1. A simple system is described for the quantitative histological assessment of three skeletal disorders. It is based on the preparation of undecalcified sections of iliac crest biopsies, photography of cancellous bone and scanning of standard prints by a sensing device attached to a digital computer.

2. Areas of uncalcified osteoid are quantified by visual inspection during photography and the skeletal lesions of hyperparathyroidism are assessed semi-quantitatively.

3. This system makes it possible to define osteoporosis and osteomalacia according to quantitative histological criteria and provides some assistance in the assessment of the severity of hyperparathyroidism.

Key words: bone histology, osteoporosis, osteomalacia, hyperparathyroidism.

Iliac crest biopsy is a well-recognized procedure for the diagnosis of haematological conditions (Hale & de Gruchy, 1959). The technique has been less frequently employed in the diagnosis of bone disease (Annotation, 1955), because currently available methods for the quantitation of skeletal histopathology (Beck & Nordin, 1960; Bordier, Matrajt, Miravet & Hioco, 1964; Garner & Ball, 1966) are tedious and time-consuming.

In the present paper we describe a quantitative method for the assessment of bone histology using previously described methods (Ball, 1957; Garner & Ball, 1966) but with an additional photo-sensitive scanning device and a computer program. Our findings in forty-one cadavers are described.

PATIENTS, MATERIALS AND METHODS

The cadavers were those of normal subjects (twenty-five male, sixteen female) aged 15–63 years. They had apparently been in good health until the time of violent deaths from vehicular
accidents, drowning, shooting or drug overdosage. Subjects who died at work of cardiovascular disease were also included. None of the subjects had a history of being confined to bed and their nutritional status was adequate as judged by history and post-mortem appearances. All specimens were taken within 12 h of death.

A 5 mm skin incision was made over the iliac crest, 2 cm posterior to the anterior superior iliac spine (Chalmers & Weaver, 1966). The periosteum was incised and bone was removed as described by Hocking (1964) with a trephine instrument (Ramsay Surgical, Sydney, Australia). A piece of bone 3 mm in diameter and up to 2 cm in length was obtained (Fig. 1).

The bone was fixed for at least 24 h in 10% formol saline, buffered to pH 7.5 with 0.5 M-sodium barbitone. The specimen was dehydrated in ethanol and placed in three changes of acetone over 3 h. This was followed by constant mechanical agitation in a 50% (v/v) acetone–Araldite mixture for 24 h and in undiluted Araldite for a further 24 h. Araldite is an epoxy resin from Ciba-Geigy Australia Ltd. It is prepared for final use by mixing Araldite M (CIBA) with HY 906 (CIBA) and HY 960 (CIBA) in a ratio (by wt.) of 50:40:1. The specimen was then placed in a fresh solution of Araldite and heated for 6 h at 37°C followed by 8–12 h at 70°C.

The hardened block was shaped with a hacksaw blade and sections were cut longitudinally at 8 μm with a Leitz base sledge microtome (Type 1300) through the diameter of the cylindrical specimens (Matrajt, Bordier & Hioco, 1967). The sections were mounted on warm slides coated with 2% gelatin solution and dried at 70°C for approx. 1 h.

The slides were immersed in 1% (w/v) aq. AgNO₃ and exposed to strong light for 10–15 min until a deep brown colour developed (Lillie, 1948). They were then rinsed in water, immersed in 5% (w/v) Na₂S₂O₃ for 1 min, rinsed in water again and counterstained for approx. 5 min with fresh 1% aq. Toluidine Blue (buffered to pH 7 with NaHCO₃). After another rinse in water, the slides were dried at room temperature. This is essentially the technique of Garner & Ball (1966). It makes calcified tissues appear brown or black whereas uncalcified tissues appear blue (Garrick, Ireland & Posen, 1971).

The slides were inspected for damage sustained during expulsion from the trephine apparatus or during cutting. Knife marks were ignored but quantitative measurements could not be taken in some 10% of specimens because of obvious distortion.

Photography of cancellous bone was performed at a magnification of 80×. A field 450 μm × 300 μm immediately adjacent to cortical bone was photographed on 35 mm high contrast film (Copex Agfa, 40 A.S.A.) at standard exposure times. Eleven further contiguous fields were similarly photographed and, after standard development, the twelve frames were printed onto a sheet of glazed photographic paper 20 cm × 15 cm (8 in × 6 in) (Fig. 2). Cortical bone, identifiable as such, was not photographed.

Scanning was performed by a ‘Gestofax’ attachment (Gestetner ES 390) to a KDF9 computer. This scanning device consists of a rotating cylinder and a sensing head which carries a lens system to focus a light beam on to an area 19 μm × 22 μm. A photo cell measures the amount of reflected light from the photograph. For any given spot being scanned, the output of the device is either a ‘yes’ or a ‘no’ depending on whether the spot is darker than a predetermined level. In this study the scanning device was programmed to regard both calcified and uncalcified bone as ‘black’ so that the output referred to total rather than calcified bone (Garner & Ball, 1966). The device was checked by means of known test frames between every two patients.
FIG. 1. Three representative cylindrical specimens consisting mostly of cancellous bone, obtained from the cadavers of women aged between 46 and 55 years.
FIG. 2. A typical set of photographs of twelve contiguous fields of cancellous bone from one subject taken on high contrast film (Agfa Copex A.S.A. 40) and contact-printed on to glossy single-weight photographic paper (Ilfobrom, 5). Uncalcified material is not distinguished from calcified material in such a print and both are regarded as 'black' by the scanning device. Original magnification ×80.

FIG. 3. The appearance of cancellous bone in severe hyperparathyroidism (undecalcified section, original magnification ×80). Surface A is relatively normal, surface B is severely affected. Cellular detail is not seen with high contrast film.
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The photograph as shown in Fig. 2 is wrapped around the cylinder and rotation commences. Every rotation of the cylinder produces some 3000 ‘yes’ or ‘no’ signals and these are recorded in groups, where every group represents one rotation of the cylinder, a strip 19 μm wide on the photograph being scanned. A series of frames is thus converted into a series of ‘yes’ and ‘no’ signals recorded on magnetic tape; nine or ten photographs (up to 120 frames) may be recorded on a full reel of tape.

The original program was devised to calculate and print out the proportion of blackness for each frame and to ignore the surrounding black areas. This made it necessary for the first frame in each column to have a white area (approx. 1.5 mm × 1.5 mm) in its bottom left corner so that the position of the column on the page could be determined. However, it is more convenient to calculate the ‘white’ areas for each frame and to adjust the program so that the areas occupied by skeletal tissue are calculated by subtraction. The percentage of ‘bone’ in each frame is printed out, then the mean and standard deviation for twelve frames, along with identification information (previously read in from punched paper tape). Both programs are available from the authors on request.

While each field was being photographed, estimation of uncalcified tissue was performed by eye with a Zeiss graticule in the eyepiece (Garner & Ball, 1966). The number of intersections overlying uncalcified material was counted and divided by the total number of intersections. This gave the area and hence the percentage area covered by uncalcified bone in each field. An attempt was also made to assess bone resorption in each field by counting the number of ‘squares’ in the eyepiece graticule containing microscopic ‘erosions’ (Fig. 3).

The following terminology was used: Total bone index was defined as the area occupied by skeletal material (calcified and uncalcified) expressed as a percentage of the total area scanned. The osteoid index was the area occupied by uncalcified bone expressed as a percentage of the area scanned. Demineralization index was the osteoid index divided by the total bone index multiplied by 100. (Demineralization does not imply that mineral has been lost.) Hyperparathyroidism, as judged by the number and distribution of erosions was classified as ‘absent’, ‘present’ or ‘severe’.

RESULTS

Table 1 shows the results of replicate determinations at different stages of the scanning procedure. Reproducibility was excellent when the same set of photographs was scanned automatically on separate occasions. The difference between duplicate determinations became greater when a second set of photographs was taken by the same observer and greater still when another observer took the second set of photos.

Good inter-specimen correlations were obtained regardless of whether the specimens came from the same iliac crest or from the opposite side. However, such good correlations could not be consistently obtained when specimens from other sites (such as sternum or lumbar spine) were compared with material from the iliac crest. The discrepancy between iliac crest and lumbar vertebrae has been noted (Chalmers & Weaver, 1967; Dunnill, Anderson & Whitehead, 1967), though some workers (Lindahl & Lindgren, 1962; Bell, Dunbar, Beck & Gibb, 1967) found a good correlation between the bone density of the axial skeleton and that of the peripheral skeleton.

Table 2 shows the reproducibility of osteoid index determinations performed by the manual
TABLE 1. Reproducibility of estimations of total bone content (calcified and uncalcified). The standard deviation of difference was calculated according to the formula: \( \text{SD of difference} = \sqrt{\frac{\Sigma d^2}{2n}} \) where \( d \) = difference between duplicate estimations and \( n \) = the number of duplicate estimations performed (Snedecor, 1952). The manual scanning was performed by the method of Garner & Ball (1966).

<table>
<thead>
<tr>
<th>No. of duplicate examinations</th>
<th>Mean bone index (%)</th>
<th>Mean difference between duplicate bone indices</th>
<th>Standard deviation of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Same photographs scanned twice</td>
<td>8</td>
<td>32.2*</td>
<td>0.41</td>
</tr>
<tr>
<td>Same section photographed twice (same observer)</td>
<td>5</td>
<td>25.3</td>
<td>1.68</td>
</tr>
<tr>
<td>Same section photographed twice (second observer)</td>
<td>8</td>
<td>23.5</td>
<td>2.11</td>
</tr>
<tr>
<td>Different bone specimens (same iliac crest)</td>
<td>14</td>
<td>24.2</td>
<td>1.85</td>
</tr>
<tr>
<td>Different bone specimens (opposite iliac crest)</td>
<td>5</td>
<td>23.8</td>
<td>1.70</td>
</tr>
<tr>
<td>Manual scanning compared to automated scanning</td>
<td>9</td>
<td>30.5*</td>
<td>2.70</td>
</tr>
</tbody>
</table>

* These high means were caused by the inclusion of two 'osteosclerotic' patients with renal failure (Garner & Ball, 1966) among these eight duplicates.

TABLE 2. Reproducibility of osteoid estimations

<table>
<thead>
<tr>
<th>No. of duplicate examinations</th>
<th>Mean osteoid index (%)</th>
<th>Mean difference between duplicate osteoid indices (%)</th>
<th>SD of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Same section (same observer)</td>
<td>8</td>
<td>0.319</td>
<td>0.09</td>
</tr>
<tr>
<td>Same section (different observer)</td>
<td>8</td>
<td>0.225</td>
<td>0.15</td>
</tr>
<tr>
<td>Different specimens (same iliac crest)</td>
<td>14</td>
<td>0.275</td>
<td>0.071</td>
</tr>
<tr>
<td>Different specimens (opposite iliac crest)</td>
<td>5</td>
<td>0.348</td>
<td>0.124</td>
</tr>
</tbody>
</table>
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Table 3 shows our results in forty-one ‘normal’ cadavers. The observed range for calcified plus uncalcified cancellous bone of the iliac crest in normal males was between 22.2% and 30.0% of the area scanned. In females the observed normal range was 17.9–30.7%. Normal subjects had between 0.06 and 0.82% of the microscopic fields occupied by osteoid seams and no normal subject showed ‘severe’ erosive changes as illustrated in Fig. 3.

DISCUSSION
Various techniques for the quantification of metabolic bone disorders are now available. They include non-traumatic procedures such as X-ray densitometry (Doyle, 1961; Nordin, Barnett, Macgregor & Nisbet, 1962; Chalmers & Weaver, 1966), gamma ray densitometry (Sorensen & Cameron, 1967; Davis, Lanzl & Cox, 1970; Goldsmith, Johnston, Ury, Vose & Colbert, 1971) and measurements of cortical thickness (Barnett & Nordin, 1960; Garn, 1970) which may be used repeatedly. Unfortunately these methods are mostly unsuitable for the examination of cancellous bone or for the detection of uncalcified osteoid, both of which require histological material. In living subjects this means a rib biopsy (Frost, 1960) which involves admission to hospital, or an iliac crest trephine biopsy which can be performed on outpatients.

Five main techniques are available for the quantitative study of skeletal biopsy material. (1) The measurement of areas covered by bone and osteoid (Garner & Ball, 1966) as employed in this study. (2) The measurement of distances between tetracycline labels so that rates of formation can be calculated (Frost, 1960, 1966). (3) Microradiography (Jowsey, Kelly, Riggs, Bianco, Scholz & Gershon-Cohen, 1965) or photon absorptiometry (Colbert, Mazess & Schmidt, 1970) of excised material. (4) Chemical analysis (Dollerup & Bohr, 1963; Chalmers & Weaver, 1966; Morgan, Stanley & Fourman, 1968). (5) Determination of bone volume by weighing (Saville, 1965) or by fluid displacement (Dequeker, Remans, Franssen & Waes, 1971). Of the five methods, the first is the simplest, especially when combined with automatic scanning. We therefore used what is essentially the method of Garner & Ball (1966), even though this has all the disadvantages of sampling procedures involving hollow apparatus.

We do not know why our values for ‘total bone’ in normal subjects are somewhat higher
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than those obtained by other workers (Bordier et al., 1964; Garner & Ball, 1966; Bordier, Miravet, Matrajt, Hioco & Ryckewaert, 1967; Sissons, Holley & Heighway, 1967; Birkenhager, van der Heyl, Smeenk, van der Suyss Veer & van Seters, 1967; Dequeker et al., 1971). In spite of the difficulties associated with photometric measurements our results were internally consistent (see Tables 1 and 2) and we can only speculate that 'healthy' Australian subjects appear to have more cancellous bone than 'healthy' northern Europeans. This applies particularly to the 'normal' subjects described by Garner & Ball (1966) who had also died from violence and whose mean age was similar to that of our 'normal' subjects.

Our results for osteoid areas in normal subjects agree with those of Garner & Ball (1966) though they are lower than those obtained by Matrajt et al. (1967) and by Woods, Morgan, Paterson & Gossman (1968).

We have not been prepared to quantify resorption or to express 'resorption surface' as a percentage of the total surface of trabecular bone (Sissons et al., 1967; Meunier, Vignon, Vauzelle & Zech, 1969), because we have not found a satisfactory definition for 'scalloping' (Fig. 3). The classical appearances are easy to recognize both in decalcified and in undecalcified material, but shallow areas of resorption may be impossible to differentiate from normal trabecular contours. An inspection of published material by Jowsey et al. (1965) suggests that microradiography suffers from the same disadvantage.

The techniques employed in the present study involve no major surgery. Special milling machines (Jowsey et al., 1965) and handgrinding of specimens (Villanueva, Frost, Inlicki, Frame, Smith & Arnstein, 1966) have been avoided. Embedding, cutting, mounting and staining procedures are within the capacity of any clinician or pathologist wishing to base a diagnosis of bone disease on objective criteria.

Histology is mandatory for the diagnosis of osteomalacia, which has been described in association with a variety of diseases (Arnstein, Frame & Frost, 1967; Paterson, Woods & Morgan, 1968; Dent, 1969; Parsons, 1969; Salassa, Jowsey & Arnaud, 1970; Garrick et al., 1971) and which may be present in a number of institutionalized elderly people (Chalmers, Conacher, Gardner & Scott, 1967). Osteomalacia is a common histological finding in renal failure (Stanbury, 1968; Ireland, Cameron, Stewart & Posen, 1969; Lumb, Mawer & Stanbury, 1971; J. P. Ingham, J. H. Stewart & S. Posen, unpublished work); it occurs in primary hyperparathyroidism (Boyce & Jowsey, 1966) and it may be present in Paget's disease (Paterson et al., 1968). In the absence of gross radiological changes (Steinbach & Noetzli, 1964) no statement concerning the prevalence of osteomalacia has any meaning unless accompanied by histological evidence of its presence and degree of severity.

Quantitative histology is one of the few parameters available for the quantitative assessment of osteoporosis. Measurements of cortical thickness (Garn, 1970; Nordin, 1971) give only limited information concerning the loss of cancellous bone (Nordin, MacGregor & Smith, 1966) which is the predominant lesion in most forms of osteoporosis (Lindahl & Lindren, 1962). X-ray and gamma ray densitometry (Nordin et al., 1962; Goldsmith et al., 1971) cannot be performed exclusively on cancellous bone. We therefore believe that the diagnosis of osteoporosis and claims concerning its prevention (Meema & Meema, 1968; Davis et al., 1970) or treatment (Rose, 1970; Fraser, Anderson, Smith & Wilson, 1971) should be supported by histological evidence of bone loss or bone gain. Bone histology may also help to prove or disprove the hypothesis (Heaney, 1965; Nordin, 1971) that osteoporosis is a form of chronic compensatory hyperparathyroidism.
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In spite of the difficulties associated with the quantification of the skeletal lesions of hyperparathyroidism, histology still provides a more sensitive index of bone resorption than all other parameters. This applies to 'primary' (Pratley, Reeve & Posen, 1972) as well as 'secondary' hyperparathyroidism (J. P. Ingham, J. H. Stewart & S. Posen, unpublished work) and whether hyperparathyroidism is present alone or in combination with other bone lesions (Lloyd, Aitken & Ferrier, 1965; Thalassinos, Wicht & Joplin, 1970). When radiological lesions are present, they are invariably accompanied by histological lesions, while histological lesions may occur in the absence of any radiological or biochemical disturbances (J. P. Ingham, J. H. Stewart & S. Posen, unpublished work).

Although the work reported here was performed on bone removed from cadavers, the method can be applied to tissue removed by biopsy from living subjects (Garrick et al., 1971).

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