6-1961

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EXPERIMENTAL MULTIBAND TETRACYCLINE MEASUREMENT OF LAMELLAR OSTEOBLASTIC ACTIVITY*

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ABSTRACT

Newly forming bone is labelled with tetracycline given in experimental animals' drinking water for three separate 24 hour periods, each 24 hour period being separated from its neighbor in time by 5 days. The first 5 day period is termed the control period, the second the experimental period. During the experimental period, an experimental drug or other experimental variable is introduced. After sacrifice, fresh, wet, undecalcified cross sections of m/3 of femurs are made and stained with basic fuchsin by Frost's methods. By suitable microfluorescence methods concentric tetracycline labelled bands are seen in the bone sections, appearing much like the growth rings of trees. The distance between the first and second tetracycline band comprises the control band; this bone grew under control conditions. The distance between the second and third tetracycline band comprises the experimental band; this bone grew under experimental conditions. The mean value of the width of the experimental band as the percentage equivalent of the control band's width is determined, using a micrometer eyepiece. This gives a qualitative figure of osteoblastic activity. A method of measuring osteoblastic activity quantitatively is described briefly.

INTRODUCTION

Previous reports described methods of measuring osteoblastic activity in man and the results of such measurements. The results have necessitated revision of some of the current ideas of the local and systemic factors governing osteoblastic activity. Little is known for certain about these governing factors. Much misinformation and frank speculation on this subject has crept into medical literature and texts in spite of the sparse and conflicting nature of the factual foundation.

If, as it seems to us, the factors governing osteoblastic activity must be studied anew, then laboratory animals must be used and a method must be available which permits accurate and unequivocal measurement of new bone formation per unit time per unit volume of the animal's skeleton. This method must be sensitive, permit direct observation of the modality to be studied and must circumvent the variables and uncertainties afflicting other methods.

In the past, ingenious attempts have been made to measure osteoblastic activity, both in animals and in man. These attempts have revolved around three basic methods. Each of these methods unfortunately is afflicted by variables which cannot be controlled or measured. Each method also has associated technical features and problems which sharply circumscribe its use.

In brief, these basic methods are:

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*Work aided by the following grants: HFH; A-4186, N.I.H.; Orthopaedic Research Education Foundation. Department of Orthopedic Surgery and Orthopedic Research Laboratory.
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(A) BALANCE STUDIES. In this method the net bodily retention or loss of a substance such as calcium is measured. Osteoblastic and osteoclastic activities are merely inferred with this method, whether measurement is accomplished with or without the use of radioisotopes. In the light of our present knowledge this method is unacceptable when the duration of the study is on the order of a week or less.

The reason is that there are other means than osteoblastic and osteoclastic activities for the production of positive or negative balance over short time periods.

(B) LABELLING NEWLY FORMED BONE. In this method a bone-seeking substance which can be identified in situ in undecalcified sections is used to identify new bone formation. This method of observing new bone formation is the oldest, it being first used with alizarin which is a component of madder root. In addition to alizarin, a few other dyes have been used and heavy metals such as lead and mercury have also been used, the latter precipitated in situ as their black sulfides. Radioisotopes have been used extensively, coupled with autoradiography.

These substances have had several drawbacks. They are toxic to the viscera when given in doses large enough for convenient working in subsequently prepared bone sections. There is also the possibility that they are toxic to the osteoblasts and alter their normal behavior.

Tetracycline bone labelling belongs to this method. As will be seen, when tetracycline bone labelling is coupled with proper sectioning and proper microscopic methodology, a powerful and versatile tool is available.

(C) BONE ACCRETION OF RADIOACTIVE BONE SEEKING SUBSTANCES. In this method the amount of a dose of radioactive substance which is later found deposited in a bone is taken as a measure of new bone formation. The difficulty with this method is that inorganic bone-seekers usually also become adsorbed on or incorporated in the crystallites already present in the skeleton at the time of administration. The latter physicochemical process should, on theoretical grounds, be a variable one from day to day and from animal to animal. This method therefore measures the effect of two variables simultaneously, without permitting a quantitative estimate of the relative importance of either.

TETRACYCLINE BONE LABELLING

We owe a debt to Milch, Rall and Tobie for their publication of the fixation of the tetracycline antibiotics in mineralizing new bone. Since their second report in 1958 we have learned the following about the physiology of this phenomenon.

During the time when the blood level of the tetracycline antibiotic is elevated most tissues are stained to varying degree by the drug. This includes most of the skeletal surfaces, such as the walls of the various vascular channels, the walls of osteocyte lacunae and of canaliculae, the crystallite surfaces in feathered bone, in new bone and the zone of demarcation of osteoid seams. (See Fig. 1)

Within 48 hours after administration of tetracyclines has ceased, all of the drug in the body has disappeared, as far as optical detection is concerned at least, with
Figure 1

Fresh, undecalified cross section adult human rib, basic fuchsin, about 300X. An Haversian system in the process of formation is seen in cross section. The Haversian canal lies in the center of the plate. The osteoid seam lies between the India Ink bars; the four bars at the bottom of the figure lie on the zone of demarcation referred to in the text. The zone of demarcation is the plane where initial mineralization of osteoid commences.

This is lamellar bone and lamellar osteoid. The formation of fibrous bone follows a slightly different route.

Tetracyclines deposited in the zone of demarcation are permanently fixed in situ. The direction of growth of new osteoid and of progressive mineralization of osteoid is centripetal, or towards the geometric center of the plate.
three exceptions. These three exceptions are tetracycline fixed (1) in the zone of demarcation of lamellar osteoid seams, (2) in newly mineralized fibrous bone, and (3) in newly mineralized hyaline cartilage. The tetracycline deposited in these three locations appears to be permanently fixed therein and disappears only when the tissue containing the drug is resorbed. The longest period of time between administration of tetracycline and subsequent demonstration in bone to our knowledge is 9 years. This was the time between the patient's receipt of the drug and his death; autopsy material revealed the bone labelled 9 years previously. Numerous examples exist in the Laboratory's files of labels 5 to 8 years old. All new bone forming during the administration of a tetracycline is labelled by the drug.

Once fixed permanently in one of the three locations above, there is no apparent diffusion of the drug into the surrounding bone. There is some fading over the years, the intensity of the 9 year old label being roughly 0.2 that of freshly deposited tetracycline, doses being assumed equal on a milligram per kilo basis.

The zone of demarcation at which labelling of newly mineralizing lamellar osteoid is proceeding has a finite thickness, on the order of 4 microns in adult humans. It is thicker in the young than in the adult. This zone progresses in the direction of mineralization a certain amount each day. This amount may be termed the thickness of lamellar osteoid mineralized per day. In rabbits there is some evidence that the tetracycline retards either mineralization, elaboration of new lamellar osteoid, or both. Evidence on this point is lacking in other animals and man at present. Nevertheless, this possibility makes it necessary in some manner to eliminate such an effect from any experimental use for measurements.

In properly prepared and mounted undecalcified sections, tetracycline may be seen in brightfield microscopy provided the dose of the drug was sufficient — over 25 mg/kilo of organism per day. Much lesser amounts may be detected by microfluorescence. Microfluorescence is necessary for accurate measurements. The orthodox but expensive ultraviolet microfluorescence illuminators are more than adequate.

For limited budgets, and for complete safety to the observer's eyes, a blue-light technique is recommended. (See Fig. 2) Photomicrography may be done with either technique.

Under microfluorescence tetracycline deposited in bone appears a golden yellow, lying in and parallel to the lamellae. (See Fig. 3) Where cement lines cut across a moiety of tetracycline labelled bone, the demarcation is perfectly sharp within the resolving power limitation of the light microscope. The intensity of fluorescence is directly related to the amount of tetracycline administered per unit time. The width of the labelled bands is related to the rate of mineralization of new osteoid and to the total duration of administration of the drug but not to the dosage per unit time.

At any single instant of time, the thickness of the zone of mineralizing bone which is being permanently labelled with tetracycline is the same as the thickness of the zone of demarcation referred to previously and is on the order of 4 microns. The border between labelled and superimposed, newer, unlabelled bone is not sharp.
Diagram of a simple microfluorescence set up which is also safe to the observer's eye, even if improperly used. The light source may be a 35 mm slide projector of 200-300 volt-ampere (Watts) capacity. Kohler illumination should be used. The blue-light filters (Wratten 47 and 47B) and the barrier filters (Wratten 8 and 9) are cemented between glass with nonfluorescing resin such as H.S.R. These filters are obtainable as 2x2 inch gelatin sheets for 50 cents each. They are manufactured by Eastman Kodak.

Standard eyepieces, objectives and substage condenser are adequate.

All photomicrographs of tetracycline fluorescence in this article were taken with this setup. Standard mounting media may be used.

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but, at the resolution of light microscopy, is fuzzy. As a result, it is easier to determine the midpoint of a tetracycline band than it is to measure its thickness. This feature is compensated for in the present method of measuring osteoblastic activity.

No detectable tetracycline remains in sections after decalcification, regardless of the method by which this is achieved. Permanently fixed tetracycline in bone does not fade appreciably in the deep freeze, but does fade slowly in ethanol and formol. Surface tetracycline stains are progressively removed by aqueous, alcoholic and dioxane solutions and are best studied by fresh, frozen sections.6,17,18,22,23,25,26

MATERIALS

The essential materials and equipment will merely be listed here. Their use will become apparent as the Method is elaborated.

\[\text{Figure 2}\]

\text{Diagram of a simple microfluorescence set up which is also safe to the observer's eye, even if improperly used. The light source may be a 35 mm slide projector of 200-300 volt-ampere (Watts) capacity. Kohler illumination should be used. The blue-light filters (Wratten 47 and 47B) and the barrier filters (Wratten 8 and 9) are cemented between glass with nonfluorescing resin such as H.S.R. These filters are obtainable as 2x2 inch gelatin sheets for 50 cents each. They are manufactured by Eastman Kodak. Standard eyepieces, objectives and substage condenser are adequate. All photomicrographs of tetracycline fluorescence in this article were taken with this setup. Standard mounting media may be used. (Reprinted by permission, Henry Ford Hospital Medical Bulletin) but, at the resolution of light microscopy, is fuzzy. As a result, it is easier to determine the midpoint of a tetracycline band than it is to measure its thickness. This feature is compensated for in the present method of measuring osteoblastic activity. No detectable tetracycline remains in sections after decalcification, regardless of the method by which this is achieved. Permanently fixed tetracycline in bone does not fade appreciably in the deep freeze, but does fade slowly in ethanol and formol. Surface tetracycline stains are progressively removed by aqueous, alcoholic and dioxane solutions and are best studied by fresh, frozen sections.6,17,18,22,23,25,26 MATERIALS The essential materials and equipment will merely be listed here. Their use will become apparent as the Method is elaborated.}
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Figure 3A

Fresh, undecalcified cross section middle third of femur of triply banded, 9 week old rat. 75X. The bright bands are tetracycline fluorescing with the set-up illustrated in Fig. 2. Periosteum is at the top, endosteum at the bottom. This was a control animal given tetracycline in drinking water for 3 separate 24 hour periods, each period separated by a 5 day interval during which no medication was received. Concentricity here is good and the resemblance to growth rings of a tree is plain. The irregular areas lying in the cortex between the triple bands and the endosteum represent pseudo Haversian internal remodelling of the cortex. This section is 60 microns thick.

The fuzzy definition of the borderline between labelled and unlabelled bone may be appreciated in this figure, as well as in Figs. 4 and 5.

On the far left, the upper 3 diagonal ink marks identify the third labelled band referred to in the text (Methods, 1); the next 2 diagonal marks identify the second label and the lower, single mark identifies the first label. The unlabelled bone between labels 1 and 2 is the control band. The unlabelled bone between labels 2 and 3 is the experimental band.

The dotted vertical lines illustrate where measurements of the thickness of the control and experimental bands would be made. A slight decrease in thickness of the experimental band is visible and is the result of the retardation of osteoblastic activity by maturation of the animal during the experiment. The animal was 6 weeks old at the beginning, 9 weeks old at the end of the experiment. This 3 week experimental period is 50% of the animal’s initial age.

Optical: Medical microscope with mechanical stage having orthogonal movements. Standard dry objectives. A micrometer eyepiece for qualitative work. For true quantitative work a calibrated micrometer eyepiece and either a calibrated net micrometer eyepiece or a calibrated Zeiss Integrating Eyepiece I will be needed.

For orthodox microfluorescence, an ultraviolet illuminator, ultraviolet filter and barrier filters are needed; these are obtainable on order from most microscope manufacturers. For blue-light fluorescence microscopy, four Wratten filters and a 35 mm slide projector are necessary. (See Fig. 2)
Diagram of femoral cross section. The shaded bands represent the control (inner) and experimental bands (outer) referred to in the text. Such perfect concentricity is seen in the adolescent, and in adult rats; rats cease growing only upon dying.

Section preparation. "Wet-or-Dry" or "Tufbak" carborundum surfaced sandpaper with waterproof adhesive, grade 320 or 360, about 20 sheets. A flat surface such as plate glass, plexiglass or cookie tin. 1% or/and 0.3% basic fuchsin in 40% ethanol. H.S.R. or other suitable low-fluorescence microscopic mountant. Acid media may not be used. Assorted containers, slips, cover glasses. Source of running water.

Supplies: Achromycin, Terramycin, Aureomycin, or other brand-name of single or mixed tetracycline-based antibiotics. All of the currently available tetracyclines or derivatives are suitable for the present work. The most water soluble ones will be the easiest to work with. A very fine toothed coping saw such as is used for cutting out children’s puzzles is necessary to saw the slabs of bone which will subsequently be ground to sections. Rodent bones are unmanageably brittle with coarse-toothed saws.

METHODS

We first describe some details which have been published in detail elsewhere. Then the multiband tetracycline technique will be outlined as a series of steps.

Fresh, undecalcified, unfixed, ground sections are made by Frost’s method from the middle third of the diaphyses of standard bones of the experimental animals. The sections should be cut reasonably accurately perpendicular to the longitudinal axis
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of the bone. The usual standard bones for this work are femur, tibia and humerus. Any long bone including rib is satisfactory from animals as large as or larger than adult cats.

The sections are stained in 1% basic fuchsin if from adult animals, and in 0.3% basic fuchsin if the animals were very young. Unstained sections may be used but the contrast of the fuchsin is helpful in relating the morphology to the tetracycline labels. Staining time and solute should be as recommended. Final section thickness should be about 50 microns.

After staining, sections should be hydrated in a tumblerful of water for 20 minutes and the surface stain then ground off both sides of the section. Sections are then dried in air and mounted in a nonfluorescent nonacidic microscopic mountant. This laboratory uses H.S.R., Harleco (Xylol). Non fluorescing media must be used with UV set-ups.

The experimental method follows as a series of steps. Where modification for other animals or purposes may be necessary, the reasons for the procedure are discussed so that modification may be done intelligently.

Figure 4

Fresh, undecalified cross section upper middle third of femur of triply banded 7 week old rat. 75X. The periosteum is above, the endosteum below. The rat was triple banded at 5 day intervals as described in the text, Methods, 1. The 3 tetracycline bands are plain, and in places exist on the endosteal as well as periosteal surfaces. Concentricity is poor and there is considerable, irregularly oriented, internal remodelling of the cortex. Poor concentricity such as this is seen in younger animals and close to the metaphyses. For this reason sections are best obtained from the middle fifth of the bone. Note that the labelled bands are broad—the result of poor correlation with the long axis of the bone. Measurements of a section such as this would have to be interpreted with caution. Measurements of the interior remodelling cannot be easily done due to variable obliquity of the new bone with respect to the longitudinal axis of the bone.
(1) **Experimental Timing**: The present experimental animals are 6 week old rats. The total duration of the experiment is broken up into a number of periods of time, each period of time being reflected in the new bone produced in the femur and seen as growth-like rings on cross section. These rings are comparable to the growth rings seen in the stump of a tree. (See Figs. 3, 4, 5)

![Figure 5](image)

**Figure 5**

Fresh, undecalified cross section middle third of the femur of 8 week old rat. 75X. Microfluorescence. Triply banded with tetracycline. Given parenteral hydrocortisone during the experimental period, leading to retardation of lamellar endosteal osteoblastic activity. The value measured was 0.3 of normal, control band width (see text, Method, 7).

There has been no periosteal (top of figure) osteoblastic activity during the entire experiment. The bands illustrated are the result of endosteal osteoblastic activity. The opposite quadrant of this section reveals periosteal banding, no endosteal banding, and a similar hydrocortisone effect.

(A) On day number 1 of the experimental period, the animals are given drinking water containing 1.0 to 1.5 gm/liter of the tetracycline antibiotic selected for use. Any new bone mineralized during this period, which should be exactly 24 hours long, will be labelled by the tetracycline. 6 week old rats drink about 20 cc/day and thus ingest about 200 mg/kilo. Plenty!

(B) From days 2-6 inclusive no tetracycline or drugs is administered. This is the control period and during this period the control band of new bone is being formed on top of the first labelled band. If injections of a drug are to be given during the later experimental period, dummy injections should be given during the control period because the physiological stress caused by the injections might affect bone growth (cortisone markedly retards lamellar bone formation).
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(C) On day 7 tetracycline is again given in the drinking water for exactly 24 hours. This produces a second tetracycline labelled band laid down on the preceding control and first labelled bands.

(D) On days 8-12 inclusive the experimental drug, surgical procedure or other change in the skeletal environment of the animals is introduced. This constitutes the experimental period and during this time the experimental band of bone is being deposited.

(E) On day 13 tetracycline is again given in the drinking water for exactly 24 hours. This produces the third tetracycline labelled band.

(F) The animal should not be sacrificed before day 16 of the experiment. The reason is that at least 3 days are necessary for more new bone to be formed to bury the third labelled band. A longer period of time before sacrifice is permissible as long as the delay is not sufficient to allow remodelling to destroy the three concentric bands. A fourth labelled band after a second control period and band may be indicated in some circumstances.

(2) Design of the Experimental Timing: The rate at which the experimental animal produces new bone governs the time intervals given in (1) above. In 6 month old rats and in 6 month old dogs the duration of each tetracycline label would be 2 days instead of one, and the duration of the control and experimental bands would be about 10 days, in some cases 15. Much longer periods of time than this will be unwise for most purposes because the total duration of the experiment is then apt to equal or exceed the time required to form the average Haversian system. This will lead to many partially labelled Haversian systems and this in turn leads to error in subsequent interpretation of measurements. This qualification does not apply if the animal does not form Haversian systems.

(3) Need for Controls: A unique feature of the present method is that the experimental animals serve as their own controls for a number of variables. For example, variation in level from which the section is cut, individual variation in reaction to food, environment, and like factors are eliminated because they exist in the same animal in the same place in the same bone for the duration of the experiment. The effect of maturation on bone growth rate is not eliminated. In young animals such as 6 week old rats, the amount of maturation occurring between the control and experimental periods might affect the normal growth rate in those periods. For this reason, in very young animals in whom the total duration of the experiment is more than 10% of their age, controls must be run. The controls should be labelled in the same manner and for the same periods of time as the experimental animals but not given experimental treatment. Measurement, calculation and correction are described below in section (7). (See Fig. 3).

(4) Sections: Immediately after sacrifice undecalcified, cross sections are made which are cut accurately perpendicular to the long axis of the femur and through the middle third. These are stained and mounted as suggested.\(^9,^{10}\)
(5) Observation of labelled Bands: Under microfluorescence the tetracycline labelled femoral cross sections will appear about as in Fig. 3. Usually 3 concentric rings of tetracycline will be seen in those parts of the periosteal and endosteal surfaces where lamellar bone apposition was active. The perfection of concentricity, and the degree to which the three rings envelop the entire circumference of the bone, depend on a number of factors, among them being the age of the animal and the level from which the sections are cut. Concentricity is imperfect in the younger animals and imperfect in the neighborhood of strong muscle attachments, again more in the young than in the old. The illustrations purposely consider the more difficult situation to deal with: the young. (See Figs. 3, 4, 5)

(6) Qualitative Measurement of Lamellar Osteoblastic Activity: Imagine that the femoral cross section being measured is divided into pie-shaped fragments by radii placed as in Fig. 3. These radii should be separated by approximately equal angles. In adult animals or in adult Haversian systems the concentric bands will surround the entire circumference of the bone or Haversian system. In young animals they will usually surround only part of the circumference. Places where the labelled bone was deposited in such irregular fashion as illustrated in the cortical interior in Fig. 4 are ignored. There is justice in this deliberate omission because the bone forming in these areas is doing so under the aegis of different control and triggering mechanisms from that formed in the making of the concentric bands.

Along each of the radii diagrammed in Fig. 3, the width of the control and of the experimental band must be separately measured with the eyepiece micrometer. If the value for the control band be designated \(c\), and for the experimental band \(e\), then the ratio of the two is expressed as \(\frac{e}{c}\), and designated as \((oba)\) for osteoblastic activity. Slide rule accuracy is all that is required. Sufficient magnification should be used in these measurements so that the distance between the first and second labelled bands is more than 5 divisions of the eyepiece micrometer. The points of measurement are from the middle of one labelled band to the middle of the neighboring band.

The \((oba)\) of all the measurements of all the sections of a single bone, such as the femur, are averaged and the mean determined. The figure obtained is designated as \((OBA)\), and is the mean osteoblastic activity of the experimental band in terms of the control band as unity and normal. Multiplying \((OBA)\) by 100 gives the osteoblastic activity of the experimental period as the percentage of normal. It is assumed that the control band width is 100% in this case.

In general about 50 or more separate determinations of \((oba)\) should be averaged, obtained from at least 3 sections of each bone and from at least 4 animals, before an experimental effect is accepted as valid. When departures of experimental osteoblastic activity from normal lie in the range of 60%-140% of normal, more animals should be run before accepting an experimental effect as valid. Greater departures than this range may be provisionally accepted as valid with the numbers given.

(7) Correction of \((OBA)\) for Maturation Effect. Assume that in a given experiment with cortisone this drug was found to retard lamellar osteoblastic activity
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in the experimental band. The measured value was 0.3, or 30% of normal. (See Fig. 5). Assume further that litter-mates of this animal used as controls to determine the maturation effect on osteoblastic activity gave a value for the second tetracycline-free band (corresponding to the experimental band in the experimental animals) of 0.8, or 80% of normal. (See Fig. 3). The true, retarded osteoblastic activity produced by the cortisone in the experimental group of animals is thus 0.3, or 0.37, or as percent 37%.

(8) Differing types of Lamellar Osteoblastic Activity: When measuring the effects of variables on lamellar osteoblastic activity, it must be realized that there are good reasons to anticipate differences in effects of some variables on Haversian, periosteal and endosteal lamellar osteoblastic activities. For this reason, these three types of osteoblastic activity should be recorded separately rather than lumped together. Lumping them together is permissible only when the total, quantitative osteoblastic activity per unit time is being measured. This is a different phenomenon entirely and is the synthesis of the quantitative contributions of the three modalities named.

Note: The method as described so far provides a qualitative rather than quantitative measure of the various forms of lamellar osteoblastic activity referred to.

When Haversian osteoblastic activity is being measured, the values for a number of Haversian systems — preferably over 20 selected by a proper sampling technique — should be averaged. Single Haversian systems are measured as though they were cross sections of very small long bones.

(9) Quantitative Osteoblastic Activity Theory: A true, quantitative measurement of osteoblastic activity measures the volume of new bone formed per unit volume of the skeleton per unit time. This is desirable in certain types of work in progress in this and other laboratories. It is more difficult to do.

The measurement may be achieved by measuring the total cross section area of sections made as recommended in Methods (4) and labelled as recommended in Methods (1). Then the total cross section area of the control and experimental bands should be measured. Because of the longitudinal grain of diaphyseal bone, the longitudinal grain of periosteal, endosteal and Haversian lamellar bone formation, and because 3 or more sections from any one bone will be measured, the results of such measurements may be reduced to unit volume measurements.

Example: Assume the total cross section area of 3 sections from a particular rat femur is 11.6 mm². Assume that the total cross section area of the control band in these 3 sections is 2.1 mm². Then the control band, in terms of a single, average mm², is 2.1 or 0.18. In other words, on the average for every mm² of cross section, there is 0.18 mm² of new bone formed in this animal. Because of the longitudinal grain and the fact that with several sections we have sampled the length of the diaphysis of the femur, we are justified in stating that for each mm² of diaphysis there is 0.18 mm² of new bone formed. This refers only to the middle third of the diaphysis. Metaphyseal formation must be separately measured by different and more complicated means.
In the above example the one thing still missing is the element of time. Assume that the control band was laid down over a 5 day period. Then in terms of new bone formed per mm$^3$ (the unit volume selected in this example) of diaphysis, per day, the figure 0.18 above is multiplied by 1 to obtain $0.036 \text{ mm}^2/\text{mm}^3/\text{day}$. If, on the other hand, we wish to express the measurement in terms of a month, the value 0.18 must be multiplied by $30 = 6$, and we obtain $1.08 \text{ mm}^2/\text{mm}^3/\text{month}$, or 1.08 mm$^3$ new bone formed per single mm$^3$ of skeletal tissue per month.

We recommend expressing results in a different manner than either of the above: the biological half-life of the bone. This means the period of time required for half the reference volume to be replaced with new bone, assuming that osteoblastic activity continued at the measured rate throughout this period and assuming that osteoclastic activity was equal. Neither of these two assumptions hinges on actual events and so neither need be verified by separate measurements. In the example above 0.18 mm$^3$ of bone was formed in 5 days; biological half-life is therefore $0.5 \times 5$, or $2.78 \times 5$, or 14.9 days. The 0.5 figure is half of the unit volume.

The figure for biological half-life always indicates the time period in which half of any reference volume of bone (such as mm$^3$, cc$^3$, liter, inch$^3$) will be formed at the rate measured. The biological half-life is thus readily convertible to other systems of measurement and, more important, permits immediate and valid comparison of measurements made at different times, in different animals and under different experimental conditions.

(10) Quantitative Osteoblastic Activity: Practice: There are many methods for obtaining the basic measurements required. Being microscopists we recommend the point counting planimetric procedure outlined by Hennig$^{24}$ and incorporated into the design of the Zeiss Integrating Eyepiece Micrometer$^{18,24,32}$ This procedure is explained in some detail in the references listed.

Briefly, the method works as follows: An eyepiece reticule containing a number of points (indicated by small crosses, or the intersections of the orthogonal lines of a net micrometer reticule) arbitrarily positioned in the reticule is superimposed on the image of an objective with N.A. between 0.3 and 0.5. Microfluorescence technique is essential. The reticule is calibrated with a stage micrometer$^{18,24,32}$ so that the exact, real area of the field being measured is known for the combination of objective and eyepiece used. (See Figs. 6, 7).

Fields are selected for measurement by a sampling technique outlined in other publications.$^{19,21}$ (See Fig. 8). Magnification is adapted to the thickness of the cortex and total cross section area of the section, being as low as the necessary resolution will permit in cross sections of human femurs, and as high as necessary in cross sections of small bones such as human rib or rat femur to fill the field with the thickness of the cortex.
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Figure 6

Diagram of reticule in Zeiss Integrating Eyepiece I (A) and of a typical net micrometer (B). The point of intersection of orthogonal lines is that referred to in the text, Methods, 10.

Figure 7

A typical net micrometer reticule superimposed on the image of a tibial cross section. There are 16 line intersections. Assume the vascular area of the section is desired, i.e., the total area composed of vascular spaces rather than bone. In the present case there are 2 “hits” on vascular channels and 14 hits on bone. If a large number of fields are measured in this fashion, and if the fields are selected by an adequate sampling procedure and if the total area of the field (which is larger than the area of the square) is known by calibration, and if the total number of fields and hits are recorded, the vascular area can be measured with good accuracy.

In measuring osteoblastic activity by the multiband tetracycline method, the same principle is used. Rotating the eyepiece without moving the section repositions the reticule points. Such repositioning may be counted as a separate field as long as all measured parts of the section are treated identically.
Each field is measured by noting where the reticule points intersect with control (or experimental) band on one tally, where the reticule points intersect with other bone on a second tally. The intersections of reticule points with the microscopic image of the section are termed "hits". During the measurement of a single field the fine motion of the microscope may not be readjusted nor may the eyepiece be rotated. Separate measurements may be obtained by rotating the eyepiece an arbitrary amount between measurements on the same location of the section, recording each as a separate field on the third tally. This procedure is permissible only if all fields measured are all also treated in the same manner. Hits on the lumens of vascular spaces are not tabulated.

This procedure is similar to that diagrammed in Fig. 7.

At the conclusion of the measurements of, for example, 3 sections from the middle third of a single rat femur, all of the hits on control band (or experimental band) are added to all of the hits in the other bone. This total represents the total section area measured. Assume the total number of hits was 2000. Divide the total hits on the control band by this number, assuming there were 158 hits on the control band.

\[
\frac{158}{2000} = 7.9\%, \text{ the proportion of control band to total bone volume.}
\]

In terms of unit volume the control band is \(0.079 \text{ mm}^3/\text{mm}^3\).

If the control band was laid down in 5 days, and we wish to state the osteoblastic activity in terms of the biological half-life of the middle third of the diaphysis, the calculation is as follows:

\[
0.5 \times 5 = 31.6 \text{ days biological half life, a real value determined on the rib of a 4 month old child. The figure 0.5 again is half the reference, unit volume.}
\]

The reader will note that in this procedure the real total area of the section is not measured. A sample of it is measured however, and this is the feature that
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necessitates an adequate sampling procedure. By suitable choice of numbers of tallies periosteal, endosteal and Haversian lamellar bone formation may be measured simultaneously. Adequate precision with this method exists when the total hits on the smallest compartment being measured are in excess of 100.

Correction for maturation effect is done as described in section (7) above, after separate measurement of control animals has been done to obtain the necessary figures.

DISCUSSION

(1) The method described is suitable for measuring lamellar osteoblastic activity only.\textsuperscript{12,14,15,16} It will not suffice for measuring the production of fibrous bone such as is produced in fracture callus, in certain bone neoplasms, or in the epiphyseal plate of growing animals. We have repeatedly referred to and emphatically again refer to this point. Too many investigators have mistakenly assumed that new bone is new bone and it is all alike. The result has been, as one example, the widespread misconception that protein anabolic hormones stimulate the formation of new lamellar bone.\textsuperscript{15,16,18} It is true that administration of estrogen leads to increased amounts of fibrous bone in mouse and bird medullary cavities. Estrogen and its homologues do not stimulate the formation of lamellar bone in any animal known to us, including mice and birds. Since human postmenopausal osteoporosis (the disease most often treated with estrogen) is a disease of lamellar bone rather than fibrous bone, this observation is pertinent.

(2) The lamellar bone produced as endosteal bone apposition, the lamellar bone produced as circumferential lamellar bone apposition and the lamellar bone produced as Haversian systems are not necessarily governed by the same local and systemic factors, or not necessarily affected to the same degree by these factors.\textsuperscript{25} This is a proper field for investigation with the technique described.

(3) It follows that endosteal and circumferential lamellar bone formation are best studied in growing animals; when growth has ceased these modes of new bone formation largely cease too. It also follows that the formation of Haversian systems, which is the mode of internal reconstruction of the cortex of most interest to us, is best studied in adolescent and adult animals; this mode of reconstruction is minor in the very young and in small rodents.

(4) While young rats, mice, rabbits and similar animals are very useful for experimentation because of their rapid growth and short life span, it should be emphasized that in many respects their skeletal physiology, anatomy and histology differ from that of man. It is therefore risky to draw parallels between such animals and man. It is quite permissible to use the laboratory animal as a testing ground, relying on human material to confirm the presence or absence of similar phenomena.

(5) The present method is of considerable value for the purpose of studying and discovering the various physical and chemical factors which affect the rate of osteoblastic activity in general. Note, however, that the measurements are on the middle third of one bone of an animal. Metaphyseal bone, bone of the axial skeleton and cancellous bone have been ignored for obvious technical reasons.
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It will shortly become possible to normalize the osteoblastic activity of a standard bone to the osteoblastic activity of the entire skeleton, thanks to the collaboration of quantitative radioisotopic techniques with our quantitative histological techniques. This normalizing factor amounts to a calibration of the present method, as well as a calibration of methods previously published. This calibration will considerably enhance the usefulness of the present method in both animal and human skeletal studies.

REFERENCES

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25. Henry Ford Hospital Orthopedic Research Laboratory: Publications (In preparation.)


