) has led to tests in vitro of many physiological compounds and certain toxic agents, including two compounds that interfere with osteogenesis. Tests of both thalidomide and lathyrogenic compounds in organotypic cultures show effects that closely resemble those known from in vivo experiments (*2*).

In the experiments reported here, tetracycline was chosen for such testing for several reasons. Earlier investigations have shown that this antibiotic is transmitted from the maternal circulation to the fetal organism where it is incorporated by the skeleton, especially by the zones of active bone formation (*3*). Its retarding effect on the growth of embryonic bones has been observed in experiments with animals and in premature infants (*4*); even a true teratogenic effect of the drug has been suggested, although this is attributable to an indirect effect (*5*). It seemed important to examine these effects under controllable conditions in vitro.

Ulnar and radial bones of 14- to 16-day-old mouse embryos were aseptically removed under a dissecting microscope and were cultivated on a Trowell-type metal screen at the interface of medium and gas (5 percent CO_2 in air) (*6*). The medium consisted of Eagle's basal medium in Earle's balanced salt solution, with 10 percent inactivated fetal calf serum and sodium bicarbonate (0.0172*M*). Purified tetracycline hydrochloride was added to the medium in various concentrations and the *p*H was checked. The bone rudiments, whether controls or treated with tetracycline, were harvested after having been cultivated for 3 to 12 days in the medium and analyzed by various methods. After measuring the length of the mineralized zone from camera lucida images (magnification × 50), the bones were fixed for histological, histochemical, or quantitative microchemical studies.

The effect of continuous treatment with tetracycline in concentrations of from 0.01 to 100 μg/ml is illustrated in Fig. 1 and Table 1. Whereas the length of the mineralized zone and the total calcium constantly increase in control cultures, tetracycline in concentrations from 1 μg/ml upward not only prevents such increase but may even lead to a shortening of the calcified zone and to a decrease in the calcium content of the previously mineralized bone.

Table 1. Total calcium of five radial and five ulnar bones (pooled), from mouse embryos, cultured in the presence of tetracycline.

Days of treatment	Calcium (10^{-2} mg)		
	Control	Tetracycline	
		1 μg/ml	10 μg/ml
0	3.00	3.00	3.00
3	3.30	2.65	2.40
7	3.75	2.30	1.95
12	5.00	1.90	2.17

Table 2. In vitro incorporation of H^3-thymidine into mouse-embryo bone rudiments, treated with tetracycline and untreated. Five bone rudiments were pooled for each determination.

Days of treatment	H^3-thymidine incorporation (count/sec)		
	Control	Tetracycline	
		1 μg/ml	10 μg/ml
	16-Day ulna		
5	45.8	49.9	23.1
	16-Day radius		
7	43.2	44.2	34.2
	15-Day ulna		
3	39.5	38.7	37.9
	15-Day ulna		
7	60.0	54.5	41.1
	15-Day ulna		
12	40.1	43.9	30.6

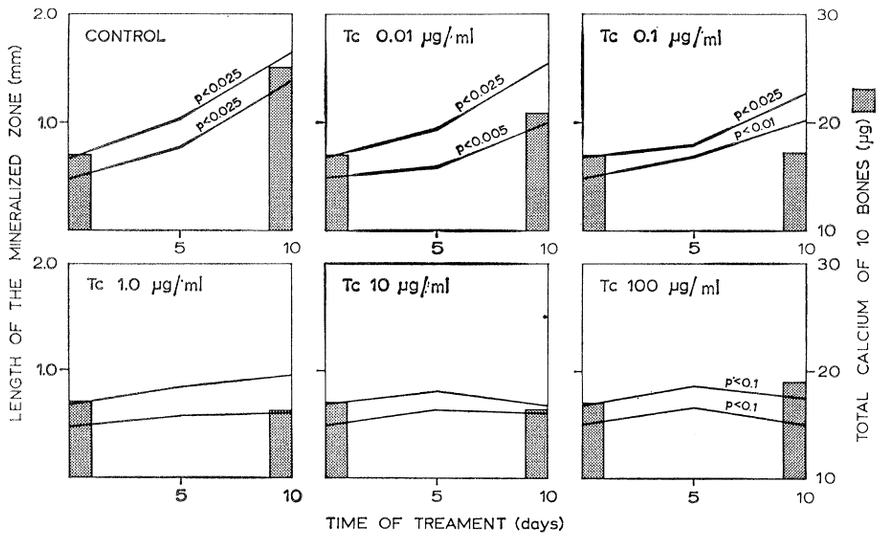

Fig. 1. Length of the mineralized zone of bone rudiments (upper row, radius; lower row, ulna) treated continuously with tetracycline hydrochloride at various concentrations. Each value represents the mean of six bones. The significance of the change is indicated by *p*-values determined by *t*-test from the individual growth rates (the absence of *p*-value shows that the change has not been statistically significant). The bars represent the total calcium of the 12 pooled bone rudiments.

In order to study the reversibility of this effect, experiments were designed in which the treatment was interrupted after various periods and subsequent mineralization was observed in normal medium. The results (Fig. 2) indicate that the inhibitory effect is reversible after short-term treatment, but that this may not be so after prolonged presence of the tetracycline. Moreover, in all experiments in which tetracycline treatment was interrupted after about 3 to 4 days, subsequent development resulted in severe malformations (Fig. 3).

The histological findings varied greatly and clearly correlated with the stage of osteogenesis at the time of explantation (and the onset of tetracycline treatment). In already mineralized rudiments, examination of a cryostat section under ultraviolet light re-

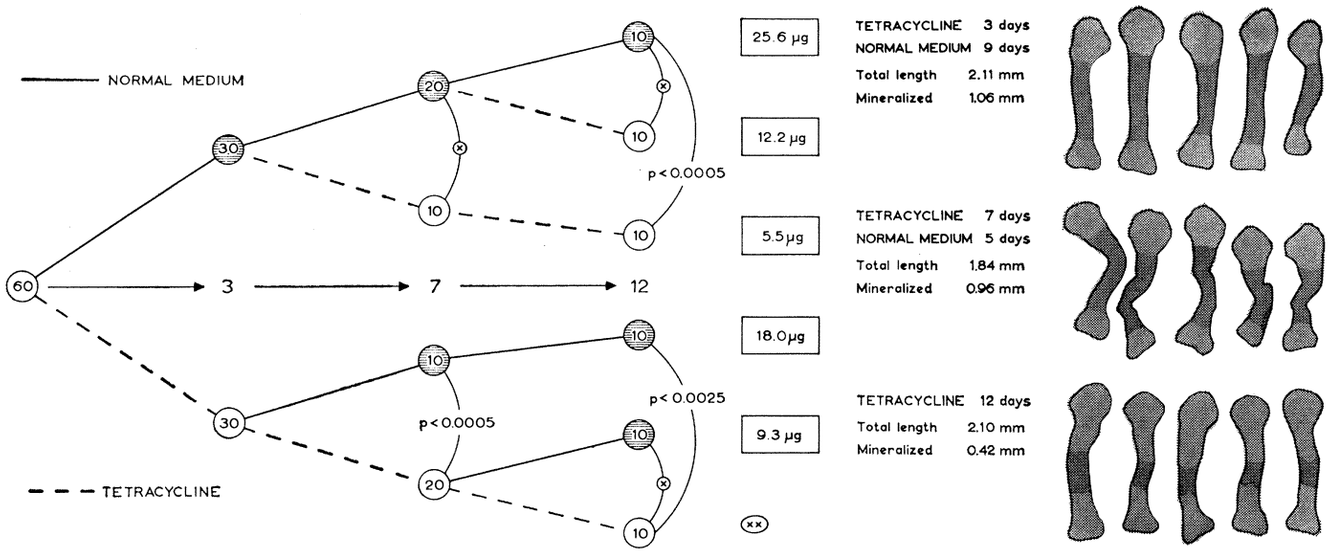

Fig. 2 (left). A scheme of the experiment described in the text showing changes in the mineralized zone of bones treated continuously with tetracycline at a concentration of 1 μg per milliliter, or transferred from tetracycline to normal medium, or vice versa. The number of rudiments for each determination is indicated, as is the mathematical significance of the changes (*x*, no statistical significance). The final calcium values in each series were determined from 10 pooled bone rudiments (*xx*, unsuccessful determination). Arrows indicate the numbers of days in culture. Fig. 3 (right). Drawings from camera lucida images of embryonic ulnar bones after a total of 12 days' cultivation in vitro. Treatment with tetracycline was either continuous or interrupted after various periods; in some instances malformed bones resulted.

vealed a bright yellow tetracycline fluorescence in the calcified zones, and subsequent staining for calcium demonstrated very close correlation with the fluorescence (Fig. 4). Against this, in bone rudiments in which mineralization was not apparent at the time of explantation, and in which tetracycline treatment completely prevented onset of mineralization, no fluorescence or calcium was detectable in the sections. This may indicate that incorporation of tetracycline or of compounds containing tetracycline is not a prerequisite for the inhibitory effect.

In order to examine the possible role of tetracycline treatment in the synthesis of DNA by the organ rudiments, the incorporation of H^3-thymidine was measured. After treatment with tetracycline for 3 to 10 days, a 3-hour pulse was given (H^3-thymidine of a specific activity of 6.7 c/mmole in a concentration of 5 μc/ml), after which the rudiments were washed and treated for 10 minutes with 5 percent trichloroacetic acid and in absolute alcohol; this was followed by treatment with hyamine hydroxide. Radioactivity was determined with a liquid-scintillation spectrometer, and the results are given in Table 2.

My results suggest that, in organotypic cultures, tetracycline in concentrations comparable with those attained in vivo by therapeutic doses (*7*) has inhibitory effects on the mineralization of embryonic bones. The mechanism of this interference is not yet clear, although earlier reports indicate that tetracycline forms a complex with calcium ions in the surfaces of apatite crystals (*8*). However, it should be stressed that my observations also demonstrated complete inhibition of mineralization in explants in which no incorporation of tetracycline was detectable (before the onset of calcification); thus it may be concluded that either the incorporation of calcium was prevented, or the calcium ions were re-

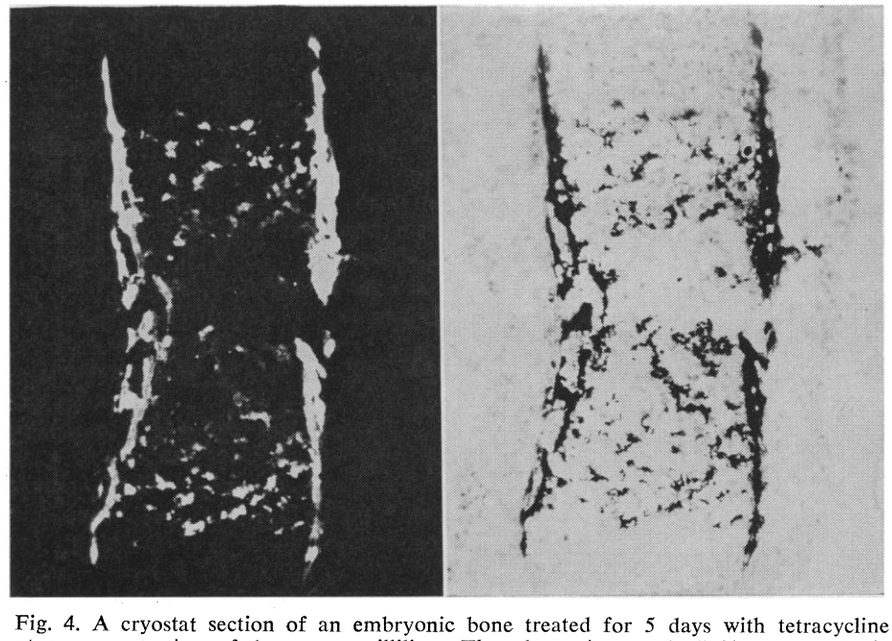

Fig. 4. A cryostat section of an embryonic bone treated for 5 days with tetracycline at a concentration of 1 μg per milliliter. The photomicrograph (left) was made in ultraviolet light and shows localization of the bright tetracycline fluorescence. The von Kossa staining (right) of the same section shows the distribution of calcium; note the close resemblance.

moved from the medium bathing the organ rudiments. Certain results of Urist and Ibsen (*8*) seem to suggest the latter alternative. Determinations of calcium in my culture medium have not, however, shown any diminution in soluble or ionic calcium during 12 days of cultivation in the presence of tetracycline at a concentration of 10 μg/ml (*9*).

Cultures of rudiments of embryonic organs seem to afford a valuable tool for the exploration and analysis of certain toxic and, perhaps, teratogenic effects of drugs. However, it should be stressed that at the present stage these conclusions are not applicable at the clinical level.

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References and Notes

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10. Work supported by the Sigrid Jusélius Foundation, Helsinki. I thank Mrs. Anja Tuomi for technical assistance.

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Productivity of Microalgae in Antarctic Sea Ice

Abstract. *Midsummer productivity of Antarctic microalgae, commonly occurring in brown sea ice along the west coast of the Palmer Peninsula, averaged more than 900 milligrams of carbon per cubic meter per hour, with an assimilation number of about 2.6. The rate of photosynthesis increased with light intensity to a maximum of about 18,000 lux, above which some inhibition was observed. The floral composition, genesis, and physiological properties of these ice communities are different from the epontic under-ice diatoms previously studied by other investigators in McMurdo Sound.*

At least two different kinds of microalgal communities exist in Antarctic sea ice which covers as much as 2 to 5 million square kilometers even in midsummer (*1*). One of these communities is the epontic diatom population that Bunt (*2*) found living on ice crystals and in the interstitial water of the ice matrix formed at the bottom surface of thick ice. This prolific flora of diatoms growing under several meters of sea ice, for example in McMurdo Sound, appears to be a shade-adapted community which can fix carbon dioxide slowly at low intensities of light (*3*). Another kind of ice population, studied by Fukushima (*4*) and Meguro (*5*), is commonly seen in yellow-brown bands of broken sea ice in the southern oceans south of the Antarctic Circle. As the weight of falling snow depresses the ice below the sea surface, sea water floods the interstices of the snow and provides a suitable place for development of a mixed diatom and flagellate community, in layers varying from 15 to 100 cm in thickness. The brown strata of ice plankton may exist as a slush during warm summer days or the layer may be frozen solid during very cold weather. We now present data concerning the amount of microalgae trapped in the ice and their photosynthetic capacity.

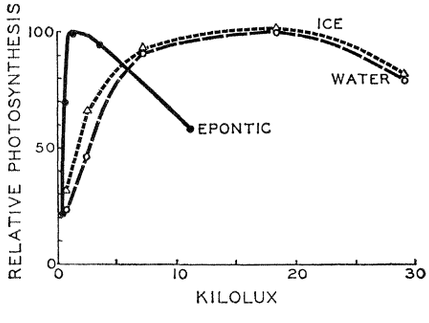

Fig. 1. Relative photosynthesis of ice and water organisms from Matha Strait, northeast of Adelaide Island, and the epontic under-ice community studied by Bunt (*3*) in McMurdo Sound.

On an expedition aboard the Argentine icebreaker *General San Martin* to Peter the First Island in February, 1965, we encountered much floating brown ice in Matha Strait and Marguerite Bay along the western coast of the Palmer Peninsula, in the vicinity of the Antarctic Circle. Samples of brown ice and sea water were obtained in plastic buckets for determination of carbon uptake by standard methods (*6*). The chlorophyll-bearing organisms were filtered onto Millipore membranes with pores 0.8 μ in diameter, then extracted in 90 percent acetone, and the pigment absorption was measured in a Beckman DU spectrophotometer aboard ship. Fixation of carbon was determined by incubation of samples in light with added $Na_2C^{14}O_3$, filtration of portions onto Millipore membranes, and counting in a Tracerlab counter (for beta particles). In order to obtain homogeneous suspensions of diatoms, portions of brown slush ice were melted in sea water and diluted appropriately to obtain practical working suspensions for experimental use. The temperature of incubation was maintained near zero with an ice-water bath.

Some typical results obtained in this study are shown in Table 1. Although the plankton was relatively abundant in these waters, the amount of chlorophyll *a* and C^{14} fixation per unit volume in the ice samples was enormous by comparison. In samples from Matha Strait, the chlorophyll *a* and C^{14} fixation of the ice organisms were approximately 30 times as great as for the phytoplankton in sea water in the same location. Chlorophyll *a* values were considerably higher than those reported in Arctic ice by Appolonio (*7*).

The assimilation numbers, expressing the milligrams of carbon fixed per hour per milligram of chlorophyll *a*,

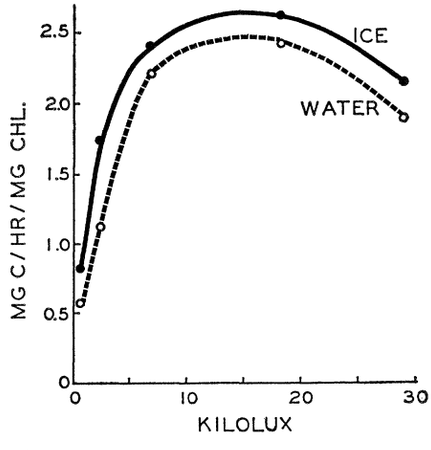

Fig. 2. Variation of assimilation numbers (milligrams of carbon fixed per hour per milligram of chlorophyll *a*) in ice and water organisms of Matha Strait, when exposed to different intensities of light (kilolux).