

Review article

Histomorphometry of bone

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SUMMARY This review of the histomorphometry of bone outlines methods of biopsy and processing of specimens in the laboratory, the basic principles of morphometry, and the measurements made in order to obtain estimates of the proportional volumes and surfaces occupied by different components of bone. Variability such as that between methods, observers and laboratories is discussed and a brief outline of automatic and semiautomatic methods of image analysis also given.

Histopathology is a subject normally considered in purely descriptive terms but a quantitative approach is sometimes of value. This is true of the pathology of bone. Bone morphometry has a role to play in the study of particular metabolic disturbances and their treatment. Group means and standard deviations are best used as a method of comparison under these circumstances. Morphometry may also be helpful in the evaluation of an individual biopsy. Care is needed over the reproducibility of techniques and the methods may be time consuming, but these aspects will almost certainly change with the development of relatively low cost semiautomatic computer-linked image analysis systems.

Bone biopsy

Several factors determine the site selected for bone biopsy, namely:

- (a) ease of clinical availability,
- (b) an area where there is active bone turnover and
- (c) adequate amounts of trabecular bone.

The iliac crest is usually chosen.^{1,2} Modern techniques of biopsy involve obtaining a cortex to cortex core of bone from the iliac crest with a wide bore trephine.³ Ideally the biopsy should be obtained from a standard site.⁴ An alternative method is to take a vertical core downwards from the iliac crest. A comparison by Visser *et al*⁵ was unable to demonstrate a systematic difference between the two techniques with respect to the measurement of volume densities. Wedges of iliac crest obtained at necropsy should include the area normally examined in biopsy specimens.

The complications of iliac crest biopsy have

recently been reported by Duncan *et al*⁶ who presented data for nearly 15 000 biopsies, three fifths of which were transiliac, the remainder obtained by a superior approach through the iliac crest. Morbidity was low with both methods, the most common problem being haematoma in patients with primary haematological diseases or those receiving heparin during haemodialysis.

Other complications included neuropathy affecting the lateral cutaneous nerve of the thigh, wound infection, pain, fracture and osteomyelitis. The overall incidence of complications in this large number of biopsies was 0.5%. Duncan and colleagues⁶ found that the large majority of patients experience little pain. A similar finding was obtained by Johnson *et al*.⁷

The experience of the person performing the biopsy plays a part in determining the suitability of the specimen for histological examination. When bone biopsy is performed as an occasional procedure, the specimen is often fragmented and inadequate.

Laboratory processing of the biopsy

The biopsy may be fixed in formalin, 70% ethanol or methanol. The latter two fixatives give better preservation of tetracycline fluorescence.⁸⁻¹⁰ It is essential to prepare undecalcified plastic embedded sections and our own preference is for methyl methacrylate sectioned at 6-7 μm on a Jung K microtome. Thicker sections (15 μm) may be used for ultraviolet microscopy of tetracycline fluorescence.

Changes in the amount of total bone and osteoid together with the activity of the cells at trabecular surfaces are the important features in metabolic bone disease. All the necessary details are detect-

able in haematoxylin-eosin stained sections but it is preferable to use several other techniques to highlight particular features. The differentiation of osteoid from mineralised bone is easily achieved using von Kossa counterstained with van Gieson, eosin, safranin or almost any other similar stain. The Goldner trichrome method gives good contrast between mineralised and unmineralised bone, as do other trichrome methods. Solochrome cyanin is used in some centres. A close correlation has been shown between results for measurements of osteoid volume when solochrome and von Kossa methods are compared.^{11,12} Comparability was less good between solochrome and trichrome, the latter tending to underestimate the amount of osteoid.^{11,13} Features at trabecular surfaces are reasonably well seen in haematoxylin-eosin and Goldner trichrome methods. Toluidine blue or thionin staining give good definition of cellular details. Osteoclasts are easily visualised by these methods, but some workers are now using cold formalin fixation, equal parts methyl/glycol methacrylate embedding medium and acid phosphatase staining for the demonstration of these cells.¹⁴

The width of the osteoid seams depends on:

- (a) the osteoblastic apposition rate, that is the rate of production of osteoid by osteoblasts,
- (b) the rate of mineralisation of the osteoid so produced by the osteoblasts.¹⁵

Clearly, an increase in the amount of osteoid relative to total bone tissue (hyperosteoridosis) is not necessarily due to osteomalacia and it is important to decide whether there is a calcification defect under these circumstances. Staining methods which demonstrate the mineralisation front include solochrome cyanin, Sudan black, cobalt salts and toluidine blue at pH 2.8.^{2,15-18} The mineralisation front appears as a granular purple line at the junction of osteoid and mineralised bone in the toluidine blue method. None of these methods is particularly reliable and the mineralisation front is best demonstrated by incorporation of a tetracycline label into the bone before biopsy.

The fluorescence of tetracyclines in bone was described by Milch *et al*¹⁹ and adapted as a means of labelling the mineralisation front by various workers.²⁰⁻²²

Tetracyclines are bound at sites of active calcification, as shown by the close anatomical relationship between tetracycline and ⁴⁵Ca deposition in bone.^{22,23} Further information and references relating to use of tetracycline labelling of bone may be obtained from the literature.^{8,10,24-29}

A single label gives information about any defect in calcification, while double-labelling enables measurement of bone mineralisation rate. The time

required between administration of label and biopsy before reproducible results are obtained has been found to be 48 to 72 h.²⁷ Tetracycline given for two or three days followed by an interval of three days before biopsy provides a suitable regimen for single labelling, while double labelling is obtained by the use of two three day courses of tetracycline separated by 10 days.

Basic principles of the morphometry of bone

It is not proposed to give a detailed account of the theory of morphometry, which is available from other sources.³⁰⁻³² Basically, the methods involve the application of probability theory to geometry by the use of estimates, rather than exact measurements. Repeated counting is used in order to make the estimates as accurate as possible. Although the measurements are made on two dimensional images (allowing for section thickness), the information derived may be interpreted on a three dimensional basis. Results are usually expressed in percentages though some workers use ratios.

Bone quantification may be performed using one of the following:

- (a) inexpensive simple eye-piece graticules,
- (b) semiautomatic instruments in which a digitising tablet is linked to a desk-top microcomputer,
- (c) fully automatic computer-linked image analysis equipment.

The following account will deal mainly with point counting and so-called linear intercept methods using eye-piece graticules.

Point counting is performed by the superimposition of a series of points on the microscope field with an eye-piece graticule and enables the estimation of areas. The principle of point counting is extremely simple. The number of points or hits occurring on a particular feature, for example, bone trabeculae is counted and expressed as a percentage of the total number of possible hits (Fig. 1). The adjacent field is counted, and so on, until a sufficient number of measurements has been made to obtain an accurate estimate of the true amount of trabecular bone as a percentage of total tissue. Thus if n_1, n_2, n_3 etc. are the numbers of points falling on trabeculae and N_1, N_2, N_3 etc. are the total possible numbers of points in each field, the area A , expressed as a percentage, can be calculated as:

$$A = \frac{n_1 + n_2 + n_3 \dots n_x}{N_1 + N_2 + N_3 \dots N_x} \times 100$$

where x is the number of fields necessary to obtain a reproducible result.

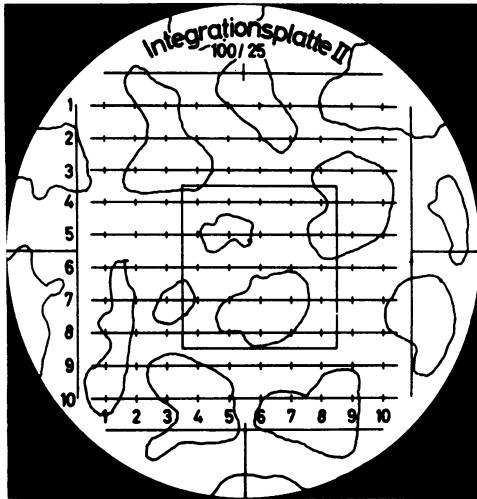


Fig. 1 Diagram to show the principle of point counting using the Zeiss Integration Plate II eye-piece graticule. If the islands are considered to be bone trabeculae, then in the small central square there are 8 out of 25 points falling on bone—that is 32% of total tissue is bone. Alternatively, 36 points fall on bone in the large square which contains 100 points—that is, 36% of total tissue is bone. (With permission of Carl Zeiss (Oberkochen) Ltd.)

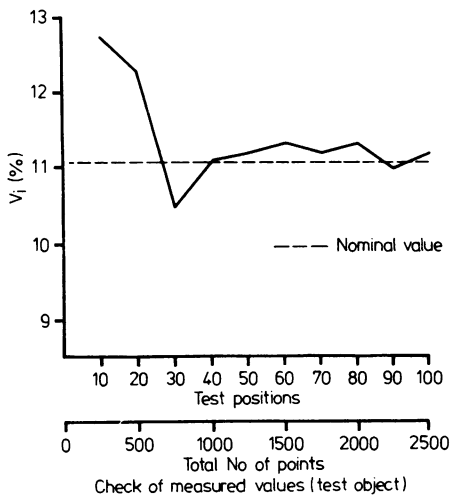


Fig. 2 Diagram to show the effect of increasing the number of fields examined and points counted on the accuracy of the estimate obtained for a volume measurement. There is considerable variation in the results obtained when small numbers of fields are measured, but the values gradually settle to a "nominal" value, shown as a horizontal line. Obviously the number of fields could be increased further, in theory until a constant value were obtained. (With permission of Carl Zeiss (Oberkochen) Ltd.)

The simplest way to overcome the problem of how much to count is to calculate the mean value after a given number of fields, count more fields and recalculate the mean value, continuing until the mean value settles to a more or less constant level. This value is described as the "nominal value" and is best understood by reference to Fig. 2.

Since measurements are made on a basically two dimensional object, the value obtained is an area, or area fraction (A_a), of the whole area of tissue counted. The Delesse principle, described by a French geologist in the middle of the last century,³³ states that area is an unbiased estimator of volume so that it is usual to express values as volumes even though areas have been measured.

The linear intercept method is used for measurements of surface area or simple length measurements. An eye-piece graticule enables the superimposition of a series of parallel lines upon the microscope field to be examined. The method is easily understood by reference to Fig. 3, which illustrates the measurement of osteoid surface using five lines. The number of intercepts, or more accurately intersections, that osteoid and total trabecular surface make with these lines is counted. Further measurements are performed on successive adjacent fields until an accurate estimate of the percentage of surface occupied by a particular feature, in this case osteoid, is obtained. Repetition until a nominal value is achieved applies in the same way as for

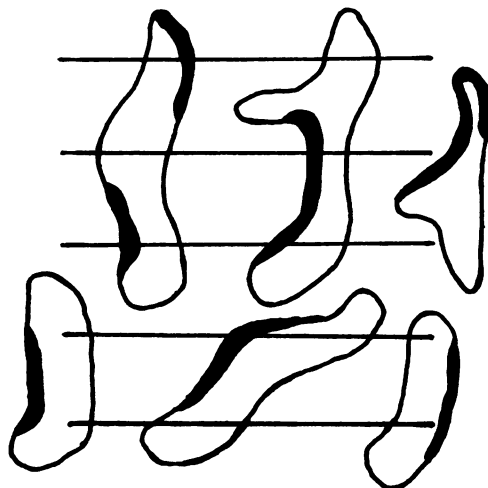


Fig. 3 Diagram to show the use of the linear intercept method for measuring surface lengths. The broad black borders represent areas of osteoid. There are 5 places where the lines intersect with osteoid-covered surface and 20 places where lines intersect trabecular surface—that is, osteoid surface is 25% of trabecular surface. This diagram is a simplified representation of the superimposition of lines in the Integration Plate II, shown in Fig 1.

point counting. Measurements of osteoid, resorption, osteoblastic surfaces may be made by this method.

The mineralisation front, as seen by ultraviolet light microscopy after tetracycline labelling of the biopsy, is also measured in this way. If double labelling of the biopsy with tetracycline is available, then it is possible to measure the distance between the two labels using a calibrated micrometer eye-piece. This measurement is performed at four equidistant points along each surface showing two fluorescent lines, as illustrated in Fig. 4, and the measurement repeated a sufficient number of times, say twenty.^{9 24 25 28} The appositional rate is calculated by dividing the mean distance between the labels (d), by the time interval between administration of labels (t). In order to obtain a true appositional rate, it is necessary to apply a correction factor since the two lines of tetracycline label are actually sectioned in random planes varying between perpendicular and horizontal. The mathematical method for the derivation of this correction factor is available in papers by Frost²⁸ and Teitelbaum and Nichols.²⁵ Frost²⁸ has recommended a correction factor of 0.74—that is, the true appositional rate may be calculated as 0.74 times the value obtained from actual measurement.

Measurements on bone biopsies

There is considerable variation in the literature with respect to the terms used for the various values obtained in bone morphometry. The volume of trabecular bone as a proportion of total tissue (bone and bone marrow) is termed the trabecular bone volume, absolute volume of trabecular bone, fractional bone volume, fractional trabecular bone volume, volume density of bone or relative trabecular volume. Surface density is strictly a measure of surface area related to volume and expressed in mm^2/cm^3 ^{3 34-36} but “surface density” and the abbreviation S_v are often used more loosely. It is vital therefore to have a clear idea of what is being expressed when examining particular results. A list of parameters and how they are measured is shown in Table 1.

The osteoid index provides a useful guide to the thickness of osteoid seams, and is derived mathematically from the osteoid volume and osteoid surface, using the following formula:

$$\text{Osteoid index} = \frac{\text{Osteoid volume}}{\text{Osteoid surface}} \times 100.$$

It has been shown to correlate well with the actual measurement of osteoid seam width.¹⁵ A rapid and fairly reliable method of assessing osteoid seams is

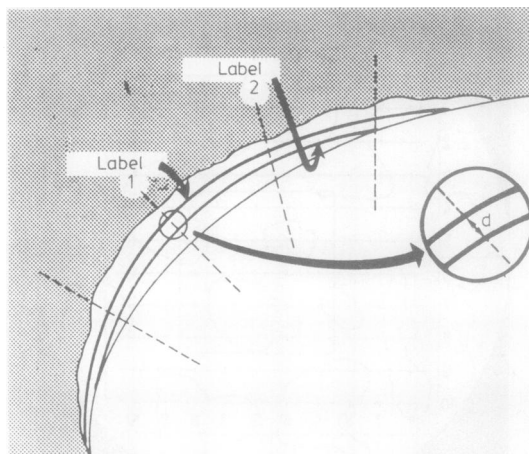


Fig. 4 Diagram to illustrate the measurement of appositional rate in bone with a double tetracycline label. The distance (d) between two fluorescent lines of incorporated tetracycline is measured with a calibrated eye-piece graticule at four equidistant points. The appositional rate is obtained with knowledge of the time interval (t) between administration of the labels—that is, appositional rate = d/t . (See text for references to correction factor required for measurement of the appositional rate.)

the use of polarised light microscopy, when the number of birefringent lamellae (bright lines) may be counted. Up to four such lamellae are present in normal bone^{37 38} so that a greater number than this is an indicator of hyperosteoidosis.

NORMAL VALUES

Definitive normal values for the measurements made in bone morphometry are impossible to give, since they vary from laboratory to laboratory. It is essential that each centre obtains a number of normal bone samples at necropsy from the iliac crest of previously ambulant cases dying suddenly with cardiovascular disease and having no other known pathology. Measurement of these specimens enables a normal range to be obtained. Examples of typical normal values are given in Table 2. Several workers have demonstrated a gradual decrease in the trabecular bone volume of normal bone with increasing age.^{2 39-41}

The mineralisation front should normally occupy more than 80% of the osteoid surface, but this decreases slightly in older subjects.² A mineralisation front of less than 20% represents a definite calcification defect. The mineralisation rate (appositional rate) as measured by double-labelling with tetracycline is normally about $1 \mu\text{m}/\text{day}$ or slightly less. Active osteoid formation by osteoblasts usually comprises around 5% of the total trabecular surface

Table 1

Trabecular bone volume	=	$\frac{\text{Volume of trabeculae}}{\text{Volume of trabeculae and marrow}}$	× 100
Osteoid volume	=	$\frac{\text{Volume of osteoid}}{\text{Volume of osteoid and mineralised bone}}$	× 100
Osteoid surface	=	$\frac{\text{Length of surface occupied by osteoid}}{\text{Total length of trabecular surface}}$	× 100
(Active) osteoblastic surface	=	$\frac{\text{Length of trabeculae occupied by active osteoblasts}}{\text{Total length of trabecular surface}}$	× 100
Resorption surface	=	$\frac{\text{Length of trabecular surface occupied by resorption lacunae}}{\text{Total length of trabecular surface}}$	× 100
Osteoclastic resorption surface	=	$\frac{\text{Length of surface occupied by osteoclasts}}{\text{Length of resorption lacunae}}$	× 100
Mineralisation front	=	$\frac{\text{Line length of mineralisation (Tetracycline fluorescence)}}{\text{Total length of trabecular surface}}$	× 100
Osteoid index	=	$\frac{\text{Osteoid volume}}{\text{Osteoid surface}}$	× 100
Appositional rate (see text, correction factor)	=	$\frac{\text{Distance between labels}}{\text{Time}}$	µm/day
Osteoclastic index	=	Estimate of numbers of osteoclasts as either osteoclasts per high power field, osteoclasts per sq mm or sq cm of tissue or osteoclasts per mm trabecular surface	

Table 2 Normal values for iliac crest bone expressed as percentages (from Melsen *et al*⁴¹)

Age (yr)	Male	Female
Trabecular bone volume		
10-19	24.5±1.7	31.8±2.7
20-29	24.6±2.3	26.8±1.7
30-39	22.9±1.1	26.5±2.4
40-49	17.4±1.9	22.5±3.5
50-59	20.4±1.2	20.1±2.4
60-69	13.7±1.2	15.5±1.8
70-79	16.2±2.4	17.0±2.0
> 80	15.4±2.8	17.7±3.2
Osteoid volume		
10-19	2.6±0.6	2.1±0.7
20-29	2.5±0.3	2.4±0.4
30-39	1.8±0.3	1.7±0.4
40-49	2.2±0.9	1.3±0.5
50-59	3.0±0.5	1.9±0.4
60-69	1.6±0.3	2.0±0.6
70-79	1.8±0.5	2.1±0.5
> 80	0.9±0.2	1.3±0.2
Osteoid surface		
10-19	17.8±2.8	14.7±1.9
20-29	18.8±2.5	16.7±1.6
30-39	14.9±2.2	14.1±2.6
40-49	17.2±5.5	14.1±1.7
50-59	20.3±1.2	19.3±3.1
60-69	16.0±2.4	19.8±3.4
70-79	18.3±2.5	18.2±3.4
> 80	12.0±3.2	12.7±0.8
Resorption surface		
10-19	3.9±0.4	3.2±0.3
20-29	4.0±0.5	3.9±0.3
30-39	3.7±0.3	4.0±0.4
40-49	4.1±0.8	3.4±0.2
50-59	4.0±0.3	5.1±0.6
60-69	4.1±0.6	3.6±0.6
70-79	5.0±0.6	4.3±0.5
> 80	6.0±0.1	3.7±0.6

and active resorption less than 1% of total trabecular surface.²

VARIABILITY

Important aspects of variability in bone quantification include differences between (a) sites in the same bone (b) bones in the same patient (c) observers (d) laboratories, as well as (e) variations in methods, such as staining techniques and magnifications used in microscopy.

There have been numerous studies in which different sites in the iliac crest were compared. Minimal differences have been demonstrated between biopsies at adjacent sites in the same iliac crest^{2,4,41,42} although differences do occur when the bone is more than 2 cm posterior or inferior to the standard site of biopsy.⁴ Comparison of results obtained from the left and right iliac crests have been performed by several workers^{4,5,11,37,43,44} and no systematic differences have been demonstrated.

The influence of staining methods on bone histomorphometry has already been mentioned, comparability being good between solochrome and von Kossa for measurements of osteoid volume, and less good between solochrome and trichrome methods. Melsen *et al*⁴ found higher values for osteoid volume and osteoid surface using Masson trichrome compared with toluidine blue.

There are several questions which arise with respect to the use of tetracycline labelling of bone. The timing of administration of label and biopsy is

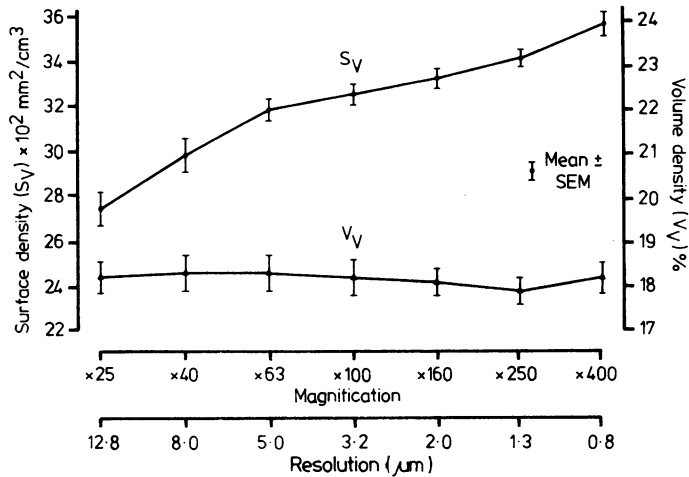


Fig. 5 Surface density (S_v) and volume density (V_v) of iliac crest cancellous bone at different magnifications, showing the systematic increase in the estimate of surface density with change in magnification and no difference in volume density. (From Olah,³⁶ with the permission of Armour-Montagu, Levallois, France.)

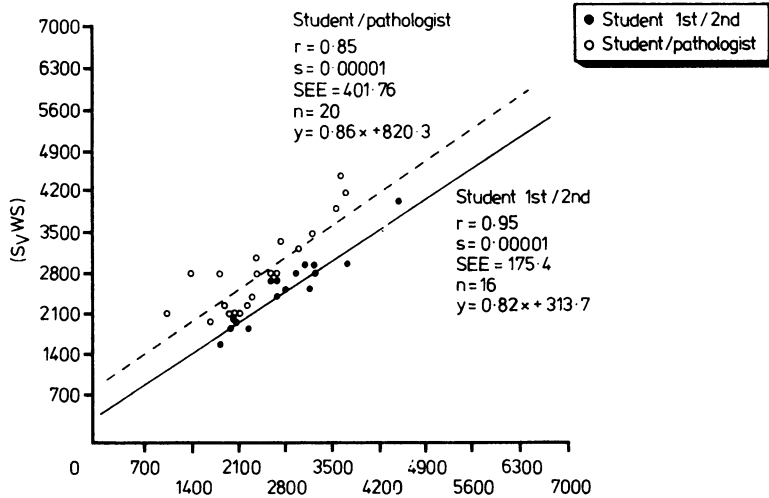


Fig. 6 Determination of the surface density (S_v) of trabecular bone. Sixteen values obtained by a student at the beginning and end of a study of 150 normal cases, 20 values of a student compared with a experienced pathologist on the same biopsies. (From Delling et al.,⁴⁵ with the permission of Armour-Montagu, Levallois, France.)

important and biopsy should normally be two or three days after the last label. If the interval between two labels is much longer than three weeks then the percentage of osteoid bearing two labels will be decreased.

The influence of microscopic resolution on the results obtained by bone morphometry has been described.^{36,37} Changes in magnification between 25 and 400 times did not influence estimations of volume (volume density), but over the same range of magnification there was a systematic increase in the estimate of surface density.³⁶ (Fig. 5)

Delling and his colleagues⁴⁵ have shown that there is greater variability in results obtained for surface measurements compared with volume measurements. This was particularly the case where cellular

details at trabecular surfaces were being evaluated, and many more fields had to be measured to obtain reproducibility under these circumstances. The relatively small amount of cellular activity at trabecular surfaces and the irregular distribution of changes are contributing factors. The same authors also performed comparisons between observers and laboratories.⁴⁵ The experience of the observer was found to be important, especially with respect to those surface measurements relating to cellular activity. The volume and surface densities of trabecular bone were not affected by observer experience in the sense that comparison of a student with himself and the student with an experienced pathologist both showed good statistical correlation (see Fig. 6). The shift in the line for the pathologist/

student comparison suggests that the pathologist was noting features which were not recognised by the student.

Differences between centres looking at the same biopsies are also of interest. When four different morphometry groups were asked to assess 10 biopsies, there was considerable variation with respect to values obtained for osteoid surface measurements though trabecular bone volume showed much smaller variation.⁴⁵

Semiautomatic and automatic methods in bone quantification

The examination of large numbers of specimens is time-consuming and tedious using the simple eyepiece graticule. Automated and semiautomated computer-linked systems offer an alternative approach. Both types of equipment have the advantage that they enable a much greater throughput of material. Disadvantages include the amount of time required setting up the equipment before sections may be examined, and the need to stain sections in a way which is suitable for image analysis. Automatic image analysers may be divided into three main categories, according to whether they work by source-plane-scanning, specimen-plane-scanning or image-plane-scanning.³²

Image-plane-scanning is the basis on which all television-linked systems operate. The best known system of this type is the Quantimet 720 and the following account is based on personal experience of its use.

Computer-linked image analysis using the Quantimet may be considered in three stages. Input is by means of a closed circuit television linked to a light microscope. The signals resulting from the scanning of the microscope image by the television camera are used to produce an image on a television screen and at the same time for computer analysis of particular features. The results of the computer analysis are displayed numerically at the top of the television screen. It is also possible to superimpose the computer analysis display as an image on the television screen.

The machine of which the author has experience has television display, standard function computer, a control system to set light sensitivity and shade correction, variable frame and scale settings, densitometer function and interfaces with a desk top microcomputer. Area, intercept, perimeter and size can be measured and a programmer module enables the machine to run automatically through a series of measurements on each field.

The Quantimet is used in our own laboratories in two different ways for the measurement of bone his-

tology. Osteoid volume and trabecular bone volume are measured using the densitometer function. Signals from the television camera are passed to a detection module in which grey level thresholds are set in 64 steps from black to white. It is important that the image being analysed can be resolved into clearly distinguishable grey levels. Our preference is to use von Kossa stained sections counterstained with van Gieson and treated in such a way that coloration of the bone marrow features is deliberately leached out. The image is resolved into black, grey and white areas corresponding to mineralised bone, osteoid and bone marrow. Careful selection of the grey level settings enables accurate detection at the correct boundary point for the three features which are to be detected and measured. The areas over which there are particular grey levels are measured and values for "white, grey and black" obtained. Raw data for the areas of osteoid, mineralised bone and total tissue are then routed to the desk top computer (output terminal). The process is repeated for adjacent fields and all the raw data accumulated in the output computer. Osteoid volume and trabecular bone volume are calculated automatically by a programme in this computer, in just the same way as would be performed in conventional point counting.

Although it is theoretically possible to obtain perimeter measurements using the same basic method, we have preferred to use the Quantimet as a semiautomatic image analyser. It is possible to outline trabecular surface, osteoid and resorption surfaces and lengths occupied by active osteoblasts and osteoclasts with a light pen (Fig. 7). The line lengths of each outlined feature are routed to the output computer for each field examined and the measurements calibrated so that it is possible to obtain an absolute value for the lengths measured. The output computer programme accumulates data for each measurement and after a previously specified number of fields has been examined, calculates osteoid surface, active osteoblastic surface, resorption surface, osteoclastic resorption surface and total length of trabecular surface. The numbers of osteoclasts seen is accumulated in the output computer as each field is examined, so that it is possible to obtain an osteoclast index, expressed as osteoclasts/mm trabecular surface.

Semiautomatic systems for quantitative analysis of histology consist of a "digitising tablet", which is an electronic drawing board, linked to a microcomputer. The microscopic field may be projected onto this drawing board or a side-arm drawing tube used. Commercially available semiquantitative equipment designed for this purpose includes the Leitz ASM, Reichert-Jung MOP and Videoplan. The digitising tablet enables the outlining of features seen in the

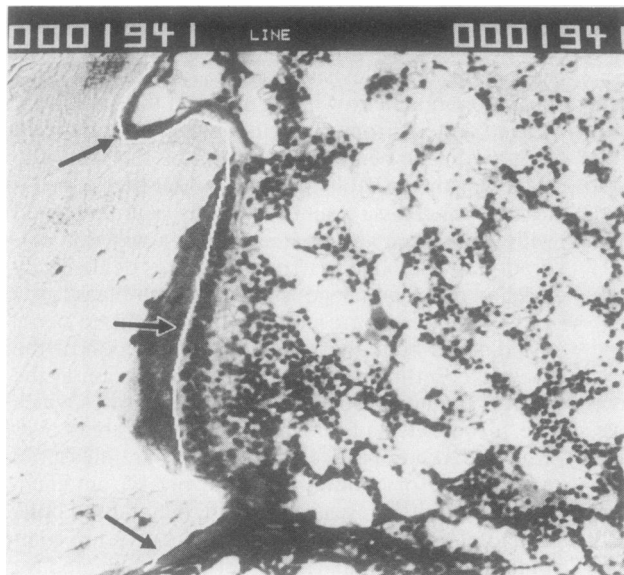


Fig. 7 Photograph of the Quantimet 720 television screen, showing the use of a light pen (white line) to outline the trabecular surface. The line is occupying 1941 picture points.

microscope field and measurements of line length, perimeter, intercept or area, are then rapidly obtained. These measurements can be expressed in absolute terms by previous calibration. Area and surface measurements for each field may be summed and the various bone morphometry parameters calculated by use of a suitable programme in the microcomputer. Semiautomatic methods are subjective, in that they rely on the drawing of features by the observer. The use of digitising tablets for bone quantification is described elsewhere.⁴⁶⁻⁴⁸

COMPARISON OF BONE QUANTITATION METHODS

A good correlation has been found between the actual volume of bone, measured by water displacement, and the point counting technique.^{38,49} A difference of 1.5% was found between the use of the Zeiss eye-piece graticule and the Quantimet method by Giroux, Courpron and Meunier.¹¹ No difference in accuracy has been found between point-counting and a semiautomatic method using a digitiser tablet.⁴⁷ Smaller numbers of fields needed to be measured using the semiautomatic method to achieve the same coefficients of variance with respect to both non-cellular and cellular (bone formation and resorption) parameters.

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