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Undecalcified preparation of bone tissue: report of technical experience and development of new methods

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Summary. For reliable quantitative and qualitative analysis of bone specimens undecalcified preparation is essential. The "conventional" technique for this purpose is embedding in methylmethacrylate. Larger bone specimens, highly sclerotic specimens, cortical bone or bone implants consisting of metals or ceramics require modifications of this technique or completely new methods. We report our experience with the undecalcified preparation of 47700 bone specimens. New techniques such as the cutting of large area sections up to a size of 5×6 cm and grinding procedures for completely artefact-free preparation which are applied in special cases are also described. A new technique of combinded two- and three-dimensional analysis of bone specimens is presented. In our experience these methods are fundamental for morphological investigation of bone.

Key words: Bone – Undecalcified preparation – Grinding technique

Introduction

Morphological investigation of metabolic bone disorders in routine diagnosis as well as for scientific purposes requires undecalcified preparations (Anderson 1982; Arnold and Jee 1954; Recker 1983). In the least 20 years a great number of biopsies including bone tumours and localised bone disorders have been investigated by means of plastic embedding in the Department of Bone Pathology of the Institute of Pathology, University of Hamburg. The great advantage of this method is the possibility to distinguish between mineralised bone tissue and unmineralised osteoid by an excellent preservation of cellular structures. Bone cells and the components of haematopoesis are stainable by the well-known conventional methods. In contrast with paraffin techniques, undecalcified preparation increases expenditure, takes longer to complete, and prevents the reliable use of immunochemistry. The preparation technique is limited by the size of material (not larger than 2×2 cm for common microtomes) and the impracticability of cutting larger specimens of cortical bone perpendicular to collagen fibres. For these reasons the development of new techniques was required to estimate such variables as the extent of femoral head necrosis, the extent of necrosis in osteosarcomas caused by chemotherapy or to estimate the changes in cortical bone after implantation of hip joint endoprosthesis. Conventional preparation requires removal of the implant. This would lead to destruction of the bone-implant interface.

In the following text we describe: our experience with the "conventional" plastic embedding procedure and some improvements in this technique; the preparation of undecalcified large area sections (up to 5×6 cm in size); the use of newly developed grinding techniques; and combined two- and three-dimensional preparation and analysis of bone specimens.

Materials and methods

In the last 20 years, we have been able to study undecalcified sections of 47700 bone specimens. The numbers of iliac crest bone biopsies, resection specimens, bone tumours, and bone implant interfaces are shown in Table 1.

Embedding of iliac crest bone biopsies in methylmetacrylate. Following Delling et al. (1972) iliac crest biopsies are dehydrated for 12 h after fixation, fat is withdrawn carefully by an ethanol-acetone mixture and a final ethanol treatment follows. This procedure seems to be very important for complete infiltration of plastic embedding medium. Furthermore the dehydrated and defatted bone tissue should be submerged for at least 24 h in methylmethacrylate. This is done in the complete methylmethacrylate composition without the presence of polymerisation starter (benzoylperoxide). Some problems may be due to the fact that the process of polymerisation takes either several weeks or happens so rapidly that an exothermic reaction with formation of bubbles occurs. This can be avoided by prefilling the gelatine capsules (diameter 24 mm) to a height of 5 mm with methylmethacrylate. Following polymerisation of this prefilled bottom the bone specimen is inserted together with the final mixture of methylmethacrylate and the softener (nonyl-

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Table 1. Bone specimens investigated from 1970 to 1990 in the Department of Bone Pathology, Institute of Pathology, University of Hamburg

Total number	47700
Iliac crest bone biopsies (metabolic bone diseases)	35000
Iliac crest bone biopsies (haematological diseases)	3500
Non-tumourous bone diseases (resected material from different areas of the skeleton)	4000
Primary bone tumours	4100
Secondary bone tumours	1100
Cases with large sections (complete tumour area reconstruction, femoral heads etc.)	800

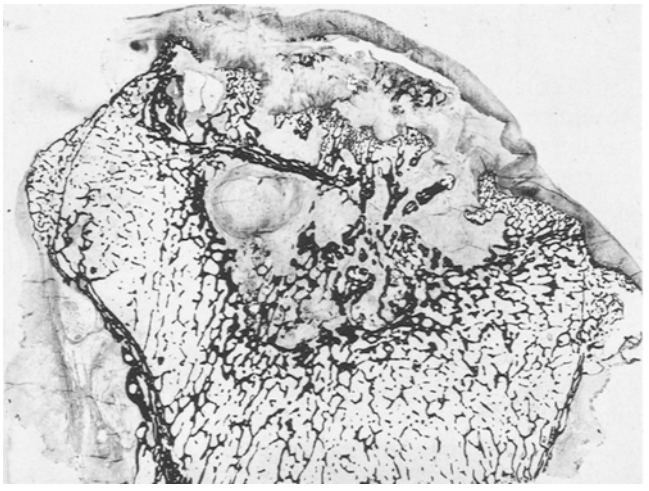

Fig. 1. Femoral head necrosis with deformation of the articular surface. von Kossa stain, 6 µm thickness

phenolpolyglycol-ether-acetate) and the starting substance benzoylperoxide (2000 ml methylmetacrylate, 500 ml nonylphenolpolyglykoletheracetat, 75 g benzoylperoxide).

Polymerisation occurs at 36° C for 24–48 h. Using this procedure polymerisation within a water bath is not necessary. When polymerisation has finished the gelatine capsules can be softened in cold water for easy removal. This is a great advantage in comparison with the use of glass tubes, where there is a considerable risk of injury for the technical assistant as a result of the requirement for mechanical destruction. The bottom of the polymerised block of methylmethacrylate is removed by a saw. The methylmethacrylate surrounding the bone biopsy is removed by manual filing and then the block is tightly fixed to the sliding carriage of the microtome. To avoid compression artefacts it is advisable to cut moist. Stretching is done with a special solution (3/5 butylglycol and 2/5 ethanol 70%) before the slice is attached to the slide by pressing down with a plastic film. After deplastination all conventional staining reactions are applicable. The most common stainings in our laboratory are Goldner's stain, toluidine blue reaction and a modification of Kossa and Giemsa's staining (Figs. 1, 2). For interpretation of tetracycline markers under ultraviolet light we cut 12-µm-thick sections and use them unstained.

Undecalcified large area sections. In the past, large bone specimens (femoral heads or bone tumours) were prepared by decalcification. This method allows quite a good demonstration of trabecular bone structure, but the specimens require such a lot of time for decalcification that cellular details are mostly destroyed. In preceding tests it could be shown that solid microtomes (for instance K-microtome; Jung, Heidelberg, FRG) were able to cut specimens up to a diameter of 5–6 cm nearly without artefact. Larger specimens could not be managed by these microtomes so that we were forced to divide bone tumours larger than 5 cm into several pieces. For this purpose we sawed resected bone tumours in slices of 4 mm in a representative area. This area was then separated into single pieces of 5 cm in length as a maximum. X-ray documentation, made of the whole specimen before sectioning into several pieces, is very helpful in reconstruction.

Specimens are dehydrated for 4 days and fat removed carefully in an acetone-ethanol mixture before restoring in ethanol. Afterwards infiltration with methylmethacrylate for at least 14 days is required. Specimens with sclerotic areas should be infiltrated for 24 days in methylmethacrylate (by addition of the softener but without starter) at 4° C. Afterwards the infiltrated bone specimens are put in plastic forms and one third filled with methylmethacrylate. To achieve the same effect as for the iliac crest biopsies by using prepolymerised tubes we insert large pieces of completely polymerised methylmethacryalate. Finally we fill the forms with methylmethacrylate monomer and cover it. Polymerisation occurs at 33° C for several days. As a result of the inability to prevent oxygen access to the methylmethacrylate, there is a smooth surface to the block. We cover the soft acrylate surface by an autopolymerisate. This leads to a firm surface and the whole block can be used later on without "blocking". When polymerisation has finished the block is removed from the form. Then it is sawn under massive water cooling to get to the plane of the embedded specimen. Now the block can be fixed to the microtome and cut with a common HK3 knife. The cutting procedure is the same as for iliac crest biopsies. The slice is removed from the back of the cutting knife by a slide of 5×7 cm in size. Drying of the sections should occur under pressure for at least 3 days. Afterwards the large area sections can be deplastinated and are suitable for all conventional stainings.

Ultrathin grinding technique. Under certain conditions it is necessary to perform a grinding procedure. In particular, it is useful

Table 2. Preparation of undecalcified bone tissue

	Iliac crest bone biopsies	Reconstruction of bone tumours	Implants
Size (maximum)	20×25 mm	50×70 mm	50×100 mm
Fixation	Neutral formalin	Neutral formalin	Neutral formalin
Dehydration	12 h	10 days	24 h
Immersion	24 h	10 days	14 days
Polymerization	24 h (36° C)	3 days (36° C)	8 h (blue light)
Cutting	Rotation microtom	K-microtom	Grinding technique
Section thickness	3–12 µm	5–12 µm	down to 10 µm
Staining	All routine staining procedures (von Kossa, Goldner, Giemsa, toluidine blue)		

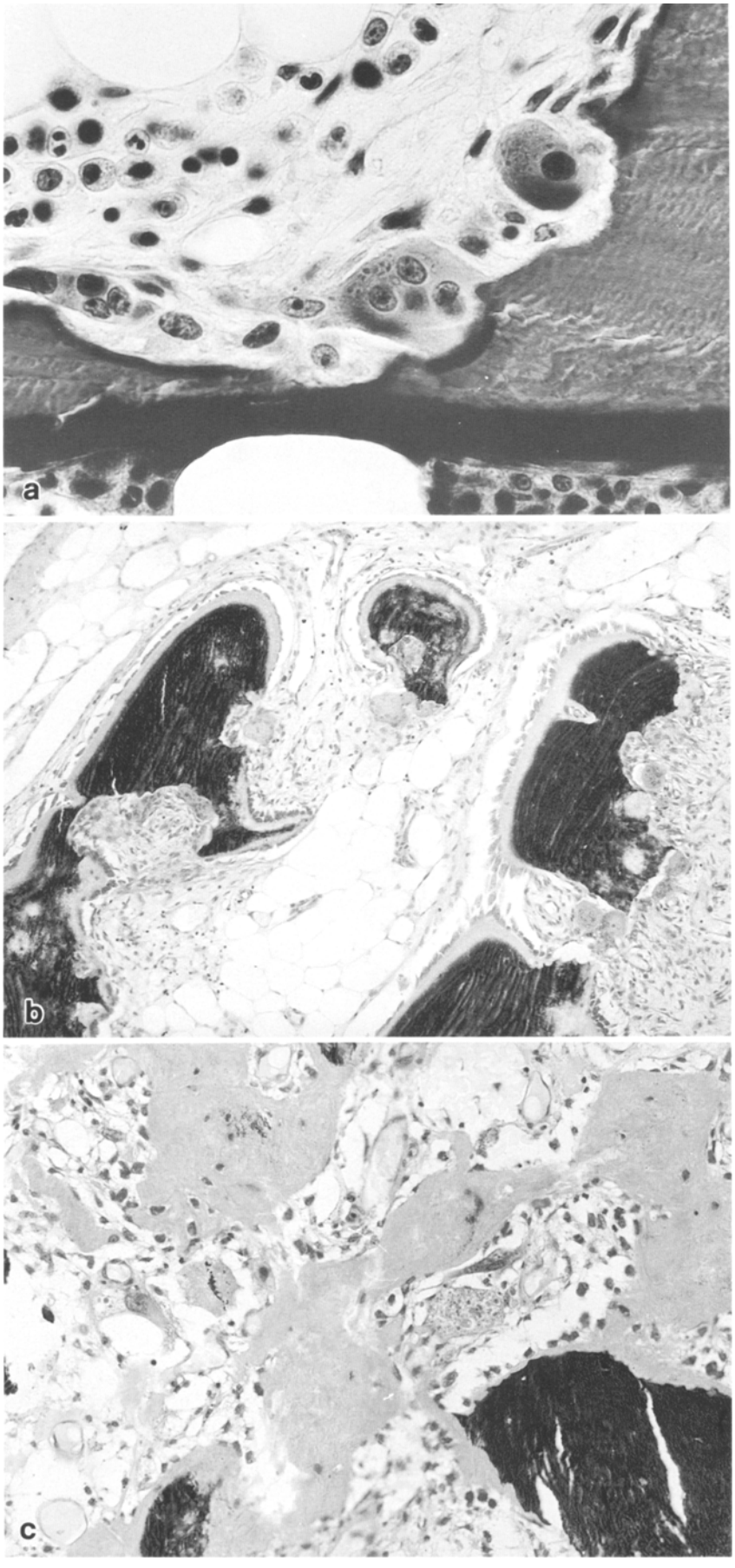

Fig. 2a–c. Demonstration of bone cells following undecalcified plastic embedding procedure. **a** Osteoclasts in normal bone tissue. Goldner stain ×400. **b** Paget' disease of bone. Toluidine blue, ×60. **c** Osteosarcoma after chemotherapy. Toluidine blue, ×160

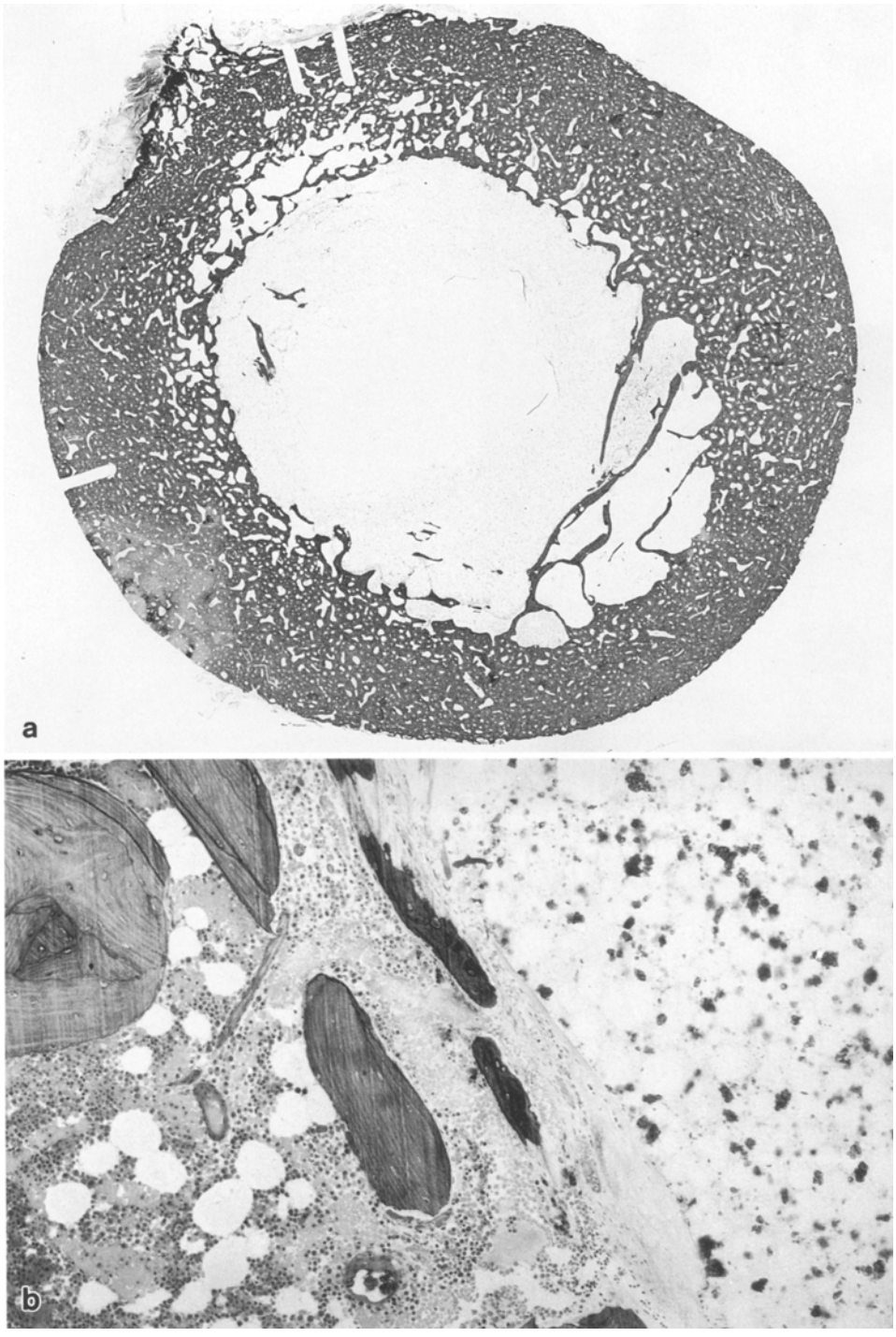

Fig. 3a. Ultra-thin grinding of a femur cross-section with a cemented endoprosthesis fixed in the marrow space. In this case, the metal stem was removed before starting the embedding procedure. Toluidine blue, 10 µm thickness. **b** Bone-cement interface. The grinding technique is best suited to preserve the bone cement (*right*) in connection with the surrounding bone (*left*). Mild fibrotic reaction could be seen. Toluidine blue, ×100

for: highly sclerotic large tumours such as sclerosing osteosarcomas, osteomas, and teeth; histological examination of specimens with metal implants with preservation of the implant plus surrounding bone tissue. Implants of any kind (cemented or cement free) can be examined in this way (Figs. 3, 4).

Bone cement consists of methylmethacrylate and therefore dissolves in the conventional plastic embedding medium. The medium used for grinding (Kulzer VLC, 7200) avoids these problems, in part because the bone cement dissolves more slowly. Moreover, if defatting and dehydration are performed by using a mixture of petroleum and gasoline instead of acetone and ethanol bone cement is preserved completely.

Specimens made using this technique are absolutely free of artefacts such as fissures, which appear to be inevitable by cutting procedures.

Grinding demands techniques totally different from those for common methylmetacrylate embedding. The basic principles of this technique were developed by Donath and Breuner (1982). Specimens up to 10 × 5 cm are cut to a thickness of 3 mm. After dehydration and carefully dissolving fat the specimens are submerged in the special plastic medium (Kulzer) for infiltration. When the polymerisation process under blue light is finished, specimens can be ground by an automatic grinding machine (Exact, Norderstedt, FRG). The minimal thickness attainable is 5–10 µm.

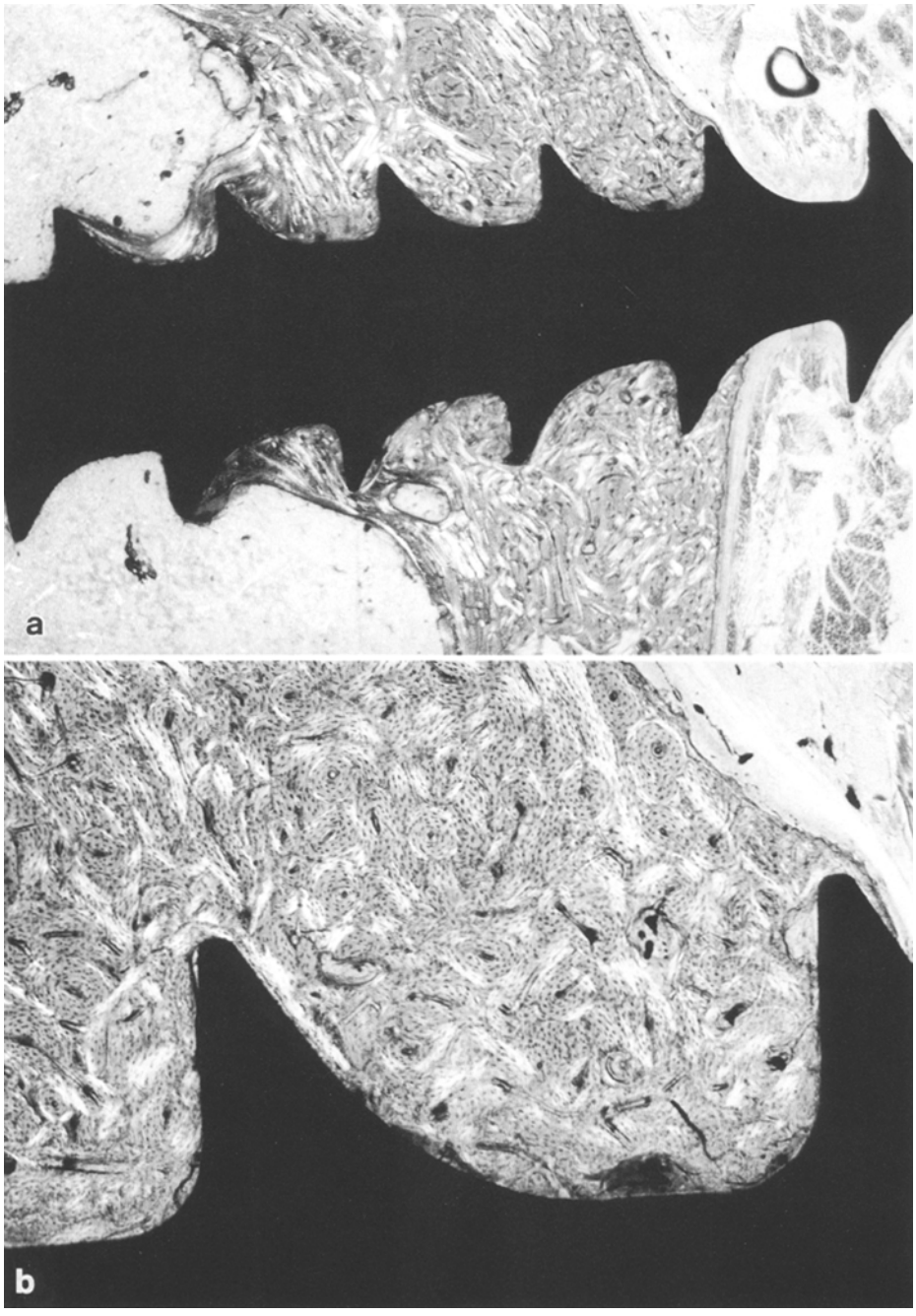

Fig. 4. a Ultra-thin grinding of a metal screw in direct contact to cortical bone. Sheep, toluidine blue, half polarized light, ×20). **b** Demonstration of the artefact-free bone-metal interface in the same section at higher magnification. ×100

Staining with toluidine blue reaction, Kossa, or other stains is possible. The grindings are excellent for quantitative analyses.

Surface stained block grindings with combined two- and three-dimensional estimation simultaneously. A great disadvantage of cutting and grinding sections is the prevention of subsequent three-dimensional analysis of bone structures. Serial sections and the utilisation of special computer programs may be one way to solve this problem. However, this method requires a lot of time and finance and the result is mainly influenced by the quality of the slices. Therefore we developed a new technique allowing two- and three-dimensional inspection of grindings 1–3 mm in thickness. For this purpose the specimens are embedded in the same way as for ultrathin grindings. After polymerisation has finished the specimen can be ground. Thereafter the surface is polished. In this way we obtain a specimen block with a thickness of 1–3 mm and a parallel-plane surface. Staining of the surface is done with toluidine blue reaction, Kossa modification or other established stainings. In perpendicular sections we could show that the staining is constantly restricted to the surface and immersion depth is 1 μm in maximum. These preparations allow the estimation of all cellular variables and structural features at the surface, as in conventional slices. Furthermore, the technique allows an impressive inspection of pre-existing vascular structures filled with erythrocytes and therefore visible without staining. In this way vascularity can be analysed in its extent, its branching, and its course as well. Moreover, trabeculae can be estimated in regard to their shape (plates or struts), orientation and interconnection by means of a stereological microscope (Fig. 5). Experiments have shown that measurements can be done directly in the third dimension with a special device (Zeiss, Oberkochen, FRG). With this method it was possible to show the perforation of struts and plates or microcallus formations (Fig. 6) (Hahn et al. 1989; Vogel et al. 1989).

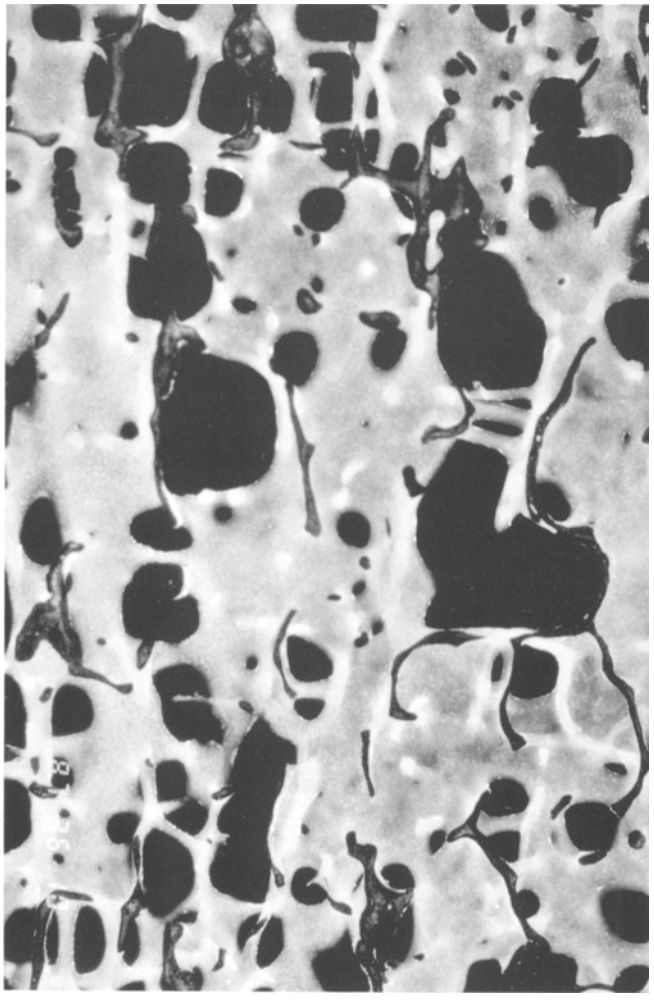

Fig. 5. Demonstration of the three-dimensional trabecular bone structure in the spine. This special preparation technique allows a combined analysis of the bone structure at the stained surface (black, two-dimensional) and the corresponding internal (white) three-dimensional bone structure. Surface-stained block grinding, Kossa modification, 1 mm thickness

Discussion

These preparation techniques for the morphological analysis of undecalcified bone specimens represent the current standard for conventional estimation of osseous and cellular structures. The application of enzymohistochemistry requires water-soluble acrylates (such as glycolacrylate, JB4 etc.). Originally these plastic embedding media were developed for soft tissues and do not have the necessary hardness. The application of immunohistochemical procedures reveals remarkable possibilities. Unfortunately up to now reproductivity could not be achieved in all cases and before routine application the solution of some problems seems to be essential (Schröder and Delling 1986).

Undecalcified preparation of iliac crest biopsies is essential for the diagnosis of mineralisation defects and other metabolic bone disorders. For example, a decalcified iliac crest bone biopsy sent to us did not allow a definitive diagnosis. Therefore another biopsy had to be taken and after undecalcified preparation the diagnosis of osteomalacia could be made and an adequate therapy was possible. Generally, osteopathies progress over a long time. Consequently preparation time of about 10 days can be tolerated. In special cases a preparation period of approximately 5 days is possible. Further reduction of preparation time influences the quality (especially of osseous structures) of the slices. The method has proved to be appropriate in the routine preparation of bone tumours where the excellent preservation of tumour cell structures and tumour-associated organic

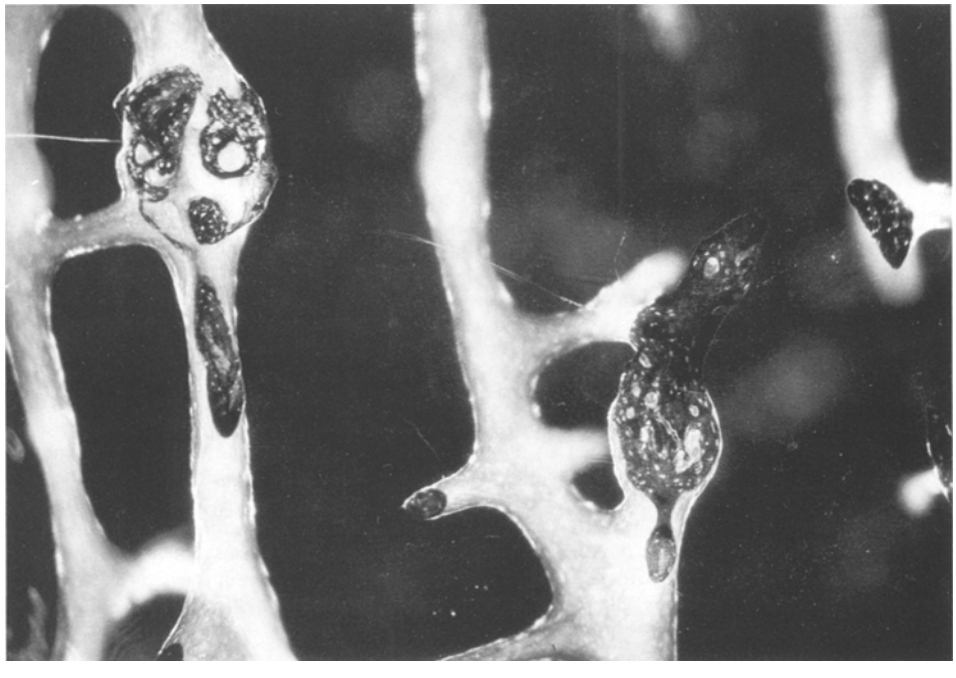

Fig. 6. Microcallus formation in the trabecular bone of a vertebral body. The microcallus might be a sign of a fracture or a stress peak in this area. The block-grinding technique offers the possibility to grind in a defined way down to the level of the microcallus formation in preparation for staining. Surface-stained block grinding, Kossa modification, 1 mm thickness

matrix are the major advantages. Decalcified preparations (e.g. highly sclerotic tumours) often require long decalcification, thus destroying a lot of cellular features. Because of the preparation time of several weeks the preparation of undecalcified large area sections is not a routine method. We use this method for special investigations (Sommer et al. 1985) only. Quantitative analysis of trabecular bone structures in the proximal femur, for example, or estimation of the complete area of osteosarcomas is possible. In particular, the combination with macroradiographs allows the estimation of the extent of sclerosing processes, mineralisations, and osteolytic destruction in bone tumours.

Preparation of undecalcified ultrathin grindings of 5–10 µm requires a lot of financial and staff support. The costs for the basic equipment are about DM 70000. Therefore this technique is reserved for special centres of bone research. The examination of bone implants (endoprostheses, hydroxylapatite, preservation of bone cement) and the morphological examination of cortical bone all require this technique. It can be combined with microradiography and it is possible to measure tetracycline double labelling. For some examinations it is more appropriate to use the surface-stained block grinding technique. The advantages compared with ultrathin grindings are the shorter time of preparation and the possibility of three-dimensional analysis. We use it to measure the real thickness of trabeculae, discrimination of struts and plates and their three-dimensional configuration. Furthermore this method make it possible to analyse the intertrabecular connections and the number of perforations in vertebral bodies and iliac crest biopsies.

If necessary it is possible to prepare an ultrathin grinding from this preparation, but their interpretation requires a lot of experience. Grindings are ideally suited for quantitative analysis, being absolutely free of stretching artefacts or fissures. The described techniques take a lot of time, but only in this way can excellent quality of the preparation and therefore reliable results be achieved.

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