TETRACYCLINE LABELLING METHODS OF MEASURING THE GROWTH OF BONES IN THE RAT

E. TAPP, MANCHESTER, ENGLAND

From the Department of Pathology, University of Liverpool

The observation of Milch and his associates in 1957 that tetracycline becomes localised in areas of new bone formation has been confirmed on many occasions (Milch, Rall and Tobie 1958; Frost, Villanueva and Roth 1960; Harris 1960; Muzii 1961), and in 1960 Harris suggested that tetracycline might be suitable for use as a marker whereby the rate of bone formation could be measured. Since then a number of workers have studied the growth of compact lamellar bone by tetracycline labelling methods (Frost 1961, 1963; Harris, Jackson and Jowsey 1962; Urist, Zaccalini, MacDonald and Skoog 1962). Difficulties, however, arise in the interpretation of any measurements made on compact lamellar bone. Osteoblastic activity is irregular in such bones and varies from one part of the bone to another, so that a large number of serial sections have to be examined to obtain a true picture of the amount of osteoblastic activity in the bone.

This report is concerned with the use of tetracycline labelling in bones which grow in a relatively simple way such as the tibia of the rat. With tetracycline labelling, the normal growth pattern of this bone both in length and in width has been determined and compared with radiological methods.

NORMAL UPTAKE OF TETRACYCLINE BY THE RAT SKELETON

Within thirty minutes after an intraperitoneal injection of tetracycline a diffuse yellow fluorescence is seen on the surface of the bone when it is examined in incident ultra-violet light. The fluorescence is particularly bright near the epiphysis. In undecalcified sections prepared from these bones tetracycline fluorescence is present throughout the bone but it is most marked on some periosteal and endosteal surfaces. A bright band of tetracycline fluorescence is also seen in the calcified cartilage at the epiphysial line.

After twenty-four hours the diffuse fluorescence disappears from the bones in general but some of the drug remains permanently localised as narrow bright yellow bands in areas of new bone formation. These bands, which are between 5 microns and 10 microns in thickness, are seen close to the periosteal and endosteal surfaces of the diaphyses of the long bones, the vertebrae and the skull (Figs. 1 to 3). In animals killed more than twenty-four hours after the injection of tetracycline the bands are farther away from the surfaces; they persist until the bone containing them is remodelled. An example of this is seen at the proximal end of the tibia, where extensive remodelling associated with the formation of the tibial tuberosity occurs.

When doses of tetracycline are given at intervals of a few days a series of bands is seen; these appear in transverse sections as concentric rings in the outer part of the cortex rather like the growth rings in a tree trunk (Fig. 4). If the time of administering tetracycline is known, measurements of the amount of bone formed between such bands provides a method, described below, for measuring the rate at which the bone is growing in width.

In animals killed at twenty-four hours there is, in addition to the periosteal bands, an irregular band of tetracycline fluorescence between 30 microns and 80 microns in thickness in the metaphyseal bone a short distance from, and parallel to, the epiphysial line (Fig. 5).
Figure 1—A bright band of tetracycline fluorescence close to the periosteal surface of the tibia. A similar but incomplete band is seen at the endosteal surface. Tetracycline had been given twenty-four hours before the animal was killed. (Unstained, ground section in ultra-violet light, ×20.)

Figure 2—A band of tetracycline fluorescence close to the endosteal surface of the tibia. The section is a longitudinal one close to the metaphysis. (The periosteal surface is not included in the photograph.) Tetracycline had been given twenty-four hours before the animal was killed. (Unstained, ground section in ultra-violet light, ×15.)

Figure 3—Three bands of tetracycline fluorescence close to the periosteal surface of the vertebral body (the upper part of the photograph). Tetracycline had been given on three occasions at seven days, four days and one day before the animal was killed. (Unstained, ground section in ultra-violet light, ×30.)

Figure 4—A series of concentric bands in the outer part of the cortex. Tetracycline had been given on five occasions: fifty-six days, forty-two days, twenty-eight days, fourteen days and one day before the animal was killed. (Unstained, ground section in ultra-violet light, ×20.)

Figure 5—An irregular band of tetracycline fluorescence in the metaphysial bone a short distance from the epiphysial cartilage (dark band). Some tetracycline is seen also in the deeper part of the metaphysis. Tetracycline had been given two days before the animal was killed. (Unstained, ground section in ultra-violet light, ×15.)
In animals killed at two or three days after the injection of tetracycline this band is farther away from the epiphysial line. The bone containing the drug is eventually reabsorbed by osteoclastic activity in the marrow cavity; this occurs after three to four days in young animals but may not occur for fourteen days in older animals (Fig. 6). The distance from the epiphysial line at which the band of tetracycline is found indicates the amount that the bone has grown in length from that epiphysis after the administration of tetracycline. This is the basis of the method for measuring the rate of growth in length of the bone at the proximal end of the tibia. The epiphysial cartilage at the distal end of the tibia is more irregular than that at the proximal end and therefore the method was not applied to the rate of growth at the distal end.

**FIG. 6**

Three bands of tetracycline fluorescence in the metaphysis. Tetracycline had been given on three occasions at fourteen, four and two days before the animal was killed. (Unstained, ground section in ultra-violet light, ×30.)

**TETRACYCLINE METHODS**

**Rate of growth in width**—Measurements were carried out on two different groups of rats, each group consisting of six animals.

The bones of the rats in the first group were labelled by administering tetracycline in a dose of 7.5 milligrams per 100 grammes body weight on three occasions at intervals of seven days from the fourteenth to the twenty-eighth day of life. In the second group the bones were labelled at intervals of fourteen days from the thirty-fifth to the ninety-first day of life.

The animals were killed forty-eight hours after the last injection of tetracycline. The tibiae were removed immediately and fixed in neutral formol-saline. After embedding in methyl methacrylate, thin transverse slices two to three millimetres thick were sawn from each block and ground down to sections 10 microns to 20 microns in thickness. The sections were mounted unstained in a non-fluorescent mountant (Fluormount) and examined in transmitted ultra-violet light.

The distance between the bands of tetracycline labelled bone varies at different points on the circumference of the bone. Thus, linear measurements would not give a true indication of the growth in general and the present study was based on measurements of the area between the rings.

Two sections were taken from each bone and photographed in ultra-violet light. The negative of each photograph was projected on to paper, and the outline of the bones and the tetracycline bands traced on to the paper. The outline so obtained was cut out to give a series of rings representing the bone between the tetracycline bands. These rings of paper were then weighed and compared with the weight of a known area of the same paper.

**Rate of growth in length**—Measurements were carried out on eight different groups of rats aged between fourteen and ninety-one days, each group consisting of four rats. The bones of each rat were labelled by administering tetracycline in a dose of 7.5 milligrams per 100 grammes body weight on two occasions, two and four days before they were killed. The animals were killed forty-eight hours after the second injection of tetracycline. Longitudinal sections of the upper half of the tibia in the mid-coronal plane were ground.
The distance between the bands of tetracycline labelled bone in the metaphysis was measured at three points in the middle third of the section. The measurements were made from the densest part of each band rather than from the edges, which were irregular and more difficult to define. The distance between the bands is the amount that the tibia has grown by the activity of the proximal epiphysial cartilage during the two days between injections. The distance from the most recently laid down band to the epiphysial line was also measured when the latter could be identified. This indicates the rate of growth for the last two days of the rat's life and serves as a check on the accuracy of the measurement between the bands, for it is usually the same as in the two previous days unless rapid alterations are occurring in the rate of growth at this epiphysis.

**RADIOLOGICAL METHODS**

Radiological methods for determining the rate of growth in width of the tibia are unsatisfactory as the distance to be measured on the radiograph is so small. Consequently the radiological measurements are confined to determinations of the rate of growth in length.

A single group of ten rats was used and the length of the tibia was measured in serial radiographs taken at intervals of seven days between the fourteenth and thirty-fifth day of life and then at intervals of fourteen days up to ninety-one days old. The radiographs were taken while the rats were anaesthetised with sufficient ether to produce good muscle relaxation, with the animal in the prone position and the tibia closely applied to the film. This reduces any parallax error to the minimum.

The distance between the proximal and distal articular surfaces of the tibia was measured in each bone on a projected image of the radiograph magnified four times. The distance on the projected image could be determined to ±0.5 millimetres. The rate of growth was then determined from the difference between the average length of the tibia at the various times.

**RESULTS**

*Tetracycline method for measuring rate of growth in width*—The time of the greatest rate of increase in the width of the tibia was between the twenty-first and twenty-eighth day during

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (days)</th>
<th>Rate of increase in cross-section (square millimetre per day)</th>
<th>Standard error</th>
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<tbody>
<tr>
<td>I</td>
<td>14 to 21</td>
<td>0.055</td>
<td>±0.002</td>
</tr>
<tr>
<td></td>
<td>21 to 28</td>
<td>0.059</td>
<td>±0.002</td>
</tr>
<tr>
<td>II</td>
<td>35 to 49</td>
<td>0.023</td>
<td>±0.002</td>
</tr>
<tr>
<td></td>
<td>49 to 63</td>
<td>0.016</td>
<td>±0.001</td>
</tr>
<tr>
<td></td>
<td>63 to 77</td>
<td>0.017</td>
<td>±0.001</td>
</tr>
<tr>
<td></td>
<td>77 to 91</td>
<td>0.015</td>
<td>±0.001</td>
</tr>
</tbody>
</table>

which period the cross-section of the tibia was increasing by 0.059 square millimetre per day. The rate fell rapidly from the twenty-eighth day to the fifty-sixth day and then remained fairly constant; there was a trivial increase in the rate between the sixty-third and seventy-seventh day, but this was not statistically significant. The average rate of increase in cross-section per day is given in Table I.
In the sections prepared from the rats in Group I (that is, at fourteen to twenty-eight days) some remodelling of the earlier bands had taken place (Fig. 7). This could result in inaccurate measurements in this age group.

**Tetracycline method for measuring rate of growth in length**—The rate of growth in length was greatest at the twenty-eighth day when the rate of growth at the proximal epiphysis was 0.23 millimetre per day. After the twenty-eighth day the rate declined rapidly to about fifty-six days and then remained fairly constant during the remainder of the period of the studies, apart from a trivial rise at seventy-seven days which was not statistically significant. The measurements are given in Table II.

In the animals studied when they were fourteen days old, the labels were not well defined and in some instances the bone containing the first label had already been reabsorbed (Fig. 8).

Measurements at this age would be inaccurate and therefore these results are not included. From twenty-one days onwards the band of labelled bone from which the measurements were made was well defined and varied in thickness from 0.08 millimetre when the rate of growth at the epiphysis was 0.23 millimetre per day to 0.03 millimetre when the rate of growth was 0.07 millimetre per day.

**Radiological method for measuring the rate of growth in length**—The rate of growth in length was found to be maximum at fourteen days when the bone as a whole was increasing in length by 0.50 millimetre per day as a result of growth at both the upper and the lower epiphysis. The rate remained high until the twenty-eighth day after which it declined rapidly. There was, however, a slight acceleration of growth between the sixty-third and seventy-seventh day. The average length of the tibia at the various times is given in Table III.
DISCUSSION

Tetracycline methods—The method for measuring the rate of growth in width appears to be a satisfactory one except in very young animals, when some difficulty is encountered due to remodelling, and in animals nearing maturity at which time the rate of growth in width is very slow. In animals between twenty-eight and fifty-six days old there are, however, narrow bands at an adequate distance apart and these enable the rate of growth in width to be measured accurately. Injections at intervals of seven days could be used in this age group but longer intervals are more satisfactory as in the older animals aged between fifty-six and ninety-one days.

The accuracy of the method for determining the rate of growth in length depends principally on the concentration of tetracycline in the band of labelled bone in the metaphysis. In bones from rats labelled after fourteen days a fairly well defined band is seen in the metaphysis and, as one might expect, the band decreases in thickness as the rate of growth diminishes. The measurements indicate that the thickness of the band is approximately one-third of the rate of growth in length per day from that epiphysis. This indicates that the level of tetracycline in the blood is adequate to label the bone for approximately eight hours following an intraperitoneal injection. This time agrees well with measurements of serum levels following tetracycline administration (Cunningham, Hines, Stokey, Vessey and Yuda 1954).

In animals fourteen days old the bands are indistinct and unsuitable for accurate measurements. The reason for this is twofold: firstly, the bone is growing so rapidly that a large amount of bone is formed during the eight hours that the tetracycline is circulating; and secondly, at this early age the metaphysial bone is less completely mineralised than in older animals and therefore most of the metaphysis is capable of taking up some tetracycline.

Tetracycline has been used in much the same way that Alizarin and radioactive isotopes such as calcium 45 and strontium have been used in the past. However, Alizarin is toxic and therefore unsatisfactory if rates of growth are to be measured (Harris, Travis, Friberg and Radin 1964). Radioactive isotopes as well as being capable of damaging bone are expensive to produce and difficult to handle. Tetracycline does not have these disadvantages; it is not toxic in the doses used here; it is relatively cheap; and no complicated apparatus is required to detect the bands. The definition of the bands of tetracycline labelled bone is comparable with the definition of the labels obtained using radioactive isotopes (Harris 1960).

Radiological methods—In the radiological method the distance between the proximal and distal articular surfaces of the tibia was measured. These are two well defined surfaces, and

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Rate of growth at the proximal epiphysis (millimetre per day)</th>
<th>Standard error</th>
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<tbody>
<tr>
<td>14</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>21</td>
<td>0.15</td>
<td>± 0.008</td>
</tr>
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<td>28</td>
<td>0.23</td>
<td>± 0.009</td>
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<tr>
<td>35</td>
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<tr>
<td>49</td>
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</tr>
<tr>
<td>63</td>
<td>0.10</td>
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<tr>
<td>77</td>
<td>0.11</td>
<td>± 0.007</td>
</tr>
<tr>
<td>91</td>
<td>0.07</td>
<td>± 0.004</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Average length of tibia (millimetres)</th>
<th>Average increase in length (millimetre per day)</th>
</tr>
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<tbody>
<tr>
<td>14</td>
<td>16.5</td>
<td>0.50</td>
</tr>
<tr>
<td>21</td>
<td>20.0</td>
<td>0.36</td>
</tr>
<tr>
<td>28</td>
<td>22.5</td>
<td>0.36</td>
</tr>
<tr>
<td>35</td>
<td>25.0</td>
<td>0.18</td>
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<tr>
<td>49</td>
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</tr>
<tr>
<td>91</td>
<td>32.0</td>
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</tbody>
</table>
easily observed when the radiograph is magnified to four times its original size. This is more satisfactory than measuring the distance between the proximal and distal epiphyseal plates (Frost, Stanisavljevic, Villanueva and Roth 1962), as the distal epiphyseal plate is difficult to define. Sissons (1953) introduced a metal marker into the diaphysis of the femur and used this as fixed point from which to measure the rate of growth of the bone from the distal epiphysis. This method, although it has the advantage of measuring the growth rate of a single epiphysis, was not used here as it is considered undesirable to introduce a foreign body into the bone, however far away from the epiphysis, when measurements on growth are to be made.

Comparison of tetracycline and radiological methods—The main advantage of the tetracycline method for measuring the rate of growth in length is that measurements can be made over a period as short as two days. The distance between the bands of labelled bone can be measured with an accuracy of 0.01 millimetre. On the other hand, using the radiological method the length of the bone cannot be measured with an accuracy greater than 0.13 millimetre, so that growth can only be investigated over much longer periods of time.

The differences in rate of growth obtained are explained to some extent by the fact that the radiological method measures the growth in length of the bone as a whole, while the tetracycline method has been used here to measure the rate only at the proximal epiphysis. The rate of growth at the proximal epiphysis is greater than half the rate of growth of the whole bone. This is in agreement with the well known fact that most of the growth in length in the hind limbs occurs at the epiphyses above and below the knee.

There is marked disparity in the rates of growth measured by the tetracycline and radiological methods in fourteen to twenty-one-day-old rats. The radiological method is likely to be inaccurate as the articular cartilage is very thick at this time and the end of the bone is more difficult to define.

The growth pattern of the normal rat—The tetracycline method enables one to construct curves showing the variation in the rate of growth in length and in width of the normal tibia. It is seen from Figure 9 that such curves have the main peak occurring between the twenty-fourth and thirty-second day and with a very slight rise between the seventieth and seventy-seventh day of life. These peaks occur at the same time as those in the curve, showing the variations in the rate of increase in weight of the animals.

These results are in agreement with Swanson and van der Werff ten Bosch (1963) who compared the rate of increase of the body weight in both males and females with the linear growth rates (derived from measurements of tail length). These workers found that the maximum rate occurred at about forty-two days (a little later than in the present study) and they associated this with puberty. They also showed that the secondary spurt in growth in body weight coincided with a similar acceleration in linear growth. The present observations demonstrate that growth in width of the tibia also follows this pattern.

The rate of bone formation and bone resorption—The tetracycline method for determining the rate of growth in width of the tibia also provides a method whereby the rate of bone formation in the periosteum can be studied.

The process of bone resorption is more difficult to study but the metaphysis is a convenient site at which this can be observed. The metaphysis can be divided into two zones. Firstly, the part nearest to the epiphyseal line consists of small, fairly straight trabeculae (primary bone trabeculae) which are closely packed together. In animals aged between twenty-one and forty-two days they extend from the epiphyseal line towards the diaphysis for a distance which is almost always about 0.37 millimetre. In older animals between forty-nine and ninety-one days the length of the primary trabeculae varies but is usually between 0.15 and 0.20 millimetre. Secondly, the part nearer the marrow cavity consists of larger trabeculae which are more widely spaced and extend into the marrow cavity for a variable distance of 1 to 2 millimetres.
In animals between twenty-one and forty-two days the rate of growth at the proximal epiphysis varies between 0.15 millimetre per day and 0.23 millimetre per day; after forty-two days the rate decreases to about 0.10 millimetre per day. It can be seen therefore that in both these age groups the length of the primary trabeculae represents about two days' growth from the epiphysial line, indicating that as the rate of growth in length diminishes so also does the rate of resorption in the metaphysis. It is clear that if the rate of growth in length at the epiphysis is established by the tetracycline method the length of the zone of primary trabeculae can be used as an indication of the rate of resorption.

The tetracycline methods in addition to providing methods of determining the rate of bone growth can be used also to study the basic processes of bone formation and bone resorption under normal conditions, and in bones under the influence of hormones or other drugs.

SUMMARY

1. Tetracycline labelling methods have been used to measure the rate of growth in length and the rate of growth in width of the tibia of the normal rat.
2. The main limitations of the tetracycline methods are that in very young animals the bands of labelled bone are indistinct and remodelling occurs quickly; in animals nearing maturity, the growth in width is very slow and periods of at least fourteen days are required to give reliable results.

3. The tetracycline labelling methods can be used also to determine changes in the basic processes of bone formation and bone resorption.

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REFERENCES


