

MODIFICATION OF LEAD METHOD FOR DEMONSTRATION
OF ALKALINE PHOSPHATASE ACTIVITY WITH LIGHT-
AND ELECTRON-MICROSCOPY.

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There have been some methods for the demonstration of alkaline phosphatase activity with a high alkaline pH optimum, using metal salts as a capture reagent¹⁾⁻⁷⁾. In Takamatsu and Gomoris' methods the precipitates of calcium phosphate were transformed into silver or cobalt phosphate for visualization with light microscope. However, one step reaction is necessary to obtain exact cytochemical results for electron microscopy. Lead ions were first used by Mölbert et al.³⁾ as the capture reagent for this purpose. However, the reaction was carried out below pH 8 and diffusion seemed to occur. Deimling, her co-worker, described a modified lead method conducting the reaction at pH 8.2-9.2 with light microscopy⁴⁾. The present author also tried the lead method and found that the concentration of potassium sodium tartrate, the chelating reagent added to make lead ions stable in alkaline solution, is critical and they used too much tartrate.

1. *Determination of concentration of tartrate in the incubating medium.* For the incubation procedure, the substrate mixtures were made as follows; 2 ml of buffer solution (2% sodium barbiturate, 0.2 M tris-maleate or AMPD), 1 ml of 2.5% sodium β -glycerophosphate, 1 to 5 ml of 2% potassium sodium tartrate or 0.5-1.5 ml of 20% tartrate. The solution was adjusted to 8.7 ml with distilled water and finally 1.3 ml of 1% lead nitrate was added drop by drop with continuous stirring, and adjusted to pH 9.2 with NaOH solution. If the solution adjusted to pH 9.2 was still turbid, it was filtered.

Small pieces of the rat kidney and intestine were fixed with 10% cold formal-calcium (pH 7.2, 4°C) which contained 7.5% sucrose for over night and washed with cold tris-maleate buffer (pH 7.4, 0.2 M) containing 7.5% sucrose for several hours. The 10 μ frozen sections for light microscopic study were incubated for

5–10 minutes. After brief washing with distilled water, the sections were immersed in diluted yellow ammonium sulfide for a few minutes, washed again and mounted with glycerin-jelly.

The incubating media and results are shown in table 1.

Table 1.

	1	2	3	4	5	6	7	8	9
2% Sod. barb.	2	2	2	2	2	2	2	2	2
2.5% glycerophosphate	1	1	1	1	1	1	1	1	1
2% K. Na. tartrate	2	2.5	3	3.5	4	5	0.5	1	1.5
(mg/10 ml of tartrate)	(40 mg)	(50 mg)	(60 mg)	(70 mg)	(80 mg)	(100 mg)	(100 mg)	(200 mg)	(300 mg)
H ₂ O	3.7	3.2	2.7	2.2	1.7	0.7	5.2	4.7	4.2
1% Pb (NO ₃) ₂	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
out-look at pH 9.2	sl. t.	sl. t.	v. sl. t.	cl.	cl.	cl.	cl.	cl.	cl.
reaction in kidney and intestine	++	++	++	++	++	++~+	++~+	±	±
out-look of solution after 24 hours.	sl. t.	sl. t.	sl. t.	cl.	cl.	cl.	cl.	cl.	cl.

t: turbid, sl.t.: slightly turbid, v.sl.t.: very slightly turbid, cl: clear.

When the substrate mixture contained less than 80–100 mg/10 ml of tartrate, they gave marked reactions in both the kidney and intestine. In the cortex of kidney, the reactions were exactly limited to the brush border of renal tubules and arterioles. Blood capillaries and glomeruli were negative. The prolonged incubation would result in further reactions in some cellular components. However, the present purpose is to determine the concentration of tartrate which gives the best results. When the concentration of tartrate exceeded 100 mg/10 ml, the reaction markedly decreased and only spotty reaction products were observed in the brush border. In the intestine, the highest grades of activity occurred at the epithelial surface, microvilli, and slight reactions also occurred at the Golgi region. However, it is not clear whether these slight precipitates showed true enzymatic activity in Golgi apparatus or not, because many fatty droplets absorbed from the cell surface and concentrated at the Golgi area often conjugate with lead ions or lead phosphate particularly at alkaline range. Goblet cells were completely negative. Diffuse staining of the epithelial cells in light brown color was observed by longer incubation than 5 minutes at room temperature.

Anyway, tartrate less than 100 mg/10 ml in the incubating medium gave a marked reaction both in the kidney and intestine. Tris-maleate buffer (although it does not have efficient buffering action at over pH 9) used in place of barbiturate

also gave similar results. The concentration of tartrate should not exceed 100 mg/10 ml. When AMPD-HCl, an effective buffering solution at pH 9.0–9.2, was used, 50 mg/10 ml of tartrate was enough to keep the solution clear. These results indicated that Mölbert and Deimling^{3), 4)} used apparently excessive amounts of tartrate. The comparison of the concentration of tartrate in each incubating medium is shown in table 2.

Table 2. Comparison of concentration of tartrate.

	Mölbart et al ('60)	Deimling ('64) (light microsc.)	Mizutani ('66)
Substrate	p. n. ph. ph.	p. n. ph. ph.	G.P.
Buffer	Tris	Tris	Barb, AMPD-HCl or Tris-mal.
K. Na. Tart.	<u>1.9 g/50 ml</u>	<u>3 g/50 ml</u>	<u>0.4 g/50 ml</u> ≥
Pb (NO ₃) ₂	Ca. 4 mM	10 mM	Ca. 4 mM
pH	7.6–7.8	8.2–9.2	9.2

The fact that 60 mg/10 ml of tartrate is necessary to keep 13 mg/10 ml of lead nitrate stable indicates that one molecule of lead ion corresponds to four or more molecules of tartrate. When citrate was used in place of tartrate, one molecule of citrate corresponds to one of lead ion⁶⁾. In addition, in the case of tartrate, the amount necessary to keep the solution clear also depends on the pH of the medium. The lower the pH the more tartrate is necessary. The explanation of the difference between citrate and tartrate demands further studies.

Recently Hugon and Borgers⁷⁾ suggested that neither citrate nor tartrate were necessary to make lead ions stable because maleate in the buffering solution was also effective for this purpose. However, as they described, their incubating medium became cloudy in 15 minutes, particularly from its surface probably due to formation of lead carbonate. Therefore, tartrate or citrate should be added to the incubating medium for longer incubation.

2. *Effects of Mg⁺⁺.* When 10 mM in final concentration of magnesium ion was added, more tartrate was necessary. However, 80 mg/10 ml was enough for the present purpose and such an incubating medium gave stronger reactions. The incubation of the sections for 10 minutes resulted in slight nuclear staining at the sites of high enzymatic activity, probably due to the diffusion of rapidly formed phosphate or lead phosphate from the active sites.

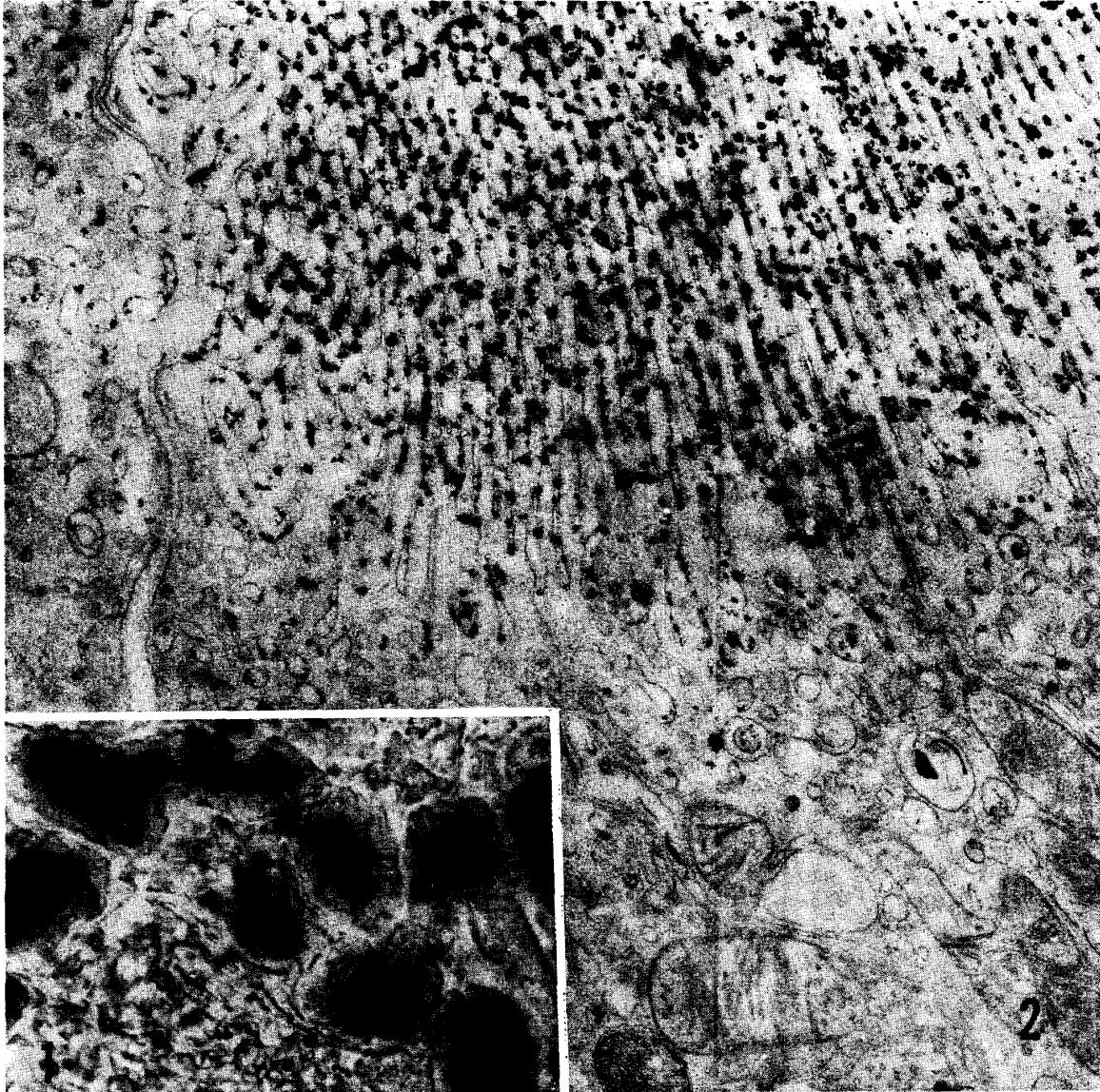


Fig. 1. Alkaline phosphatase activity at the rat Kidney. Formol-Ca-fixed tissues incubated in the substrate mixture which contained 80 mg/10 ml tartrate for 10 min. Marked reaction occurred mainly in the brush border of renal tubules.

Fig. 2. Forty micron frozen section of glutaraldehyde fixed tissues was incubated for 5 min. The reaction products are seen in the brush border and continuous small vesicles.

Thus the following incubating medium is recommended:

0.2 M AMPD*-HCl buffer (pH 9.0-9.2)	2 ml	
2.5% Na. β -glycerophosphate	1 ml	
2% K. Na. tartrate	2.5 ml	
distilled water	2.7 ml	
(or 0.1 M MgSO ₄ 1 ml and H ₂ O 1.7 ml)		
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1% lead nitrate	1.3 ml	drop by drop with continuous stirring

Adjust pH to 9.2 with NaOH. If barbiturate is used, amounts of tartrate is increased to 3.5 ml and distilled water is decreased to 1.7 ml.

3. *Electron microscopy.* For electron microscopic studies, small tissue slices were fixed promptly after removal with 3-4% glutaraldehyde buffered to 7.4 with cacodylate⁸⁾ for 1 hour, and washed with buffer solution. Forty micron frozen sections were incubated in the above mentioned incubating medium for 5-10 minutes at room temperature, post-fixed with 1% OsO₄ for 1 hour, dehydrated with graded alcohol and embedded in Epon. Fig. 2 shows that fine reaction products appeared at the membrane of brush border of the rat kidney. Further details of fine structural localization of alkaline phosphatase activity will be described later.

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* 2-amino-2-methyl-1,3-propanediol