Histochemical Journal **25**, 446–451 (1993)

The von Kossa reaction for calcium deposits: silver lactate staining increases sensitivity and reduces background

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Received 29 October 1992 and in revised form 23 November 1992

Summary

The classical von Kossa method has been modified: the high silver nitrate concentration in the original was replaced by 0.05% silver lactate with hydroquinone remaining the reducing agent of choice. The present modification stained calcification nodules with a sensitivity comparable to the original von Kossa reaction, but resulted in a reduced background staining in cultured osteoblasts. The method works well also with plastic- or paraffin-embedded tissue sections.

Introduction

The von Kossa reaction (von Kossa, 1901) remains the routine method for demonstrating calcium deposits in tissue (Pearse, 1972). The method is based on the substitution of tissue calcium, bound to phosphates, by silver ions and the subsequent visualization of silver ions by hydroquinone reduction to metallic silver. The reaction is generally regarded as specific for calcium, although numerous pitfalls exist (Pearse, 1972; Puchtler & Meloan, 1978). Both von Kossa (1901) and Puchtler & Meloan (1978) in their thorough revision of the method concluded that only yellow precipitates can be considered specific.

We have demonstrated that positive von Kossa staining correlates well with calcifications in the extracellular matrix in cultures of human osteoblasts as demonstrated by electron microscopy (Kassem *et al.*, 1992). In these cultures the von Kossa stain usually yielded identifiable mineralization nodules; however, in some cultures a more diffuse staining was seen. Examination of the slides was often confounded by background staining, and we were not able to distinguish between different colour intensities in the staining of the cultures. The present paper describes modifications of the von Kossa method. A procedure is suggested that results in reduced background staining – and possibly increased specificity, as will be argued later.

Materials and methods

OSTEOBLAST CULTURES

Osteoblasts were cultured from fresh human trabecular bone explants as previously described (Robey & Termine, 1987; Eriksen *et al.*, 1988). The cell lines fulfilled immunohistochemical, enzymatic (reactions for alkaline phosphatase, osteocalcin, type 1 procollagen propeptide), and morphological criteria for osteoblasts. Human fibroblast cultures established from dermal tissue fragments were used as controls.

The cells were cultured in multi-well dishes. Before fixation half of the cultures (both osteoblasts and fibroblasts) were stimulated with β-glycerophosphate (10 mM) for 5 days to induce mineralization (see Kassem *et al.*, 1991, 1992).

Before fixation in glutaraldehyde (see below) the cultures were washed in buffer.

TISSUE SECTIONS

Tissue obtained at autopsy was fixed in 3% aqueous glutaraldehyde in a 0.15 M phosphate buffer (pH 7.3). The samples were pericardial tissue with obvious macroscopic calcifications and thyroid tissue without evidence of calcification. Samples were embedded in Epon and cut by conventional procedures.

STAINING PROCEDURES

Following a number of pilot experiments we decided to test four different staining approaches. All experiments were performed in triplicate. Culture dishes and Epon sections were exposed to the procedures indicated below.

von Kossa reaction

1. Immersion for 20 min in 2.5% silver nitrate
2. Rinse in distilled water
3. Immersion in 0.5% hydroquinone for 2 min
4. Immersion for 2 min in 5% sodium thiosulphate
5. Rinse in distilled water

Culture dishes were counterstained by 0.1% Toluidine Blue or left unstained and thereafter embedded by standard procedures. Epon sections were counterstained by Toluidine Blue.

Silver nitrate and autometallography (AMG)

1. Immersion for 20 min in 1% silver nitrate
2. Rinse in water and sodium thiosulphate, as above
3. Exposure of culture dishes and sections to AMG for 20 min, as described by Danscher (1984). AMG amplifies silver deposits in sections by adding new layers of silver, derived from silver lactate in the developer, to already existing deposits.

Silver lactate and hydroquinone

Immersion for 40 min in 0.05% silver lactate. Hydroquinone and further processing as for the von Kossa reaction, above. 0.5% silver lactate for 20 min yielded similar results.

Silver lactate and AMG

Immersion for 40 min in 0.05% silver lactate. Further processing as for 'silver nitrate and autometallography (AMG)', above.

Results

CALCIFICATIONS IN TISSUE SECTIONS

Irrespective of the staining procedure, sections with macroscopic calcifications (pericardium) were positive when stained with silver (Fig. 1). Sections exposed to silver and subsequently developed by AMG disclosed a cellular silver staining compatible with a previously described counterstain method for silver staining of epoxy-embedded tissue sections (Danscher, 1983). AMG procedures did not result in increased sensitivity. Comparing silver nitrate (von Kossa) and silver lactate favoured silver lactate-stained sections because of the reduced background staining. Thyroid tissue without macroscopic calcifications exhibited no calcium deposits with either of the two staining procedures.

CALCIFICATIONS IN OSTEOBLAST AND FIBROBLAST CULTURES

Calcification nodules were present in all osteoblast cultures exposed to β-glycerophosphate, whereas non-stimulated osteoblasts and fibroblasts were devoid of nodules (Fig. 2, a–c).

Development by AMG resulted in cellular background staining as was the case with tissue sections.

The average areas covered by calcification nodules in sections stained by silver lactate or silver nitrate were identical, indicating no differences in sensitivity of the two von Kossa variants.

Sections exposed to silver nitrate demonstrated substantially more background staining, particularly in non-

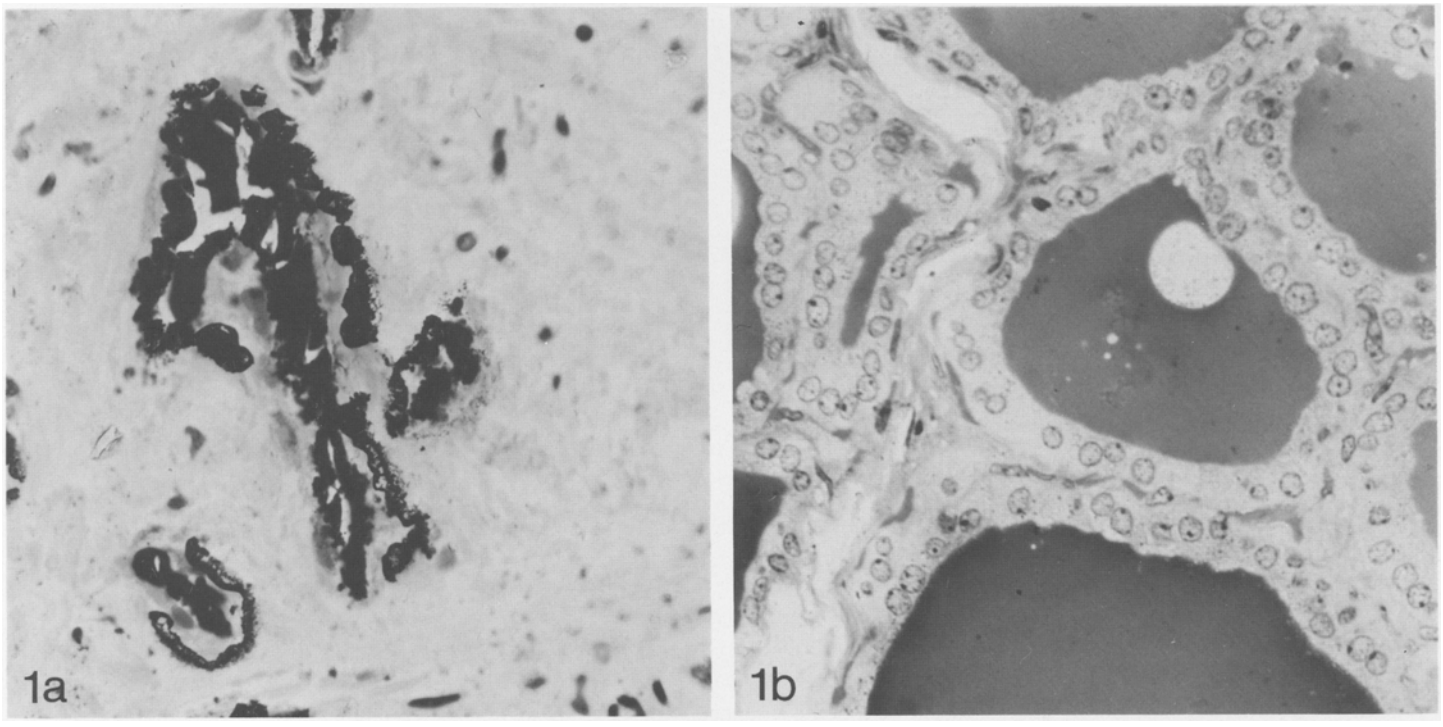


Fig. 1. (a) Calcified pericardium stained with silver lactate and developed by hydroquinone. von Kossa-positive areas are easily defined. Toluidine Blue (×600). (b) Thyroid tissue without evidence of calcification, treated as the tissue shown in (*a*) (×480).

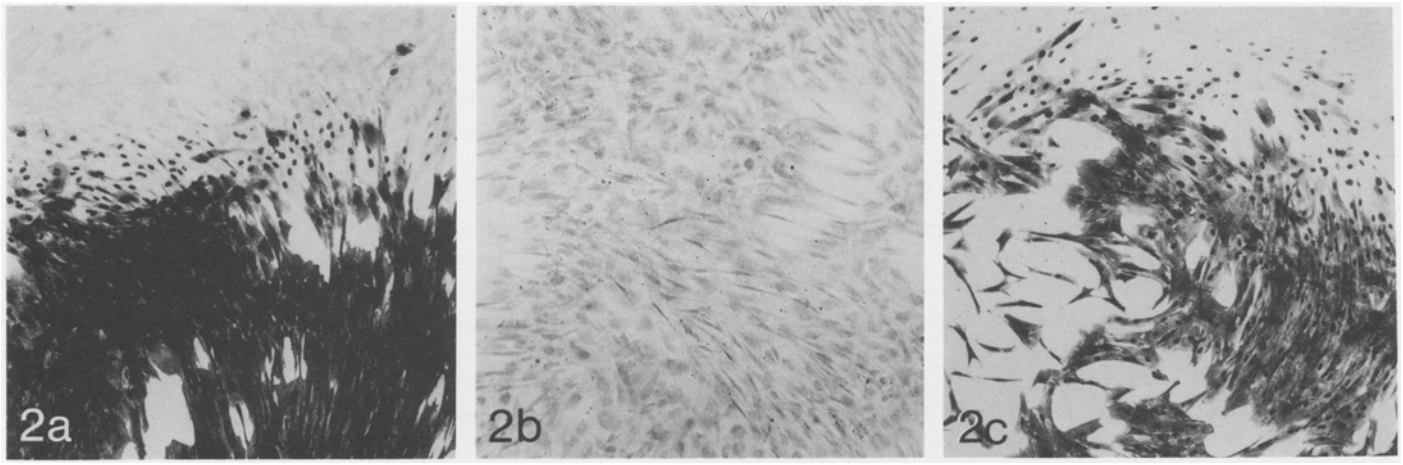


Fig. 2. (a) Osteoblast culture stimulated with β-glycerophosphate (as phosphate ion donor); stained with 1% silver nitrate and developed with hydroquinone. The border of a calcification nodule is shown. No counterstain (×40). (b) Parallel culture to the one shown in (a), but not stimulated with β-glycerophosphate; no evidence of calcification. Toluidine Blue after silver nitrate and hydroquinone (×40). (c) Parallel culture to that shown in (a) stained by von Kossa's original procedure using 2.5% silver nitrate; no counterstain (×40).

mineralized areas, than did sections exposed to silver lactate (Fig. 3a,b). Furthermore, in mineralized areas, silver nitrate-stained sections revealed a staining pattern involving intracellular colouring with a remarkable staining of nuclei and cell membranes (Fig. 4a). In silver lactate-treated sections this non-specific staining was far less prominent (Fig. 4b,c). The macroscopic appearance of a calcification nodule is shown in Fig. 5.

Discussion

In this study the ability of the von Kossa reaction – as well as the proposed modification replacing silver nitrate with silver lactate and maintaining hydroquinone as the reducing agent (see Materials and methods) – to visualize mineralization was indirectly confirmed. Sections with macroscopic calcifications were always positive, as were osteoblast cell cultures after stimulation with β-glycerophosphate, whereas control sections, unstimulated osteoblast cultures and control fibroblast cultures were all negative. Attempts to enhance the staining response by autometallographic development of silver-stained mineral deposits did not result in increased sensitivity and produced a cellular background stain comparable with that seen in the silver lactate counterstaining for epoxy-embedded tissue (Danscher, 1983) which hampers interpretation of the reaction.

The technical improvements observed after substituting silver lactate for silver nitrate as silver ion donor included higher specificity and a markedly decreased

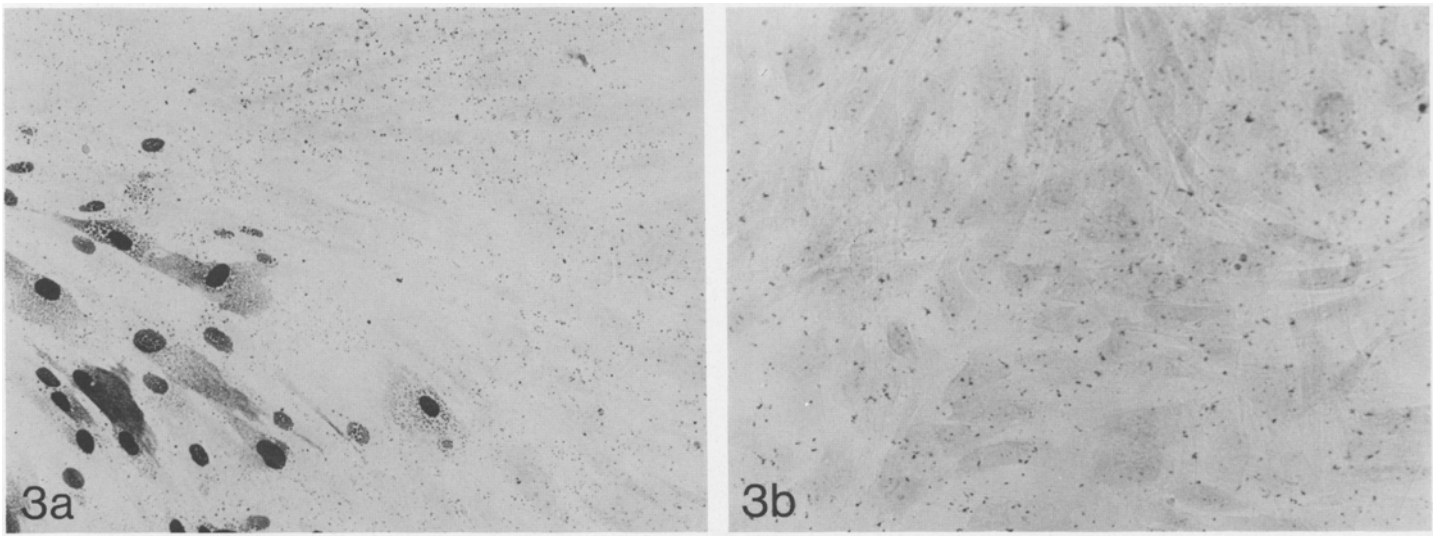


Fig. 3. (a) An osteoblast culture outside calcification nodules stained with silver nitrate and subsequently exposed to hydroquinone. A faint background and non-specificly stained cells are seen. Toluidine Blue (×150). (b) Parallel culture stained with silver lactate. Cellular staining is not present, and the background is somewhat diminished. Toluidine Blue (×150).

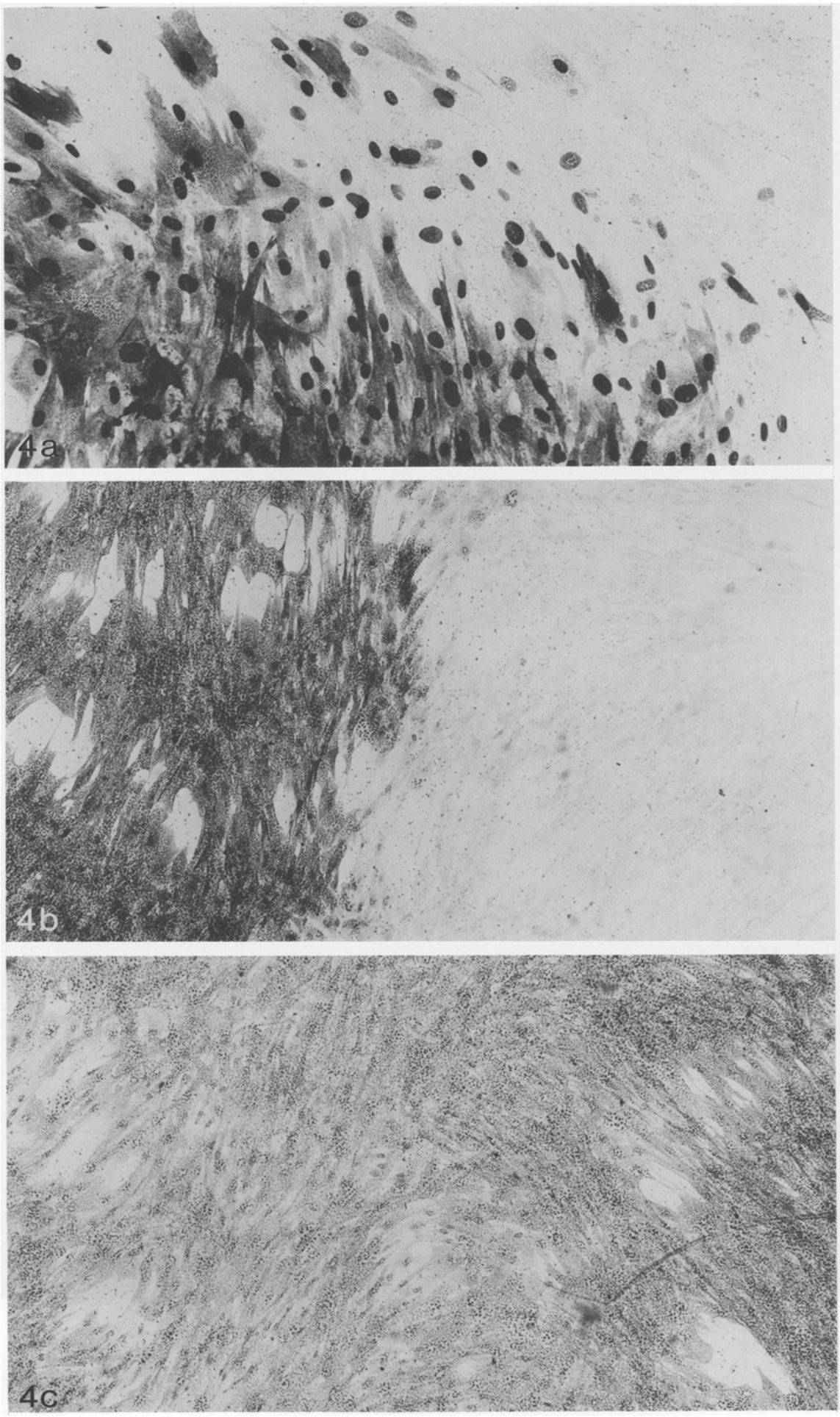
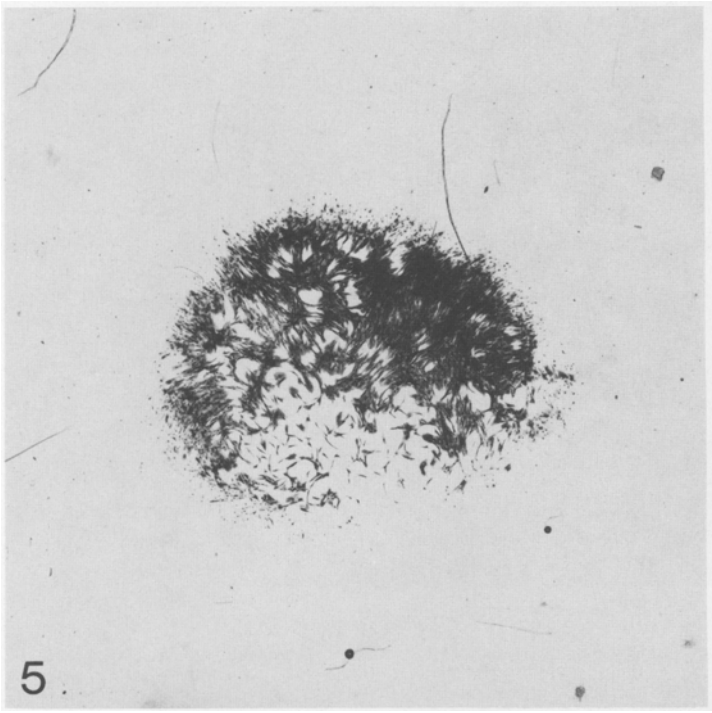


Fig. 4(a–c)



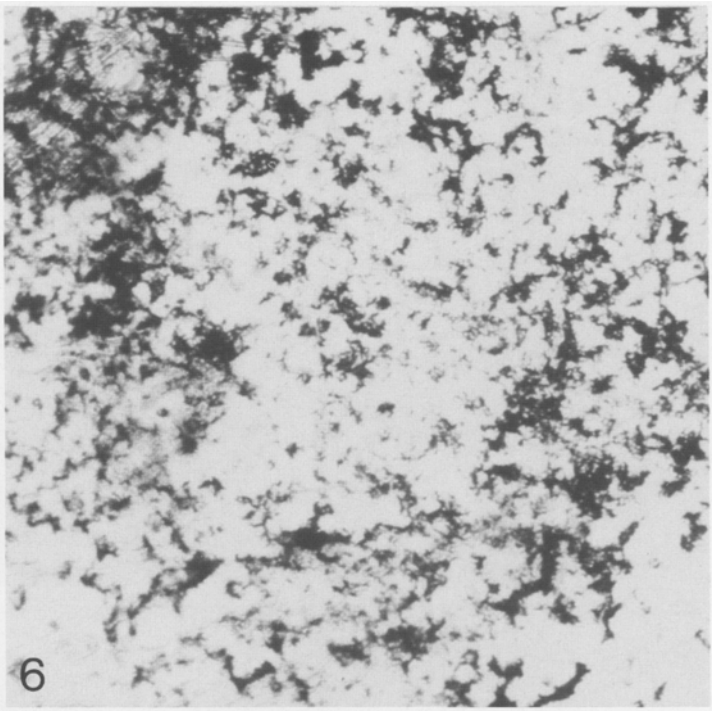


Fig. 5. β-Glycerophosphate-stimulated osteoblast culture after silver lactate and hydroquinone (silver lactate and hydroquinone procedure). The borders of a calcification nodule can be easily identified. No counterstain (×11).

Fig. 6. Stimulated osteoblast culture, grown in parallel to the one shown in Fig. 5. von Kossa staining using silver nitrate; no counterstain (×30). Occasionally this procedure produces a diffuse staining of an entire culture plate with the pattern shown in this micrograph. This pattern corresponds less well with that expected for calcification nodules compared with the nodule seen in Fig. 5.

background. Silver lactate was introduced as Ag^+ donor in autometallographic reactions (see Danscher, 1984) in order to moderate the activity in the developer. Since silver lactate has a dissociation coefficient lower than that of silver nitrate, the release of silver ions to be deposited or reduced can be regulated. The probability of non-specific reactions decreases substantially (Danscher, 1984).

Thus, by employing silver lactate, the definition of mineralized areas was much easier and we have not experienced problems with diffusely stained culture slides as is sometimes seen with silver nitrate (Fig. 6). Further, the original procedure seems to result in a remarkable staining pattern suggestive of intracellular staining, especially of nuclei. This cellular staining occurred only in areas with evidence of extracellular mineralization and may be caused by metabolic alterations of osteoblasts by the surrounding calcifications, or it may be the result of cell death in areas of mineralization, also exposing silver-positive cytoskeletons. In either case, the staining is not compatible with earlier studies (Kassem *et al.*, 1992) on identical osteoblast cultures. Ultrastructural examination of calcifications in β-glycerophosphate-stimulated osteoblasts revealed that mineralization was exclusively extracellular and neighbouring cells appeared vital with no signs of degeneration. For these reasons we find it likely that the 'cellular' staining patterns are non-specific, despite the fact that they are almost exclusively present in, or close to, von Kossa-positive areas. This cellular pattern was much reduced with the use of silver lactate.

Although a reduction of silver ions chemically or photochemically by light- or UV-light exposure was suggested to be unnecessary by Pizzolato & McCrory (1964), we have maintained the original procedure with hydroquinone. All experiments have been performed at room temperature and in daylight in order to simplify the use of the procedure.

Fig. 4. (a) β-Glycerophosphate-stimulated osteoblast culture stained by 1% silver nitrate and subsequently exposed to hydroquinone. At the margin, as well as within the calcified area, a cellular stain with visible cell nuclei is seen. 'Mineralization' in cell nuclei is considered an artefact. No counterstain (×150). (b) Parallel culture to the one shown in (a), but stained with 0.05% silver lactate. Cell nuclei are no longer prominent. Toluidine Blue (×80). (c) Same staining as in (b). The centre of a calcification nodule (×110).

A possible explanation of the chemical events taking place during the von Kossa procedures could be as follows. The 3–6 nm thick and up to 60 nm long hydroxyapatite crystals $(Ca^{2+}_{10-x}(H_3O^+)_{2x}—(PO^{3-}_3)_6\ (OH^-)_2)$ cause silver ions to adhere to their surfaces (as is known with so-called bone-seeking ions, e.g. strontium). Most likely, as predicted by von Kossa, silver ions will also be able to substitute calcium bound to phosphates in the outermost layers of the crystals. After silver ion exposure the sections are exposed to a reducing substance (hydroquinone). If amplification to visible silver deposits is to take place it is necessary that 'catalytic sites', as known from photographic techniques, be present. Such sites can, in the case of the von Kossa method, be either metallic silver atoms (a minimum of three within nanometres (James, 1939)) or silver sulphide molecules (probably only two molecules (Zieger, 1938)). Disulphide bridges are abundant in the collagenous fibres always found in close relation to calcified areas (Kassem *et al.*, 1992) and reducing substances will yield SH groups and consequently (in the presence of silver) silver sulphides. The three prerequisites for all silver enhancement (autometallographic or photographic) – free silver ions donated from the surface of hydroxyapatites, a reducing substance (e.g. hydroquinone), and catalytic sites (most likely silver sulphides) – are thus present. Since the donors of silver ions to the procedure are hydroxyapatite surfaces, it is suggested that the von Kossa method, as well as the present modification, is a true visualization of mineralizations.

Both von Kossa (1901) and Puchtler & Meloan (1978) stated that only yellow silver deposits can be considered positive. In our experience, the original von Kossa reaction, as well as the silver lactate modification, results in mostly black but occasionally brownish precipitates. The spectrum of colours seen with silver deposits (yellow to black) was demonstrated by Liesegang (1911) to be dependent on the size of the silver grains present in the sections. The colour quality is thus dependent on the amount of silver ions on the hydroxyapatite crystals and, most importantly, on the time of exposure to reducing agents (Danscher, 1983). Thus there is no justification for discussing the specificity of the von Kossa method in relation to the colours of the preserved precipitates.

From the present experiments, and from previous ultrastructural studies, we conclude that the von Kossa reaction remains a reliable tool for the demonstration of mineralization in tissue sections and in cell cultures. The original procedure can be improved by the use of silver lactate instead of silver nitrate.

References

DANSCHER, G. (1983) A silver method for counterstaining plastic embedded tissue. *Stain Technol.* **58,** 365–72.

DANSCHER, G. (1984) Autometallography. *Histochemistry* **81,** 331–5.

ERIKSEN, E. F., COLVARD, D. S., BERG, N. J., GRAHAM, M. L., MANN, K. G., SPELSBERG, T. C. & RIGGS, B. L. (1988) Evidence of estrogen receptors in normal human osteoblast-like cells. *Science* **241,** 84–6.

JAMES, T. H. (1939) The reduction of silver ions by hydroquinone. *J. Am. Chem. Soc.* **61,** 648–52.

KASSEM, M., RISTELI, L., MOSEKILDE, L., MELSEN, F. & ERIKSEN E. F. (1991) Formation of osteoblast-like cells from human mononuclear bone marrow cultures. *Acta Pathologica et Microbiologica Scandinavica (APMIS)* **99,** 269–74.

KASSEM, M., RUNGBY, J., MOSEKILDE L. & ERIKSEN E. F. (1992) Ultrastructure of human osteoblasts and associated matrix in culture. *Acta Pathologica et Microbiologica Scandinavica (APMIS)* **100,** 490–7.

LIESEGANG, R. E. (1911) Die Kolloidchemie der histologischen Silber-Färbungen. *Kolloid Beihefte* **3,** 1–46.

PEARSE, A. G. E. (1972) *Histochemistry: Theoretical and Applied,* 3rd edn. p. 1405. Edinburgh: Churchill Livingstone.

PIZZOLATO, P. & MCCRORY, P. (1964) Light influence on von Kossa's silver calcium reaction in the myocardium. *J. Histochem. Cytochem.* **10,** 102–10.

PUCHTLER, H. & MELOAN, S. N. (1978) Demonstration of phosphates in calcium deposits: a modification of von Kossa's reaction. *Histochemistry* **56,** 177–85.

ROBEY, P. G. & TERMINE, J. D. (1987) Human bone cells in vitro. *Calcif. Tiss. Int.* **37,** 453–8.

VON KOSSA, J. (1901) Über die im Organismus künstlich erzeugbaren Verkalkungen. *Beitr. path. Anat.* **29,** 163–202.

ZIEGER, K. (1938) Physikochemische Grundlagen der histologischen Metodik. *Eill. Forschungsber.* **48,** 55–75.