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Technical methods

Control of rapid nitric acid decalcification

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Decalcification is commonly employed in most histopathology laboratories for the microscopical examination of bone and other calcified tissues. Plastic processing without decalcification may produce superior results in terms of eliminating shrinkage and for demonstrating osteoid versus mineralised matrix[1–3] but may give poor cytological detail and is a much longer process. The diagnosis of non-metabolic diseases of bone such as infection and tumours requires good cellular morphology and a quick result to allow rapid therapeutic intervention for optimal patient care. In addition, many modern treatment regimens for bone tumours require a rapid report on an entire slab of resected bone to allow assessment of the benefit or otherwise of preoperative drug treatment. For these reasons a rapid method of decalcification which gives excellent and reproducible results was devised.

Many alternative decalcification regimens have been proposed,[4] but most of them have some unsatisfactory characteristics. In an attempt to reduce the commonly encountered artefacts of tissue shrinkage and adverse staining results obtained with rapid decalcification in strong mineral acids such as nitric acid many laboratories have utilised the much slower decalcification achieved with formic acid or EDTA.

Most unsatisfactory results with decalcification can be attributed to overexposure to the agent used due to inadequate control procedures. The commonly used control procedures using the ammonium oxalate chemical test[5] and its modifications are accurate in experienced hands[6] but are easily abused. Furthermore, although control using *x* rays is simple and accurate it is expensive in equipment and consumables for smaller laboratories and is only realistic for use at 24 h intervals.

Normal mechanical methods of "testing" using "bending or probing" cause artefact and are not considered by any serious bone laboratory for control purposes. To achieve rapid throughput of calcified tissues many laboratories resort to using mineral acids for decalcification but the control at intervals of 10–20 min is difficult to achieve accurately with the chemical tests and expensive with *x* rays.

A simple, cheap, and accurate method of controlling acid decalcification is to measure the weight change of the tissue as it is decalcified.[7] The endpoint is clear and the result is a controlled rapid decalcification over a few hours without adverse alteration to staining properties and with minimal shrinkage using 5% or 10% aqueous nitric acid (Figs. 1 and 2).

Verdenius and Alma[7] in their investigation of decalcifying methods used weight loss as an indicator of the rate at which calcium salts are removed. Their technique and that of Richardson[6] weighed the block in air and could be prone to error depending on the amount of fluid allowed to evaporate during weighing. It is surprising that the control of decalcification using the weighing method has not been more widely used in the light of their clear documentation of its suitability.

Calcium may expect to leave tissue in a complex manner depending on many variables including the decalcifying agent used, the amount of calcium in the tissue, overall surface area to volume ratio, and, in particular, the block's smallest dimension. In addition, the decalcifying agent has to enter the block as the mineral salts travel to the exterior and it would not be surprising if the formula describing the loss of calcium was rather complicated.[8]

Verdenius and Alma found the rate of decalcification to be directly proportional to the size of the reaction surface, while Kiviranta *et al*[9] described the ratio for 2 mm bone slices by the formula:

$$y = 54{\cdot}58 \cdot x^{0{\cdot}41}$$

where y is the percentage of calcium extraction from the bone slice and x the duration of demineralisation in days.

Examination of many examples of weight loss curves indicates that the loss of calcium follows fairly accurately an exponential decay over the major portion of the curve and the weight of calcium in the block at any time during decalcification can be described by the formula:

$$W_{(t)} = W_{(o)}e^{-\lambda t}$$

where $W_{(t)}$ = weight at time t, $W_{(o)}$ = original weight, and λ = factor depending on block and decalcifying agent.

Towards the end point of decalcification the gradient of the curve becomes progressively less nega-

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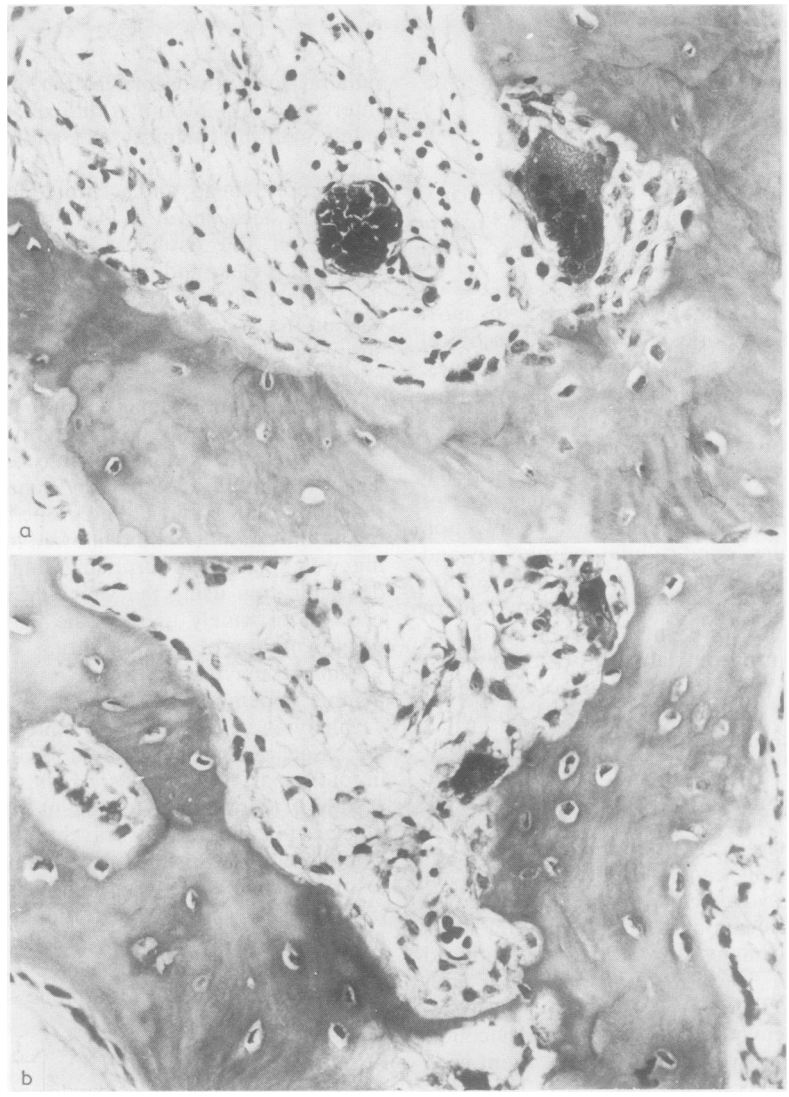


Fig. 1 *Areas of reactive bone showing well nucleated bone with some osteoblasts and marrow fibrosis. (a) Formic acid*[10] *(which also shows a solitary large osteoclast). (b) 5% aqueous nitric acid (with two small osteoclasts). Haematoxylin and eosin. Original magnification* × *560.*

tive and may turn positive. At this time the "real weight" of the block in acid may be zero or negative due to the formation of gas bubbles on the surface of the block and necessitating the use of an additional glass weight to hold it under the surface of the acid.

It is a simple task for a microcomputer to read an electronic balance, plot a graph of weight loss, and calculate the state of calcium extraction from blocks. It should also be possible to forecast the end point with improving accuracy as decalcification progresses and averaged values of λ may be applied to successive forecasts. Forecasting the end point accurately would allow planning of subsequent processing to suit the laboratory, particularly in cases of urgent biopsy material.

Method

1 Suspend tissue to be decalcified from an accurate balance capable of weighing to ±1 mg.

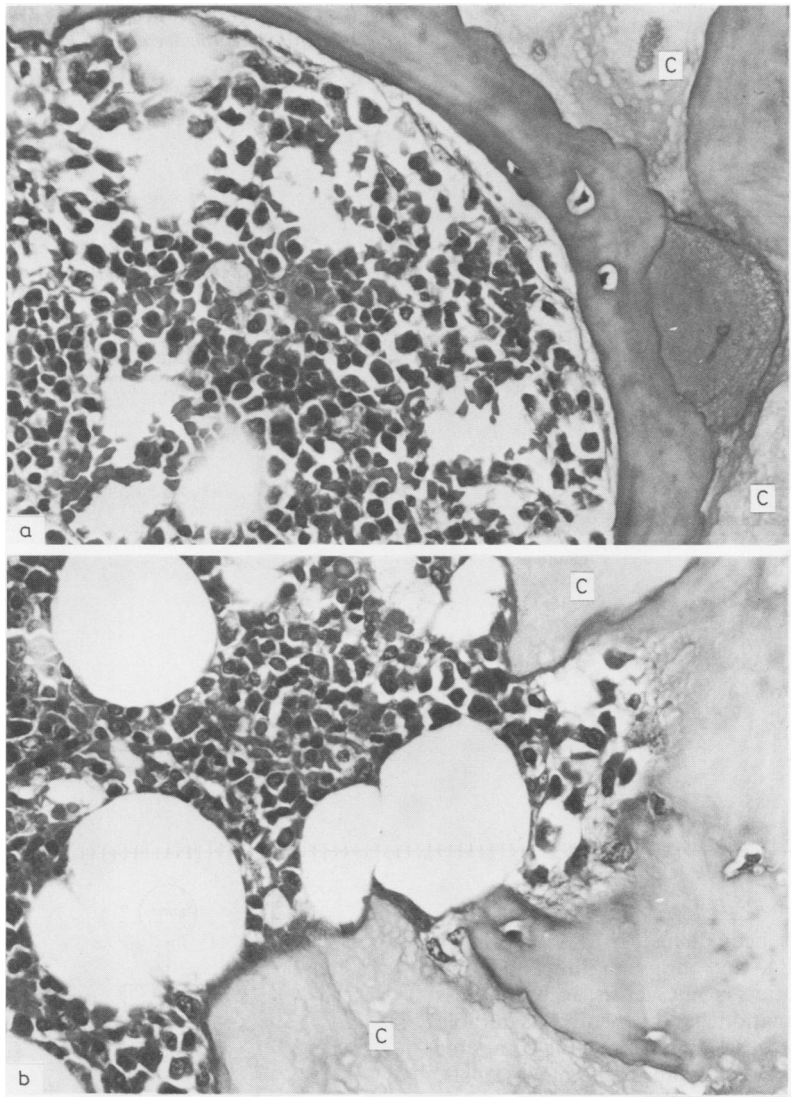


Fig. 2 *Edge of an intramedullary chondroma showing cartilage (C) bone and haemopoietic marrow. Note the lack of shrinkage of cells from the bone surface. (a) Formic acid*[10]*. (b) 5% aqueous nitric acid. Haematoxylin and eosin. Original magnification* × *900.*

2 Cover the tissue with 100 times its volume of 5% or 10% aqueous nitric acid and obtain the weight immediately.
3 Weigh the block at intervals of 5 min initially and 10 min as the weight change is reduced.
4 Construct a graph of the weight change of the tissue as in Fig. 3.
5 As the end point of decalcification is approached the negative gradient of the graph decreases and in most cases turns positive.

This simple method lends itself to control and forecasting of the end point of decalcification manually or using an electronic balance outputting the sample weight to a small microcomputer. As a control procedure this technique is of great help for other rapid decalcification techniques or fluids or indeed control of any procedure involving change of equilibrium conditions detectable as a weight change.

An Acorn Atom linked to an Oertling KB23 elec-

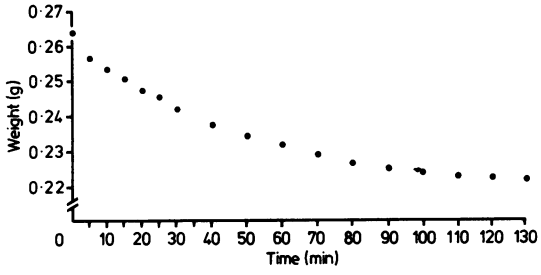
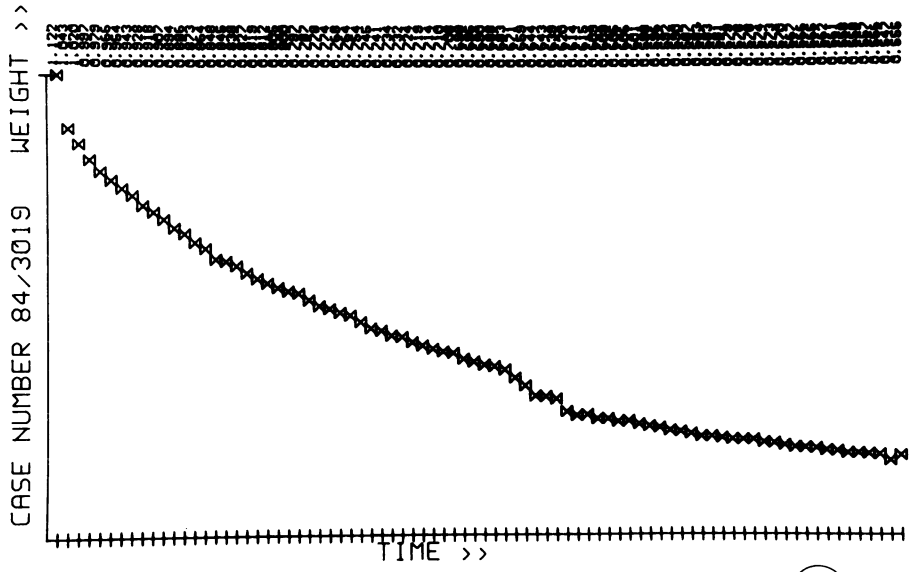


Fig. 3 *Manually drawn weight loss chart of 15 × 6 × 3 mm block of vertebrae decalcified in 5% aqueous nitric acid.*

Fig. 4 *Computer drawn weight loss chart of a 45 × 21 × 5 mm block of rib decalcified in 10% aqueous nitric acid. Total time 6 h 50 min, intervals 5 min.*



tronic balance has been in use in this laboratory since January 1984, and although the end point forecasting subroutine is not yet as accurate as is desired the combination has been of considerable help. Set up as in stages 1 and 2 above the computer programme will take the readings at 5 min intervals, construct the graph, forecast the end point, and suggest to the user when the end point is reached (Fig. 4). A BASIC programme, of which the flowchart is shown in Fig. 5, is easily written or copies suitable for use on an Acorn Atom may be obtained on disc or cassette for £3·50. A fully documented listing of the programme and details of the interconnecting cable between computer and balance are available from the authors on receipt of a stamped addressed envelope.

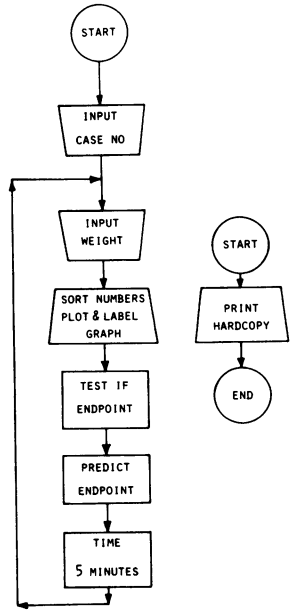


Fig. 5 *Flow chart of basic programme to measure and plot sample weight change.*

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Method for the morphometric analysis of arterial structure

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Changes in arterial structure are seen in many diseases and their morphometric quantitation has been used as an objective measure of such alterations. A critical review of techniques for arterial mensuration has been presented by Cook and Yates.[1] They described a planimetric method for the assessment of arterial structure using the perimeter of the internal elastic lamina as a reference value to indicate the true size of vessels, which were fixed without perfusion. They noted that linear measurements made on partly collapsed vessels were of dubious value as the degree of contraction and collapse is an inconsistent phenomenon. Their method did not, however, take account of the intimal component of the arterial wall. The fact that intimal thickening is often a patchy process affecting the circumference of a vessel in an irregular manner makes isolated linear measurements of little value for accurate and reproducible assessment of this lesion. The method described here allows reproducible measurements of arterial structure to be made on histological sections. It uses an adaptation of the method of Cook and Yates to transform area measurements of intima and media to fit the mathematically "circularised" measured perimeter of the internal elastic lamina.

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Theoretical considerations

A muscular artery is shown diagramatically in Fig. 1 and an ideal circular vessel in Fig. 2. The perimeter of the internal elastic lamina, L, is assumed to be constant after collapsing down from its expanded (perfused) state. The area of the media, A1, and intima, A2, are also assumed to be constant after collapsing down from a stretched (perfused) state. Direct linear measurements of lumen radius, intimal thickness, and medial thickness are unreliable if made in vessels of this type.

The radius of the ideally circular contour of the

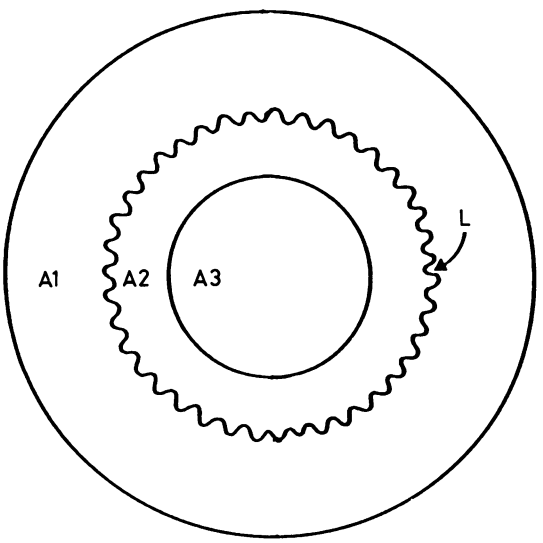

Fig. 1 *Contracted muscular artery*