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Histochemistry

Menadiol diphosphate, a new substrate for non-specific alkaline phosphatase in histochemistry and immunohistochemistry *

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Summary. Menadiol diphosphate was introduced as a new substrate for nonspecific alkaline phosphatase, following a search for new and less expensive substrates, which give a more sensitive response and are easily synthesized in the laboratory. Menadiol released by phosphatase action can be assayed by its reduction of tetrazolium salts, or it can be coupled with diazonium salts; alternatively, the phosphate can be trapped by metal ions. The synthesis and purification of menadiol diphosphate are described, and it was shown to be sufficiently stable for qualitative and semiquantitative histochemistry, as well as for the immunohistochemistry of enzymes and cytoskeletal proteins with nonspecific alkaline phosphatase as the enzyme label. For qualitative as well as semiquantitative histochemistry and immunohistochemistry, the best results were obtained by applying the method with nitro-blue tetrazolium (NBT) to acetone-chloroform pretreated cryostat sections. Tetranitro-blue tetrazolium (TNBT), benzothiazolylphthalhydrazidyl tetrazolium (BSPT) and various diazonium salts were less suitable. Fast Blue BB and VB produced satisfactory results. Ce^{3+} ions and the DAB–Ni–H_2O_2 procedure yielded better results than Ca^{2+} ions in the Co–$(NH_4)_2S$ visualization method. The NBT method with menadiol diphosphate is superior to existing methods employing azo, azoindoxyl or tetrazolium salts and to metal precipitation methods. The Ce^{3+} technique and the NBT/menadiol diphosphate method give similar results, and appear to be of equal value. In qualitative histochemistry and immunohistochemistry the NBT/menadiol diphosphate method resulted in higher quantities of precisely localized stain. Semiquantitative histochemistry with minimal incubation revealed more favorable kinetics for the menadiol diphosphate method, especially when using NBT.

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Introduction

Non-specific alkaline phosphatase (E.C. 3.1.3.1) can be studied in mammalian and non-mammalian tissues by qualitative histochemical means, using natural and synthetic phosphates, e.g. indoxyl, naphthol AS, naphthol, nitrophenol or methylumbelliferone phosphates, with an azo, azoindoxyl, or tetrazolium salt, as well as by metal precipitation methods (Lojda et al. 1979; Halbhuber et al. 1988). Quantitative histochemistry of the enzyme was performed by the azo and tetrazolium procedures (Gutschmidt et al. 1980; Van Noorden and Jonges 1987; Ruhnke 1990). Apart from peroxidase, the most popular and efficient enzyme label for light microscopic immunohistochemistry is non-specific alkaline phosphatase. The activity of this enzyme is mainly visualized, either by using azo methods with naphthol AS substrates, or by using 5-Br-4-Cl-3-indoxyl phosphate with NBT or TNBT (Luppa et al. 1986).

In the present communication we report a new substrate for non-specific alkaline phosphatase, i.e. menadiol diphosphate, and we describe its advantages for the study of non-specific alkaline phosphatase in qualitative and semiquantitative histochemistry and in qualitative immunohistochemistry.

Material and methods

Animals, tissue pretreatment. Adult female and male Wistar rats (body weight 150–200 g) were bred on the premises, kept in an artificial light-dark cycle (light 7.00 a.m.–7.00 p.m.) at 21 + 2° C in Macrolon cages, with free access to Altromin (Lippe, FRG) rat diet and tap water. The animals were sacrificed between 3.00 and 4.00 p.m. under ether anaesthesia. The organs were removed, mounted on cork plates (covered with wet filter paper), wrapped with plastic foil, frozen in liquid nitrogen and stored in sealed plastic bags at −25° C until use. Sections were cut in a cryostat (model 2800 N, Reichert Jung, Nussloch, FRG) at −25° C for histochemistry (4–20 μm) and immunohistochemistry (4 μm). The sections were air-dried, pretreated without thawing in an acetone-chloroform mixture (1:1; v/v) for 5 min at −25° C, fixed in 0.5% glutaraldehyde buffered with 0.1 *M* cacodylate, pH 7.2 for 1 min

at 4° C, rinsed for 5 min in tap water and for 1 min in distilled water. Alternatively, the sections were freeze-dried and mounted with 0.5% celloidin on albuminized glass slides according to Lojda et al. (1979).

Synthesis of menadiol diphosphate. Menadione (5 mmol) was dissolved in approx. 200 ml diethylether and shaken three times with an aqueous solution of sodium dithionite. The ether solution was then washed twice with saturated sodium chloride solution containing some sodium dithionite and dried using anhydrous sodium sulphate. The ether was removed in a N_2 atmosphere at 50° C. Phosphoroxy chloride (approx. 4.4 mmol) dissolved in 80 ml pyridine was added to the remaining menadiol in an ice bath. After 1 h the pyridine was removed (see above); a further 80 ml pyridine were added and the procedure for removal repeated. The flask was placed in an evacuated desiccator containing H_2SO_4 for 12–24 h. Ice-cold distilled water (200 ml) was then added to the flask with ice-cooling, and the flask was shaken until all solid material had dissolved. Using saturated $Ba(OH)_2$, the pH of the solution was adjusted to 9 and the precipitated $Ba_3(PO_4)_2$ discarded. An equal volume of absolute ethanol was then added to the clear solution, which was stored in the refrigerator for 24 h. The precipitated barium salt of menadiol diphosphate was removed by filtration, washed with 50% ethanol, absolute ethanol and ether, and dried in an evacuated desiccator. The barium salt was dissolved in 150 ml distilled water and transferred to a DOWEX 50 WH^+ column. The eluate was collected and concentrated by freeze-drying. The menadiol diphosphate crystallized after standing several days over NaOH under vacuum in a desiccator.

Purity controls. These were performed using high performance thin layer chromatography (HPTLC). A freshly prepared solution (5 m*M*) of menadiol diphosphate was spotted (0.2 µl/mm, 6 s/µl) as a band on an activated (120° C, 30 min) KG 60-F_{254} HPTLC plate (10 × 20 cm; Merck, Darmstadt, FRG) using a Linomat IV (Camag, Muttenz, CH) and nitrogen as carrier gas. After air-drying for 15 min the plates were developed in one dimension in a linear chamber (Camag) with methanol/acetonitrile (9/1, v/v; 2 cm). After drying for 15 min at room temperature, the same chromatogram was developed with a second mobile phase of chloroform/methanol/glacial acetic acid ((5/3/1; v/v; 8 cm). The bands were visualized by phosphorescence quenching at 254 nm using a UV-lamp (Universal-UV-light; Camag), and the hRf values were determined according to Stahl (1967).

Incubation media. The disodium salt of menadiol diphosphate was dissolved at a concentration of 0.5–10 m*M* in 0.1 *M Tris*-HCl buffer, pH 9.4 containing 5 m*M* $MgCl_2$. To this buffered substrate solution were added 0.25–40 m*M* tetranitro BT (TNBT), nitro BT (NBT) or benzothiazolylphthalhydrazidyl tetrazolium (BSPT) together with 1–4 m*M* phenazine methosulphate (PMS) or methoxy-PMS (mPMS) (tetrazolium salt method). Alternatively, the tetrazolium salt was replaced by 0.5–1 mg Fast Blue, B, BB, or VB/ml, or by 30–60 µl hexazonium New Fuchsin (HNF)/ml (azo method). The pH of the solutions containing HNF was adjusted with 1 NaOH. In the Ce-diaminobenzidine-nickel-hydrogen peroxide (DAB–Ni–H_2O_2) method of Halbhuber e al. (1988), the substrates in the original method were replaced by 3 m*M* menadiol diphosphate. In the modified Ca–Co procedure of Gomori according to Lojda et al. (1979), 2-glycerophosphate was replaced by the same molar concentration of menadiol diphosphate.

Extraction experiments for the formazan were performed at room temperature overnight with dimethyl formamide (DMF), dimethylsulphoxide (DMSO) or acetone.

In addition, the results obtained with sodium salt of menadiol diphosphate were compared with the qualitative azo, azoindoxyl, metal precipitation and tetrazolium salt procedures for nonspecific alkaline phosphatase reported by Lojda et al. (1979), and with the azo method using 1- or 2-naphthyl phosphate and hexazonium New Fuchsin as described by Gossrau (unpublished method).

Controls were performed with heated sections, using incubation media free of substrate, diazonium and tetrazolium salts and metal ions, or using complete incubation media containing either 1.5 m*M* levamisole or 10 m*M* L-phenylalanine to inhibit extraintestinal and intestinal non-specific alkaline phosphatase.

Qualitative histochemistry (endpoint incubation) was carried out for 5 to 30 min at 30° C in a shaking water bath in Coplin jars containing 25–50 ml prewarmed double filtered incubation medium (Ce and Ca method) or in moist incubation chambers using the droplet technique according to Lojda et al. (1979). After incubation, the sections were first rinsed in tap water, then in distilled water and afterwards embedded in glycerol jelly.

Semiquantitative histochemistry (continuous kinetic incubation) was performed using the direct azo, azoindoxyl and tetrazolium methods with naphthol AS, indoxyl and menadiol phosphates as substrates with minimal incubation, i.e., incubation of the tissue sections under the microscope until the first appearance of reaction product in the intestinal and renal brush borders or heart muscle capillaries, using the Perspex ring technique as given by Lojda et al. (1979). For this purpose the Perspex rings were fixed with vaseline onto the glass slides around the tissue sections and closed with cover slips. The reactions were monitored with either a conventional Zeiss light microscope at a 10-fold magnification, or using a Zeiss stereomicroscope at a 6.5-fold magnification at room temperature.

Immunohistochemistry was carried out with anti-keratin and anti-vimentin antibodies as described by Gossrau et al. (1988), and with polyclonal anti-dipeptidyl peptidase IV-antibodies according to Hartel et al. (1988), using goat anti-rabbit alkaline phosphatase immunocomplexes for antibody detection, and employing all the histochemical procedures given above to reveal alkaline phosphatase activity after 5, 10, 15 and 30 min reaction at room temperature.

Source and quality of chemicals. The chemicals were all of analytical or highest purity grade and were obtained from Boehringer (Mannheim, FRG), Merck (Darmstadt, FRG), Sigma (Munich, FRG), Serva (Heidelberg, FRG), Dakopatts (Hamburg, FRG) and Medac (Hamburg, FRG).

Results

Thin layer chromatography, purity controls

One main band (hRf-value 30) and two very adjacent and much smaller bands (hRf-values 84 and 95) were separated (Fig. 1).

Tissue pretreatment

The greatest degree of staining in unfixed air-dried, or in acetone-chloroform pretreated cryostat sections was

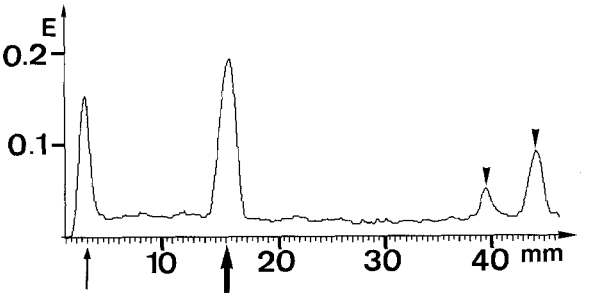


Fig. 1. Thin layer chromatogram. Large arrow menadiol diphosphate, *arrowheads* presumed menadiol monophosphates. *E* extinction, *small arrow* origin

achieved with menadiol diphosphate, in combination with tetrazolium salts (Fig. 2) or diazonium salts, or as a substrate for the metal precipitation technique with Ce or Ca ions. With both metal precipitation methods (Fig. 3), good results were also obtained after short fixation in very dilute glutaraldehyde. Using freeze-dried celloidin-mounted sections and the tetrazolium salt or azo procedure, localization of the final reaction product was somewhat more precise than in air-dried or acetone-chloroform-pretreated sections; the amount of stain, however, was lower.

Qualitative histochemistry

Incubation media, reaction principles. Irrespective of the reaction principle, 3 m*M* concentrations of menadiol diphosphate produced the greatest amounts of stain; at highly active sites, e.g. in the small intestinal and renal brush border or myoepithelial cells of oral salivary glands, adequate staining was obtained with concentrations as low as 0.75 or 1.5 m*M*. However, with both the tetrazolium and azo techniques, the final stain may diffuse. *Tetrazolium method.* At all concentrations, NBT (Fig. 4) produced the greatest amounts of formazan with no signs of diffusion. With TNBT or BPST the yield of formazan was lower and its diffusion higher. An NBT concentration of 1 mg/ml incubation medium was optimal. PMS or mPMS were without effect. Extraction experiments of the NBT-formazan with DMF, DMSO or acetone revealed practically no extractable formazan. *Azo procedure.* Using 1.0 mg of Fast Blue salt/ml or 60 µl hexazonium New Fuchsin/ml of incubation medium, more stain was generated than with 0.5 mg/ml or 30 µl/ml. However, diffusion also occurred when Fast Blue BB or VB were used; otherwise these reagents gave the best results. The quality of localization, the number of stained sites and the amount of dye formed were always inferior in the azo methods, compared with methods employing NBT, TNBT or BSPT. *Metal salt precipitation.* The Ce procedure yielded more stain and more stable final reaction product (amplified and intensified DAB brown) than the Ca technique; localization was precise with both ions (Fig. 5) and equal to that obtained with the tetrazolium method using NBT.

All *controls* yielded negative results.

In summary, the best results were obtained with the tetrazolium method using 3 m*M* menadiol diphosphate and 1 mg/ml NBT in 0.1 *M Tris*-HCl buffer, pH 9.4, containing 5 m*M* $MgCl_2$. This was therefore the method of choice. The Ce and Ca procedures produced less satisfactory results.

Comparison with the other qualitative procedures for non-specific alkaline phosphatase

The azo methods using naphthol or naphthol AS substrates and hexazonium New Fuchsin produced amorphous dyes, whereas microcrystalline final reaction products were observed with menadiol diphosphate, in combination with either NBT, or Ce or Ca ions. Microcrystalline reaction products were also formed when naphthol AS phosphate was used as the substrate with Fast Blue B for simultaneous coupling, and when indoxyl phosphate was used as the substrate in the azoindoxyl reaction or in the tetrazolium salt technique. There were slight differences in the stability of the final reaction products. Thus azo-dyes from hexazonium New Fuchsin were the most stable, followed by those of Fast Blue B, Fast Blue VB, the formazans, and intensified DAB brown, while the dye from Co sulfide was the least stable. On the other hand, due to the low or even absent background staining and the dark blue-black colour, the formazans produced by menadiol or indoxyl were more easily detectable than the azo, azoindoxyl or DAB dyes.

Semiquantitative histochemistry

Using the tetrazolium salt method with NBT, with brief or continuous incubation, formazan appeared somewhat earlier in the small intestinal and renal brush border and in capillary endothelial cells of the heart, than with the tetrazolium procedure using 5-Br-4-Cl-3-indoxyl phosphate. With the azo method using 1-naphthyl phosphate and Fast Blue B, or 1- or 2-naphthyl phosphate and hexazonium New Fuchsin, the reaction was even slower. It was slower still when naphthol AS-TR (or AS-BI) phosphate was used as substrate with Fast Blue B or hexazonium New Fuchsin, and when 5-Br-4-Cl-3-indoxyl phosphate was used with Fast Blue VB for the azoindoxyl reaction.

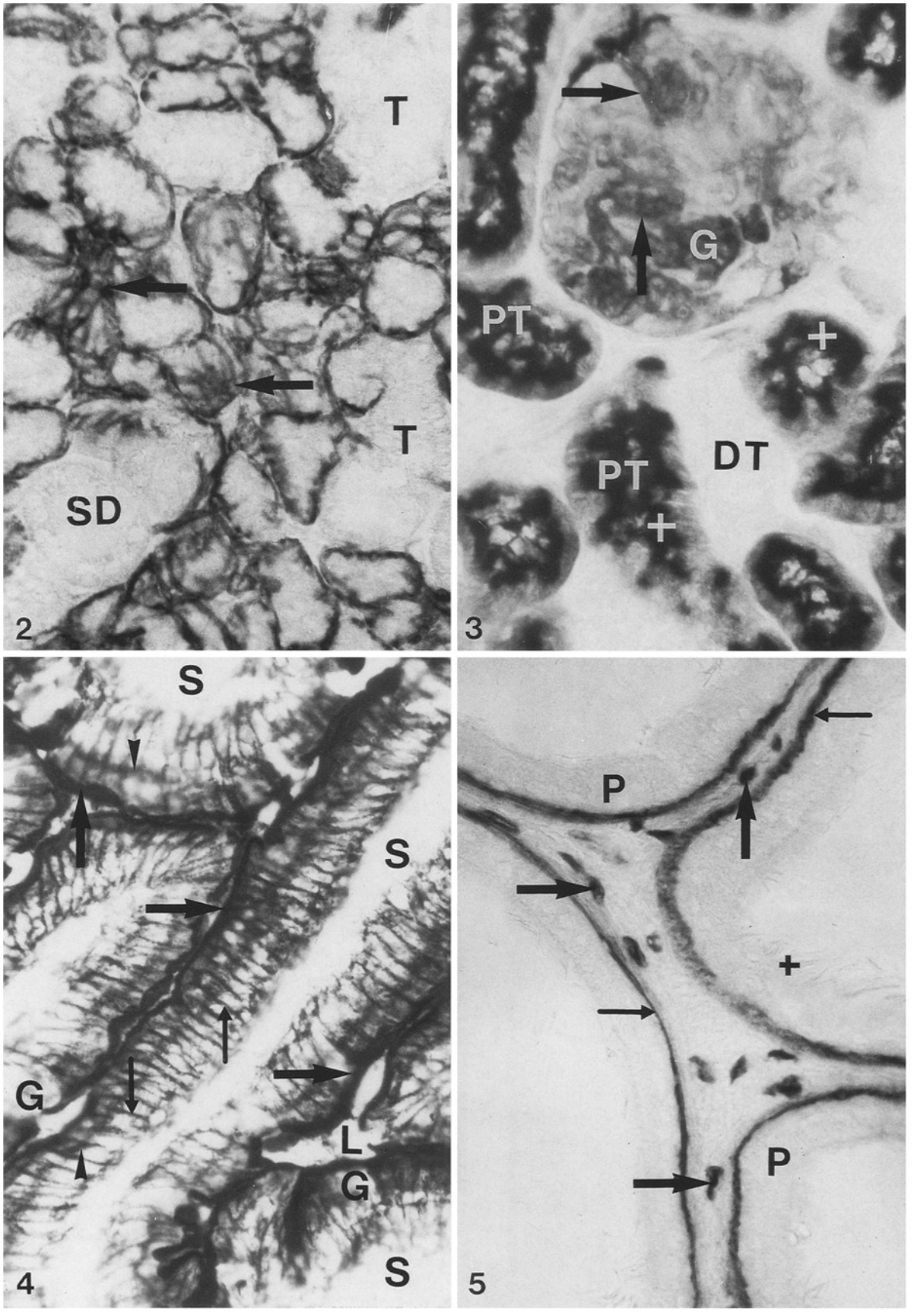
Immunohistochemistry

The following systems: 1. tetrazolium salt with 5-Br-4-Cl-3-indoxyl phosphate; 2. the azo method using naphthol AS-TR (or AS-BI) phosphate and Fast Blue B, or hexazonium New Fuchsin (HNF); and 3. 1- or 2-naphthyl phosphate in combination with HNF, all revealed immunoreactive sites for keratin, vimentin and dipeptidyl peptidase IV in the kidney and liver. The same immunoreactive sites were revealed with a similar or greater staining intensity by the tetrazolium method with menadiol diphosphate and NBT (Figs. 6, 7), and by the Ce or Ca–Co technique (Figs. 8, 9; Gossrau et al. 1988; Hartel et al. 1988).

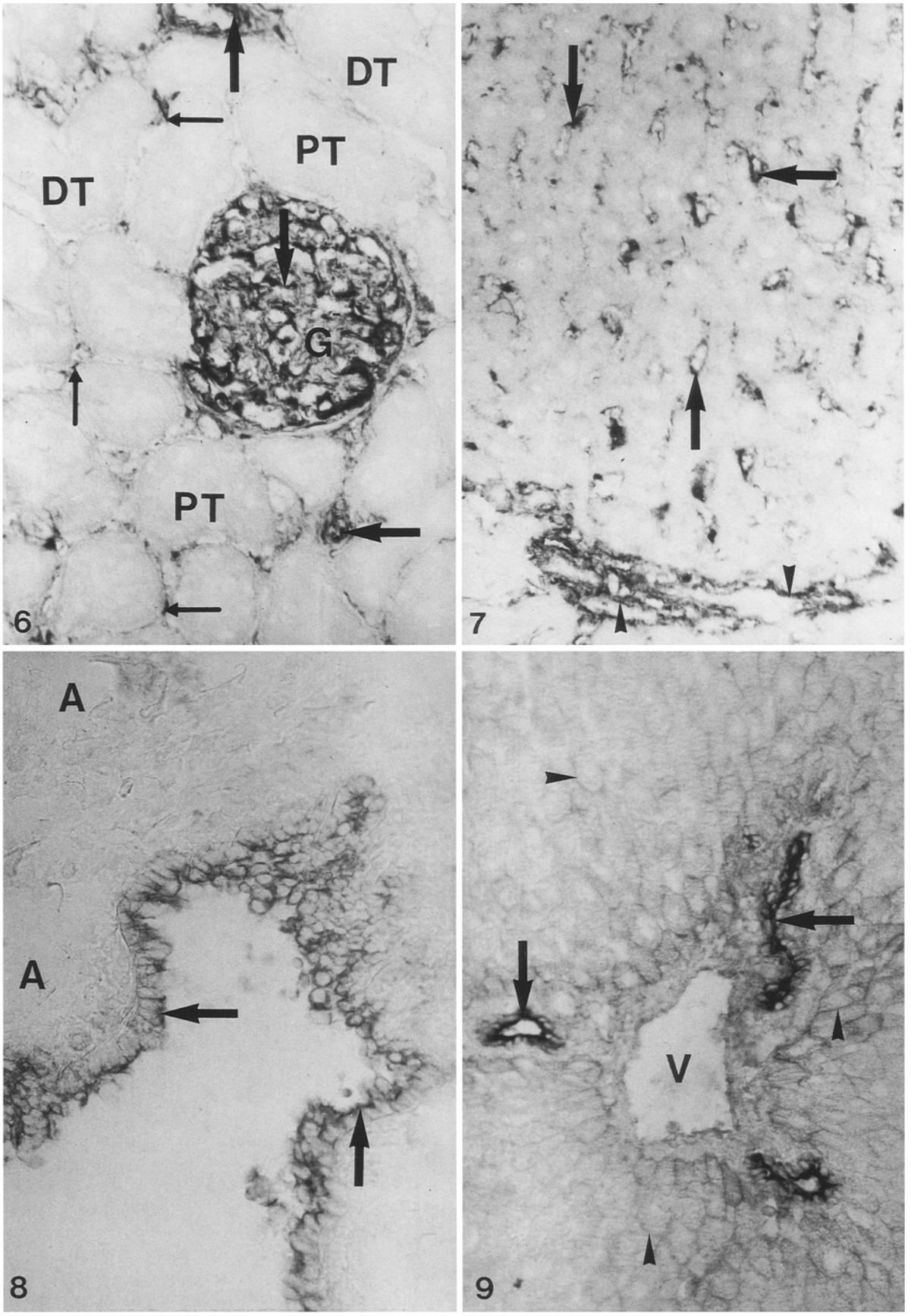
Discussion

In the present study, the disodium salt of menadiol diphosphate was introduced as a new chromogenic substrate for the qualitative and semiquantitative histochemistry of non-specific alkaline phosphatase, and for qualitative immunohistochemistry employing non-specific alkaline phosphatase as the enzyme label. We synthesized our own menadiol, and this was shown to be sufficiently pure by thin layer chromatography.

Compared with the available natural and synthetic substrates for non-specific alkaline phosphatase, mena-



Figs. 2–5. Activity histochemistry of non-specific alkaline phosphatase. **Fig. 2.** Submandibular gland, acetone-chloroform pretreated section. Reaction product in myoepithelial cells (*arrows*). *T* tubules, *SD* secretory ducts. **Fig. 3.** Kidney, glutaraldehyde fixation. Staining of endothelial cells (*arrows*) in a glomerulus (*G*) and in the brush border (+) of proximal tubules (*PT*). DT distal tubule. **Fig. 4.** Jejunum, NBT method. Reaction product in the brush border (*large arrows*), lateral plasma membrane (*small arrows*) and Golgi apparatus (*arrowheads*) of enterocytes. *S* villous stroma, *G* goblet cells, *L* intestinal lumen. **Fig. 5.** Epididymis, Ca–Co technique. Stain is seen in capillaries (*large arrows*) and smooth muscle cells (*small arrows*) in the wall of the distal epididymal duct. *P* principal cells, + sperms. *Magnification* of all figures ×400



Figs. 6–9. Immunohistochemistry with non-specific alkaline phosphatase as label. **Figs. 6, 7.** NBT method, vimentin visualization. **Fig. 6.** Kidney. Immunoreactivity in vascular endothelial cells (*thick arrows*) and connective tissue elements (*thin arrows*). *G* glomerulus, *PT* proximal tubules, *DT* distal tubules. **Fig. 7.** Liver. Immunoreactivity in sinusoidal endothelial cells (*thick arrows*) and endothelial cells of portal vessels (*arrowheads*). **Figs. 8–9.** Ca – Co technique, keratin visualization. **Fig. 8.** Lung. Immunoreactivity (*arrows*) in bronchiolar epithelial cells. *A* alveoli. **Fig. 9.** Liver. Immunoreactivity in epithelial cells (*arrows*) of bile ducts and hepatocytes (*arrowheads*). *V* interlobular vein. *Magnification* of all figures × 400

diol diphosphate has several advantages. Menadiol diphosphate is hydrolysed at the same rate or even more rapidly than the indoxyl, nitrophenyl and methylumbelliferyl substrates, which are considered at present to be the most rapidly hydrolysed compounds (Halbhuber et al. 1988); furthermore, both parts of the substrate molecule can serve for visualization of the enzyme activity, i.e. menadiol for the reduction of tetrazolium salts or simultaneous coupling with diazonium compounds, and phosphate which can be trapped by suitable metal ions, such as Ce or Ca ions. Whether Fe^{3+} ions may also be used in a metal salt procedure at alkaline pH has to be investigated.

In contrast, the naphthol substrates were shown to be useful for routine non-specific alkaline phosphate histochemistry only in simultaneous azo-dye procedures (Lojda et al. 1979), although the phosphate liberated from these substrates may serve in a metal salt method, using Gomori's Ca–Co principle to visualize the enzyme activity; furthermore, naphthol AS is nearly water-insoluble and fluoresces (Burstone 1962; Gossrau 1983; Raap 1988). In practice, however, neither the metal salt principle nor the fluorescence methods have proved to be suitable procedures for light microscopic non-specific alkaline phosphatase histochemistry (Lojda et al. 1979; Halbhuber et al. 1988; Raap 1988).

As a substrate in chromogenic procedures, indoxyl phosphate is as versatile as the menadiol compound, while the azoindoxyl version should be even better than the azo method in combination with menadiol phosphate. However, diazonium salts are less satisfactory than tetrazolium salts as visualization agents for menadiol phosphate or indoxyl phosphate. In addition, the synthesis of menadiol is simple and it uses low-priced precursors. When it becomes commercially available, menadione should therefore be much cheaper than indoxyl phosphate. Thus, the metal salt procedure, as well as the tetrazolium procedures with the menadiol phosphate have advantages over those with the indoxyl compound. Several companies have expressed an interest in this new substrate, which should be commercially available in the near future.

In combination with Ce or Ca ions, indoxyl phosphate is inferior to menadiol diphosphate, producing a final stain that is less well localized and produced in smaller amounts (Gossrau, unpublished observations), possibly because indoxyl molecules interfere in the trapping process. In the tetrazolium procedure with indoxyl phosphate, the formation of indigo can interfere with the production of formazan, resulting in two final reaction products. This will happen especially at sites with high activities of non-specific alkaline phosphatase, e.g. in the small intestinal and renal brush borders. It cannot be reliably prevented by the addition of exogenous high affinity electron acceptors such as phenazine methosulphate with and without polyvinyl alcohol (Van Noorden and Jonges 1987); this is easily shown by extraction experiments with, e.g. heated dimethylsulphoxide or dimethylformamide. The solvents elute formazan from the tissue sections, leaving the non-extractable indigo at the site of origin.

When using the tetrazolium technique with indoxyl phosphate, the generation of two types of final stain is a disadvantage in cytophotometric measurements of non-specific alkaline phosphatase in tissue sections; the existence of two absorption maxima may complicate or even prevent reliable measurements. This is in contrast to the tetrazolium method with menadiol phosphate, where only formazan is generated, and this can easily be transformed into units (Van Noorden and Gossrau 1990), which permit a direct comparison of the cytophotometric data and biochemical values, using the same or different alkaline phosphatase substrates. A detailed study of all the potential applications of menadiol phosphate for quantitative histochemistry is now in progress in our laboratories.

A further advantage of menadiol phosphate over the indoxyl substrate is that two phosphate groups (instead of one in the case of indoxyl phosphate) are available for trapping metal ions, making the metal salt procedure with the menadiol phosphate especially sensitive. This high sensitivity is seen in our results with the Ce and Gomori's Ca–Co-procedure, which indicate the potential of menadiol phosphate for non-specific alkaline phosphatase ultracytochemistry. Further studies are required on the potential applications of this system, and on the applications of menadiol diphosphate in the formazan procedure for the analysis of molecular forms of non-specific alkaline phosphatase (Moss 1982), following their separation by different techniques, e.g. isoelectric focusing.

The biochemical and histochemical advantages of menadiol phosphate in the catalytic (activity) histochemistry of non-specific alkaline phosphatase, also apply to the qualitative immunohistochemistry of this enzyme when it serves as the enzyme label. We can therefore assume that this compound will become the substrate of choice for immunohistochemical purposes, either in the tetrazolium or in the Ce-diaminobenzidine-H_2O_2 procedure.

Finally, menadiol represents a non-specific moiety to which other specific residues may be linked such as acetate, glycosides and sulphate. Since menadiol substrates can also be used over a wide pH range, they open a new field of methodological and applied hydrolase histochemistry. For non-specific acid phosphatase or non-specific esterases, the tetrazolium method with menadiol phosphate or menadiol acetate has already been shown to be valid for the analysis of these enzymes in situ (Dikow and Gossrau 1990). Whereas menadiol sulphate gave less satisfactory data, our preliminary results for the detection of extra- and intracellular glycosidases were rather promising (Dikow and Gossrau, unpublished data). If the water-insoluble (or sparingly water-soluble), fluorescent menadione (Undenfriend 1963), which is formed from menadiol, can also be exploited, then fluorescence histochemistry of phosphatases, non-specific esterases and glycosidases should also be possible using menadiol substrates.

In conclusion, menadiol phosphate represents a useful new substrate for the detection of non-specific endogenous or exogeneous non-specific alkaline phosphatase

in light microscopic activity histochemistry or immunohistochemistry. Compared with existing chromogenic compounds, it has more advantages than disadvantages. Furthermore, menadiol compounds might become a real alternative to the naphthol and indoxyl substrates in chromogenic hydrolase histochemistry in general, because they can be linked to many residues other than phosphate.

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