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THE EXISTENCE OF A ZONE OF FINITE THICKNESS DURING TETRACYCLINE LABELING OF BONE*

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Introduction

As *in vivo* tetracycline bone labeling becomes more widely used, refinements in methodology are required to cope with the increasing number of factors which disturb measurements of bone turnover. One of these factors was called the *t*hickness of the *i*nstantaneously *s*taining *z*one of tetracycline labeling (TISZ) by Frost.[1]

Briefly, TISZ is this: at any moment the zone at the calcification front (zone of demarcation, *ligne frontière*) which accepts tetracycline has finite thickness so that the final thickness of the actual label (W) is a function of at least three variables: the thickness of the TISZ, the duration of the labeling period (t), and the appositional rate (M) of new bone formation. The latter is analogous to the thickness of the layer of ice that is added daily at a skating rink by the addition and freezing of new water.

If the phenomenon exists, measurements of the bone appositional rate done on a single label are inaccurate, and it would be better if this rate were determined between two or more separate time markers, i.e., the multiple labeling method.[2]

This study was designed to establish the presence or absence of the TISZ effect; it established that it exists. A systematic study of its magnitude was not (and will not be) attempted.

Materials

An 11-year-old, medically healthy boy received a tetracycline antibiotic orally for three days in March, followed by a drug-free interval of 15 days; he then received it for eight more days in April. A subsequent hip disarticulation for a primary soft tissue thigh sarcoma provided his tibia for examination.

Methods

Undecalcified, 70-micron-thick sections, made of the tibia by hand-grinding under running water,[3] were stained with Frost's basic fuchsin.[4] Tetracycline is deposited with calcium at sites of active bone formation, and in undecalcified sections will fluoresce under ultraviolet or blue light.[5] By measuring the widths of the fluorescent bands and dividing by the number of days the drug is given (i.e., three or eight, depending on the identity of the band), a value for the rate of new bone apposition is obtained. In a multibanded site, measuring from the center of one band to the center of the next and dividing by the proper number of days

$$(\text{here } \frac{3}{2} + 15 + \frac{8}{2} = 20.5)$$

will yield a third evaluation of this rate. It is easier for the microscopist to locate the middle rather than the inner and outer edges of a given band, for typically these fade off gradually over a distance of 0.5 to 1.5 microns. Thus, estimates of their location are affected by one's adaptation to darkness and by the intensity of the light source, as well as by the passband width and absorbency at midband of the filters used in fluorescence microscopy.

Thirty-three doubly labeled osteons were evaluated. The width of each band plus the distance between their centers gave three separate values in each osteon for the rate of new bone aposition. Each determination was made at four equally spaced intervals around the circumference of the marker and the mean reported as the value.

The data are listed in Table I. Table II shows the results of two methods of finding the value of TISZ, using the data in Table I. The standard deviations of TISZ were found and are noted in the Discussion and in Table III.

Results

The single-band measurements are higher than the double-band ones, as shown in Table I. If no factor tending to make single-band measures larger than the double-band ones existed, then half of an infinte number of single-band measures should be higher and half lower than the mean of the double-band values. The X^2 test of the data shows that the possibility that such a factor does *not* exist is only one in 100 for the March label alone, and one in 200 for the April label alone, so for both together it should be on the order of one in 20,000.

In other words, measurements of the appositional rate are systematically larger when found on single bands than when found between two separate markers.

Discussion

I: *Finding the Value of TISZ:* Assuming TISZ is a constant, then the rate at which new widths of bone are layered onto older ones (M), can be calculated by substituting the values derived from the widths of the two separate bands into the following formula, and setting up and solving simultaneous equations to eliminate the common factor, k. This procedure works because k is the same in each of the two labels the boy received, but the duration of the two labels was significantly different.

Table I

March Label (8 days)			April Label (3 days)			Double Label (205 days)	
μ Thickness	M	± (M.V.D.)	μ Thickness	M	± (AV.D.)	(μ) Thickness	M
13.5	1.69	+	5.3	1.77	+	30.0	1.46
8.1	1.01	+	2.7	.90	+	17.6	.86
10.5	1.31	—	5.9	1.97	+	27.5	1.34
9.6	1.20	+	2.3	.77	—	20.1	.98
9.7	1.21	+	3.4	1.13	—	23.8	1.16
10.7	1.34	+	4.4	1.47	+	22.8	1.11
9.9	1.24	+	3.4	1.13	0	23.2	1.13
8.4	1.05	—	3.4	1.13	—	27.2	1.33
9.3	1.16	+	3.1	1.03	+	16.1	.79
11.3	1.41	—	5.1	1.70	+	30.9	1.51
9.2	1.15	+	4.5	1.50	+	22.5	1.10
8.2	1.02	—	3.5	1.17	+	22.3	1.09
11.9	1.49	+	3.1	1.03	+	18.0	.88
13.3	1.67	+	3.8	1.27	+	25.6	1.25
8.1	1.01	+	3.3	1.07	+	16.2	.79
9.1	1.14	—	4.3	1.43	+	24.0	1.17
10.9	1.36	+	4.7	1.57	+	23.3	1.14
9.0	1.13	+	2.7	.90	—	21.0	1.02
8.0	1.00	+	3.2	1.07	+	19.7	.96
11.73	1.47	+				23.7	1.16
12.13	1.52	—	5.9	1.97	+	32.7	1.60
8.7	1.09	+	2.8	0.93	—	20.1	.98
15.3	1.91	+	6.9	2.30	+	36.0	1.76
17.1	2.14	+	8.8	2.93	+	41.0	2.00
9.6	1.20	+	4.8	1.60	+	23.9	1.17
17.6	2.20	+	8.5	2.83	+	43.3	2.11
13.8	1.73	+	6.0	2.00	+	28.0	1.37
11.6	1.45	—	4.3	1.43	—	30.0	1.46
8.6	1.08	+	4.5	1.50	+	15.0	.73
16.4	2.05	+	5.3	1.77	—	37.1	1.81
7.6	.95	+	3.2	1.07	+	19.3	.94
8.0	1.00	—	3.6	1.20	+	26.5	1.29
7.7	.96	—	4.0	1.33	+	22.0	1.07
354.56	44.34	24/9	146.50	48.84	25/7	830.40	40.52

Average:

10.74	X:	1.34	4.44	X:	1.48	25.16	X:	1.23

$\Sigma X = 44.34$	$\Sigma X = 48.84$	$\Sigma X = 40.52$
$\Sigma X^2 = 63.54$	$\Sigma X^2 = 81.11$	$\Sigma X^2 = 53.44$
Coefficient of Variation .26	.35	.27
S.D. ÷ .35		± .33

This lists the raw data, including a) the mean thickness in microns of each label, b) the appositional rate computed by dividing each band thickness by the appropriate period of time, c) and at the bottom the means, S.D. and coefficient of variation.

TABLE II
CALCULATION OF k (I.E., TISZ)

A: Calculated M_c, assuming k is constant, using each individual label as the basis of determination, and setting up and solving simultaneous equations:

1) $W = M_c (b) + k$

  a) $10.74 = M_c (8) + k$

-b) $4.44 = M_c (3) + k$

       $6.30 = M_c (5)$ $\therefore M_c = 1.26 \mu / day$

2) $W = M_c (8) + k$

  a) $10.74 = 1.26 (8) + k$ b) $4.44 = 1.26 (3) + k$

       $k = 10.74$ $k = \ \ 4.44$

           -10.08 -3.68

              $.66$ $.76$

B: Evaluation based on double label

$M_o = 1.23$
(meas.)

$W = M_o (t) + k$

$W/t = M_o + \dfrac{k}{t}$ $\therefore W/t - M_o = \dfrac{k}{t}$

1)   April $M = 1.48 \mu / day$ (3 days)

            1.48

        -1.23

         $.25 \mu / day$ $\therefore k = .25 \times 3 = .75 \mu$

2)   March $M = 1.34 \mu / day$ (8 days)

          1.34

        -1.23 $= k/t$

         $.11 \mu / day$ $\therefore k = .11 \times 8 = .88 \mu$

     A) This shows the method of finding both M and k by the use of simultaneous equations, based on measurements only of the widths of individual bands.

     B) This shows how k can be obtained by the use of measurements of the thickness of single bands and the mean appositional rate found by the double-band method, which eliminates k entirely from the measurement. If k did not exisit the result of such a procedure would tend to be zero, unless there were a large systematic error in making the actual measurements. The chance that such an error is responsible for the findings in the text is judged to be remote.

TABLE III

SD OF k FOUND BY METHOD B, TABLE III

3 days X k/t = k	8 days X k/t = k
April M — Double M = k/t	March M — Double M = k/t
$\Sigma X^2 = 45.81$	$\Sigma X^2 = 106.02$
$\Sigma X = 25.14$	$\Sigma X = 36.32$
N = 33	N = 33
SD = ± .899	SD = ± 1.42
Mean k = .76μ	Mean k = 1.1μ
Calculated = .75	Calculated = .88

This summarizes the data on determination of k by method (B) of Table II. The measurements are in excellent agreement with those obtained with the use of simultaneous equations. The variance is typical, or perhaps even somewhat smaller than is typical. Note that this is intracase variance. Intercase coefficients of variation for this parameter would typically be on the order of 1/10.

1. The basic equation is W = M$_{(calculated)}$ (t) + k*— or in words: width of label = rate X time plus a constant.

Substituting numerical values for the March and April labels gives:

10.74μ = M$_c$ (8) + k (second label)

—(4.44μ = M$_c$ (3) + k (first label)

Setting up and solving the simultaneous equtions (see Table III, A) gives:

M$_c$ = 1.26μ/d

Now k is found by substituting back into the original equation:

10.74 = 1.26 (8) + k

k = .66μ

The calculated M$_c$ of 1.26 microns per day agrees well with the value derived by the double-band method (which is 1.23μ/d), which is the distance between the centers of the labels divided by the time, a procedure which eliminates k from the problem (Fig. 1) and simplifies equation 1 to the following:

2. W = M$_{(observed)}$t

Substituting numerical values we get (see also Table III, B):

25.16 = 20.5M$_o$

1.23 = M$_o$

Given M$_o$ from the double-band technique, k can be found another way, starting with this relation:

3. W = M$_o$ (t) + k

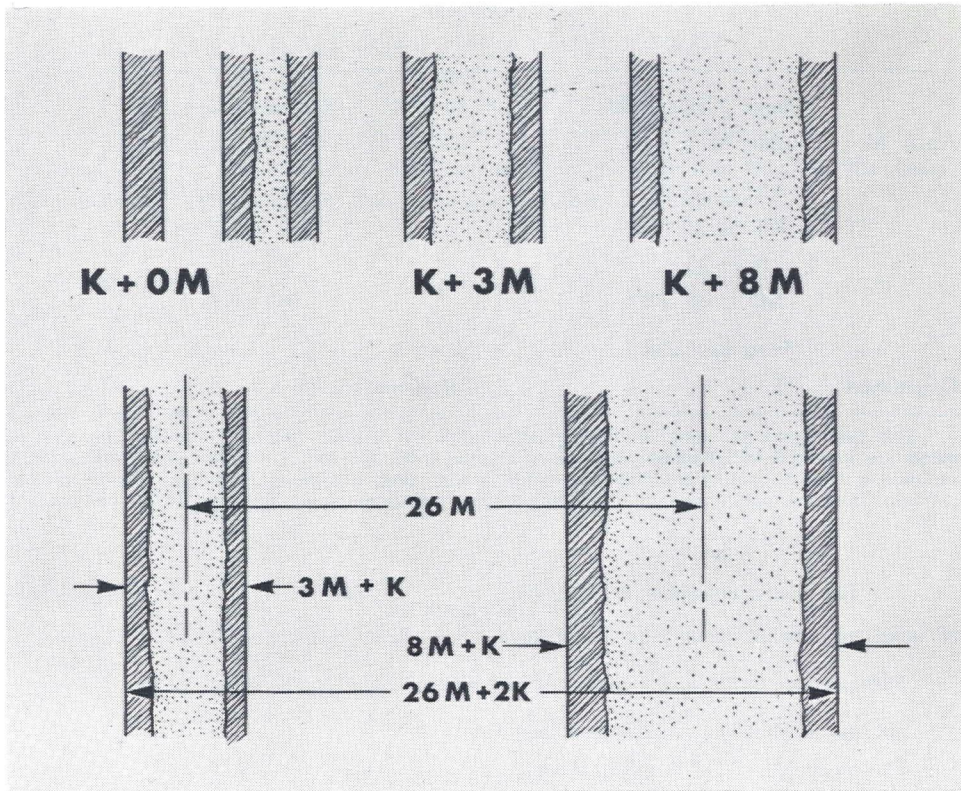*K equals TISZ in these equations, and is substituted for brevity.

Figure 1

This sketch shows the relation between k, the widths of the individual bands, and the distance between the centers of two sequential bands. The numbers represent the actual days involved in the labeling of the patient discussed in the text.

At the top is shown how k (i.e., TISZ) gradually becomes an increasingly thick label with each passing day. $K + o$ means the thickness of the label at the moment it is first deposited. $K + 3$ and $K + 8$ show the thickness after three and eight days of continuous labeling.

At the bottom is shown the relationship of the labels in the boy discussed in the text.

Dividing through by (t), and rearranging, we get:

$$W/t = M_o + k/t$$

$$W/t - M_o = k/t$$

Substituting the April W/t of 1.48 and the March W/t of 1.34, we get:

April	March
$1.48 - 1.23 = k/t$	$1.34 - 1.23 = k/t$
$.25\mu/d = k/3$	$.11\mu/d = k/8$
$k = .75\mu$	$k = .88\mu$

One standard deviation of this k value was $\pm .9\mu$ for the April's M and $\pm 1.42\mu$ for the March's M.

II. The tetracycline antibiotics deposit permanently in the zone called TISZ, which is why they can be used to measure the appositional rate (M) of new bone formation as a function of (t) the known duration of the labeling period, and (W) the observed thickness of a tetracycline band, or of the layer of bone between two such bands. The disturbing effect of TISZ on measurements of the appositional rate is eliminated by multiple-banding techniques.[2] The M_c found by eliminating TISZ through the use of simultaneous equations ($1.26\mu/d$) is substantially the same as the M_c ($1.23\mu/$) derived from the double-band technique, which eliminates TISZ at its source.

Two ways of finding the TISZ were shown. In this boy, TISZ was between .66 and .88μ, which is less than the 3μ previously estimated.[1]* The standard deviations are not disturbing, since there can be great variation in the width of a tetracycline band about a single osteon. The wisdom of making many measurements and taking their average is demonstrated by the agreement of these two independent methods (less than 15% relative difference).

III. *Conclusion:* The phenomenon called TISZ by Frost exists. This establishes the wisdom of using multiple tetracycline labels in studying the rate of new bone formation.

REFERENCES

1. Frost, H. M.: Measurement of human bone formation by means of tetracycline labeling. Canad. J. Biochem. Physiol. 41:31-42, Jan. 1963.

2. Frost, H. M., Roth, H., Villanueva, A. R., and Stanisavljevic, S.: Experimental multiband tetracycline measurement of lamellar osteoblastic activity. Henry Ford Hosp. Med. Bull. 9.312-329, June 1961.

3. Frost, H. M.: Preparation of thin, undecalcified bone sections by rapid manual method. Stain Tech. 33:273-277, Nov. 1958.

4. Frost, H. M.: Staining of fresh, undecalcified thin bone sections. Stain Techn. 34:135-146, May 1959.

5. Milch, R. A., Rall, D. P., and Tobie, J. E.: Fluorescence of tetracycline antibiotics in bone. J. Bone Joint Surg. 40A:897-910, July 1958.

*We have performed a similar study in a doubly labeled osteoporotic woman with similar results, although a larger TISZ was found in her.