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"FRACTIONAL LABELING": THE FRACTION OF ACTIVELY FORMING OSTEOONS THAT TAKE TETRACYCLINE LABELS IN NORMAL HUMAN BONE*

T. RUSH, M.D.,** D. PIROK, D.D.S., M.S.,*** AND H. M. FROST, M.D.****

INTRODUCTION

The report of Milch et al that tetracyclines label sites of active new bone formation,¹ led to the wide use of this phenomenon as an in vivo tissue marker. With the aid of this marker many previously inaccessible properties of bone remodeling could be studied in man, as well as in lower animals. These properties include the absolute rates of bone formation and the size of the various contributions to remodeling of the progenitor cell population and of the physiologically specialized cell populations (i.e., osteoclasts and osteoblasts).

This marker also permitted the development of a systematic and truly quantitative histological scheme of analysis of labeled bone.² In toto, this analysis is a distinct analytical tool. It would be expected that a new tool would unearth previously unknown problems and one of these, which was uncovered during the studies just referred to, is the subject of this report.

In 1960, Frost reported that not all bone-forming centers which had osteoid seams would “take” tetracycline labels, although all labeled sites of lamellar bone formation did have associated osteoid seams.³ Subsequently, others in the H.F.H. O.R.L. showed that in some disease states, unlabeled seams were a major feature of the histology.⁴,⁵,⁶ These unlabeled seams, on inspection, appear to be a minor facet of the histological picture in healthy humans under age 20 (and in dogs and monkeys of equivalent age), but this has not been thoroughly evaluated for any age group. Consequently, the significance of these unlabeled seams in disease is not clearly defined.

Here a systematic study of this phenomenon is reported. While it would have been desirable to investigate it by doing biopsies of normal people after deliberate

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tetracycline labeling, the practical difficulties in doing this on a large scale are insurmountable. Accordingly, an indirect, basically statistical approach to the problem was adopted. What we did was to compare the mean number of osteoid seams counted in cross sections of rib taken from 257 metabolically normal persons with the mean corrected number of tetracycline-labeled bands counted in 64 of them. (Correction of the latter was necessary due to multiple labels with tetracycline.) These data were then corrected for the effects of continued remodeling. (This remodeling removes an increasing fraction of bands with the passage of time after their deposition in the bone.) We then estimated the fraction of the seams that retained tetracycline at the time of labeling.

**MATERIALS**

Table I, in its upper half, breaks down into decades the ages of 257 people in whom the mean number of osteoid seams per mm² of cortical cross section area had been previously determined; in its lower half the subgroup of 64 tetracycline labeled persons is similarly divided. Eleven additional cases were not used because we could not tell with confidence how many times they had been labeled.

Rib samples were taken from these persons either at thoracotomy for nondebilitating anatomical abnormalities (we are indebted for this material to Drs. C. Lam, R. Tabor and T. Gahagan of the Thoracic Surgery Division, H.F.H) or at autopsy following unexpected sudden death (We are indebted here to Dr. E. S. Zawadski, of the County Medical Examiner's Office, Wayne Co., Mich.). None of the patients had known metabolic bone disease, congestive heart failure, diabetes, renal failure or chronic illness, nor had any been treated systematically with endocrine agents, salicylates, X ray or cytotoxins.

<table>
<thead>
<tr>
<th>Case Material</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
</tr>
<tr>
<td><strong>Male</strong></td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td><strong>Seams/mm²</strong></td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td><strong>Seams/mm²</strong></td>
</tr>
</tbody>
</table>

*Typical coefficient of variation in this parameter: 0.5.*
FRACTIONAL LABELING

Figure 1
Tetracycline labels glowing under the fluorescence microscope, as seen in undecalcified cross sections. Any single white ring, regardless of its thickness, is a band. The distance between any two known points in time, identified with the help of a band, is a label. The period of time during which any given band was deposited is an epoch.

In most cases the middle third of the 6th rib was sampled; in some the middle third of the 5th or 7th rib was taken. From the standpoint of the remodeling parameters studied here, this sampling difference is not significant.

METHODS

1) Definitions

(a) A tetracycline band is a single ring of tetracycline in a single moiety (such as a single haversian system or osteon) of bone. See figure 1.

(b) A tetracycline label is any interval of time whose beginning and end is marked in the tissue, in part or entirely, by a tetracycline antibiotic. Thus a label could be the time interval between the middles of two sequential deposits of tetracycline, or between the middle of a single tetracycline band and the middle of the calcification front or zone of demarcation, or from one edge to the other of a single band.

(c) A labeling epoch will be as Sedlin et al defined it: a period of time in the past during which tetracycline was given continuously, so that individual bone-forming centers accepted a single corresponding band, whose thickness increased with increasing duration of the epoch as well as with increasing speed of apposition of new bone at the individual bone-forming site of focus. 

257
Figure 2

The basic data are shown in graph form. A: seams per square mm. cortical cross section area in the 257 normals. B: bands per epoch per mm\(^2\) of cortical cross section area in the 64 labeled cases. B: the seams in the 64 labeled cases.

2) **Sections** Accurately oriented, 50-70-micron-thick undecalcified cross sections were made of the rib samples by hand-grinding under running water,\(^4\) were stained with the tetrachrome bone stain,\(^6\) and mounted in H.S.R. for permanent reference. Labels in undecalcified sections mounted in H.S.R. in 1956 (before tetracycline labeling was known) still show no evidence of fading. More than 1000 sections were used in this study.

3) **Seams/mm\(^2\)** The number of seams involved in haversian remodeling of the compacta was measured in all 257 cases (precision: 2%). Dividing the number of seams in each case by the cortical cross section area in mm\(^2\) (precision: 0.5mm\(^2\)) gives the mean number of seams/mm\(^2\) of cortical cross section area. These data are listed in Table I, 4th and 7th rows. The cortical areas were measured by the grid method, which is both rapid and the most accurate of the available techniques for making such measurements. These data are being reported separately; curve A in figure 2 shows the means.\(^7\)

4) **Band Counts** The total number of tetracycline bands in haversian systems was counted in each section of each of the 64 people who had received tetracyclines. This was done under blue-light fluorescence microscopy with a Zeiss photofluorescent microscope equipped with an Osram 200-watt high-pressure mercury burner and glass excitation and barrier filters.

\(^6\)Available as a single, prebuffered powder by the name “Osteochrome” from Harleco, Philadelphia.
5) **Number of Labeling Epochs** By inspection of all available sections of each labeled case, it was determined how many times each case had been given a tetracycline antibiotic. Sedlin has described the procedure involved in this determination. Since the number of labels was not known independently for any of these subjects, it is possible that the determination of this number is too low. The magnitude of this uncertainty is on the order of 10% of the mean for the group. There were 103 labeling epochs in these 64 subjects, or a mean of 1.6 per case.

6) **Bands/mm²/Epoch** By dividing in each case the value of the band count by the number of labeling epochs and the cortical areas, we obtained the mean number of bands deposited per labeling epoch/mm². These data are given in Table II, 3rd row. This could then be compared with the number of seams/mm² in the whole group of 257 subjects to estimate the fraction of systems that took labels; these data are in the 4th row of Table II.

7) **Corrections** With increasing age of a given band, and with increasing speed of bone turnover, there is an increasing probability that remodeling will partially or wholly remove a band after it is deposited. In the first instance an incomplete band will remain, and many such were actually seen in the material. In the second instance no trace of the band is left. Accordingly, the number of bands actually counted must tend to be less than were actually deposited by some unknown factor. This factor will vary from 0% for cases labeled within a month or so of skeletal sampling to a major percentage for cases labeled 10 or more years before sampling.

<table>
<thead>
<tr>
<th>Table II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline Labeled Data in 64 Subjects</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age</th>
<th>10-19</th>
<th>20-29</th>
<th>30-39</th>
<th>40-49</th>
<th>50-59</th>
<th>60-69</th>
<th>70-79</th>
<th>Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number sections in group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>31.8</td>
</tr>
<tr>
<td>Bands per epoch per mm²</td>
<td>.81</td>
<td>.42</td>
<td>.29</td>
<td>.22</td>
<td>.33</td>
<td>.34</td>
<td>.36</td>
<td>.41</td>
</tr>
<tr>
<td>S.D.</td>
<td>±.47</td>
<td>±.302</td>
<td>±.14</td>
<td>±.13</td>
<td>±.22</td>
<td>±.2</td>
<td>±.17</td>
<td></td>
</tr>
<tr>
<td>S.D.</td>
<td>.16</td>
<td>.09</td>
<td>.05</td>
<td>.04</td>
<td>.07</td>
<td>.07</td>
<td>.06</td>
<td></td>
</tr>
<tr>
<td>Ratio: bands, to seams in whole group of 257</td>
<td>.45</td>
<td>1.02</td>
<td>1.3</td>
<td>.57</td>
<td>.80</td>
<td>.65</td>
<td>.55</td>
<td>.83</td>
</tr>
<tr>
<td>Ratio: bands, to seams in subgroup of 64</td>
<td>.88</td>
<td>1.6</td>
<td>1.5</td>
<td>1.6</td>
<td>.96</td>
<td>1.4</td>
<td>1.5</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Data for the normal rate of haversian remodeling in this skeletal sampling site are available and are summarized in the 2nd row of Table III.  Epker found 46 months to be the mean period of time intervening between labeling and sampling in a group of 41 subjects on whom tetracycline labeling information was available.

We have used this data to compute a correction factor for each decade which, when multiplied by the actual band counts, provides a new, corrected value. First, the decimal fraction of the bone that was not turned over in 46 months at the rates given in row 2, Table III, was computed. For the first two decades this rate is changing rapidly, so the turnover was based on the interpolated rate existing at 23 months before skeletal sampling. These values are given in the 3rd row, Table III. Then the ratio of bands to seams was computed on the basis of the seams/mm² existing 46 months before skeletal sampling. These values are shown in the 4th row, Table III.

Finally, the decimal fraction of the seams that “took” labels was computed and is shown in the 5th row of Table III.

RESULTS

1) Bands/Epoch/mm² Compacta As the 3rd row of Table II shows, the means ranged from .81 in the second decade to .22 in the fifth. The shape of the curve of the mean (curve B, figure 2) is similar to that of curves previously published for normal osteoid seams by the H.F.H. O.R.L. Except for the fourth decade, the bands per epoch per mm² run consistently less than the osteoid seams per mm².
in the 257 subjects (figure 2, curve A). The variance in these figures is large, the coefficient of variation by decades averaging .8. One factor in this variance is probably the variable interval of time intervening between labeling and skeletal sampling.

2) Seams/mm² The mean number of seams in the 64 labeled subjects was compared to the mean in all 257 subjects. This comparison (Fig. 2, curves A, and B, and 4th and 5th rows of Table I revealed that the labeled cases appear to be a different group of subjects, for their seams average half the normal count save for one of the seven age decades studied.

3. Corrected Bands/Epoch/mm² The data corrected for turnover effect on the apparent fraction of seams that take labels is shown in the 5th row of Table III. There is an insignificant decrease with increasing age after age 60. A lifetime average of 90% of systems with seams take labels.

DISCUSSION

1) The data show that (i) normally some actively forming osteons fail to take a tetracycline label; (ii) normally the proportion failing to take one does not appear to change to any major degree with age.

2) Why do some systems not label? There are three inclusive and mutually exclusive possibilities: (i) the nonlabeling systems have permanently stopped making bone; (ii) they are merely temporarily inactive, and were the label given at a different month, then different systems would have failed to accept labels; or (iii) both.

Table IV
Normal Histodynamic Parameters for Human 6th Rib, Corrected for Fractional Labeling*

<table>
<thead>
<tr>
<th>Age</th>
<th>Parameter</th>
<th>Units</th>
<th>0-10</th>
<th>11-20</th>
<th>21-30</th>
<th>31-40</th>
<th>41-50</th>
<th>51-60</th>
<th>61-70</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Appositional Rate</td>
<td>Microns/day</td>
<td>O</td>
<td>1.6</td>
<td>1.4</td>
<td>1.2</td>
<td>1.1</td>
<td>1.0</td>
<td>.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>Same</td>
<td>Same</td>
<td>Same</td>
<td>Same</td>
<td>Same</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Radial Closure Rate</td>
<td>mm/year</td>
<td>O</td>
<td>.55</td>
<td>.48</td>
<td>.44</td>
<td>.40</td>
<td>.36</td>
<td>.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>.47</td>
<td>.41</td>
<td>.37</td>
<td>.34</td>
<td>.31</td>
<td>.28</td>
</tr>
<tr>
<td></td>
<td>Osteon Formation Period**</td>
<td>years</td>
<td>O</td>
<td>.12</td>
<td>.14</td>
<td>.15</td>
<td>.17</td>
<td>.19</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>.14</td>
<td>.16</td>
<td>.18</td>
<td>.20</td>
<td>2.2</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*90% labeling is assumed throughout life. The mesenchymal cell activation frequency, the bone formation rate and the resorption rate are not affected by these corrections.
**Based on an osteon wall thickness of 0.068mm.
While we have conclusive proof that (ii) does occur in specific osteons, there is no proof yet that it is the correct general explanation for the phenomenon to which this study was addressed.

3) How can we use the present information? It seems to have two applications. A) Normal values for the local bone appositional rate have been published for both haversian and cortical endosteal bone forming foci. To obtain normal values for radial closure rates, the appositional rate figures must be multiplied by the age-respective fractional-labeling figures in this article. When this is done, slightly lower values for normal radial closure rates and osteon formation periods are obtained.

Table IV lists those normal data previously published by the H.F.H. O.R.L., which are altered by the findings of this study. The correct values are given in the lower rows, the older ones in the upper. Since the variations around the mean of 90% labeled (bottom row, Table III) are not significant, it is assumed in computing the corrected data that the per cent labeled is 90% over the entire span of life. This correction is so small that it does not alter the meaning of any results which we have published or which we have in press.

B) A second reason for interest in this phenomenon is more basic, and is predicated on our present belief that fractional labeling occurs because all bone-forming centers tend to alternate between two extreme states of activity: minimum and maximum. If true, such behavior could be caused by analogous changes in blood flow through the capillary that supplies new bone-forming centers. However, it could also be a manifestation of a kind of autonomous synchrony in the behavior of the osteoblasts in each center. The latter is possible, because (a) these centers are studied in cross section; (b) the geometry of the structures is cylindrical; (c) the osteoblasts at a single cross sectional level of a single osteonal bone-forming center were generated (at least as functional entities) approximately within the same week; (d) the time scale for cell turnover and aging in man is greatly prolonged compared to that in synchronous cultures of microorganisms, so that comparable degrees of relative synchrony can exist in the presence of large differences in the absolute period of cell turnover. If this is synchronous cell behavior, it is hard to underestimate its usefulness and importance as a model system of in vivo human cell behavior, which can be studied with assurance that the act of study cannot disturb the cells whose behavior is the object of study.

4) It is puzzling that the labeled subgroup of 64 subjects had significantly lower mean seam counts than the whole group of 257 cases. There are three possible explanations: (i) the labeled cases may have differed from the nonlabeled ones to begin with, i.e., this is a sampling problem; (ii) the disease for which they were given the tetracycline, or the drug itself, caused a persistent change in their bone cell dynamics; (iii) a large error exists of which we are ignorant. We favor (ii) at present. It seems desirable that others should study this phenomenon, for if it is a reflection of a persistent abnormality in dynamics caused by a disease, it is a new phenomenon in human physiology.
FRACTIONAL LABELING

While it might seem possible that the tetracycline, or the illness for which it was given, caused an increased number of labeled bone-forming sites in these 64 subjects, this can be discounted on the basis of known time factors. It is established that any labeled bone-forming site (in normal haversian remodeling) must first have been a site of resorption for a period of one to three months. Thus, any remodeling initiated by an illness or drug must take one to three months to progress to the new bone formation stage. Most of the illnesses treated with the tetracyclines were of shorter duration than this, and so could not have caused an increase in the number of labeled bone forming sites.

SUMMARY

A comparison was made between the number of bone forming sites identified by the presence of osteoid seams, and the number identified by the presence of tetracycline labels. The data were corrected for the effects of remodeling and for the changes in number of remodeling centers that occur with age. It was found that seams normally exceed labeled systems in number, and normally 90 out of 100 seams “take” a tetracycline label.

REFERENCES
