Studies on Histochemical Methods for Hard Tissues

I) Sectioning of Hard Tissues With or Without Decalcification

Akitoshi Sugimoto, Toshio Yagi, Kinji Okamoto, Fusazo Taguchi, Haruo Hiraoka, and Kazuo Sakamoto

Dept. of Oral Pathol., Osaka Univ. Dent. Sch., Osaka, Japan
(Director: Prof. Dr. T. Terasaki)

For histochemical studies on hard tissues, bones and teeth, ground sections and decalcified sections have mainly been used. But, the application of histochemical staining reactions on these sections is much restricted. In other words, preparation of the ground sections sufficient to microscopical observation is associated with considerable technical difficulties, and most of various decalcification methods using acids are usually quite unsatisfactory, if histochemical reactions for enzymes, nucleic acids, and polysaccharides are proposed.

The present research is designed, at first, to test various decalcification methods for enzymes, nucleic acids, and polysaccharides. In addition, a new method for sectioning of undecalcified hard tissues and the effects of the histochemical staining reactions in these sections are studied.

Materials and Methods

A) Sectioning With Decalcification

A proximal end of the tibia and the kidney of adult Wistar rats were used in this experiment. Slices of these tissues were fixed in the following cold three fixatives overnight: 10% neutral formalin, 80% ethanol, and 100% acetone, and washed in water for 1 hour, then, decalcified at 4 °C in the following decalcifying fluids for 3 days, changing daily;

a) 5% EDTA-2Na salt (ethylenediamine tetraacetic acid-disodium salt) aqueous solution adjusted with NaOH at pH 7.45,6,7.
b) 5% EDTA-2Na salt in 10% neutral formalin solution adjusted with NaOH at pH 7.4.
c) 5% EDTA-2Na salt in M/15 phosphate buffer adjusted with NaOH at pH 7.4.
d) 5% EDTA-4Na salt aqueous solution adjusted with citric acid at pH 7.48.
e) Greep's fluid9,10,11: mixture of equal parts of 2% formic acid and 20% sodium citrate, pH 4.9.

On completion of decalcification, the tissues were washed for 30 minutes in 2-3 changes of distilled water, and incubated at 37 °C in 1% sodium diethylbarbiturate solution for 24 hours to reactivate the enzymes12. After washing for 2-4 hours in 3-5 changes of distilled water, dehydration through ascending alcohols, and clear in xylene, the materials were embedded in paraffin (m. p.
52 °C). The tissue sections were cut at 6μ, and stained for alkaline phosphatase (ALP-ase) and acid phosphatase (ACP-ase). Hematoxylin and eosin (HE) stain, pyronin-methylgreen (PMG) stain and periodic acid-Schiff (PAS) reaction were carried out, also.

B) Sectioning Without Decalcification

The tissues studied were a proximal end of the tibia and the incisor of adult Wistar rats. They were fixed in cold 80% ethanol and 100% acetone for 24-48 hours. After dehydration as usual, the slices were treated as follows:
1) Transfer to 50/50 mixture of absolute ethanol and n-butylmetacrylate for 2 hours.
2) Transfer to butylmetacrylate monomer for 2 hours.
3) Transfer to 80/20 mixture of butyl- and methyl-metacrylate containing 10 mg/ml catalyst (2,4-dichlorobenzoyl peroxide) for 2 hours.
4) Transfer to gelatinous capsules where final polymerization is carried out in 40 °C oven for 17-20 hours.

The methacrylate embedded tissues were sectioned with Jung microtome (K type) at 6-8 μ thickness, using No. 3 or 4 steel knife. Plastics of the sections were removed with xylene, acetone or toluene, and the sections were hydrated. The histological and histochemical staining reactions were applied as follows; HE stain, PMG stain, PAS reaction, Calcium reaction.

Results and Discussion

A) Sectioning With Decalcification

Results are summarized in Table 1.

<table>
<thead>
<tr>
<th>Fixative</th>
<th>Decalcifying Fluid</th>
<th>ALP-ase</th>
<th>ACP-ase</th>
<th>PMG</th>
<th>PAS</th>
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</thead>
<tbody>
<tr>
<td>10% neutral formalin</td>
<td>5% EDTA-2Na aq. soln.</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>80% ethanol</td>
<td>5% EDTA-2Na aq. soln.</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>100% acetone</td>
<td>5% EDTA-2Na aq. soln.</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>80% ethanol</td>
<td>5% EDTA-2Na in formalin phosphate buffer</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>80% ethanol</td>
<td>5% EDTA-4Na aq. soln.</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>80% ethanol</td>
<td>Greep's fluid</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>100% acetone</td>
<td>5% EDTA-2Na in formalin phosphate buffer</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>100% acetone</td>
<td>5% EDTA-4Na aq. soln.</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>100% acetone</td>
<td>Greep's fluid</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Notes: -- : negative, ± : traces or irregular, + : weak, ++ : moderate, ### : intense reaction.
ALP-ase and ACP-ase reactions: Of three fixatives tested, a concernible difference was not shown. Comparing the effects of 5 decalcifying fluids on histochemical staining reaction in the ethanol and acetone fixed materials, the enzyme activities are best preserved in case of 5% EDTA-2Na salt in neutral formalin (Fig. 1), while the activities were slightly reduced in 5% EDTA-2Na salt aqueous solution and Greep's fluid (Fig. 2), and considerably damaged after treatment in two others.

PMG stain: In this staining reaction, sections treated with 5% EDTA-2Na salt in neutral formalin solution showed the most intense reaction, although the reaction was more or less reduced in some sections, because some of nucleic acid may be extracted from the tissue during the decalcification.

PAS reaction: The reactions were similar in all cases of 3 fixatives and 5 decalcifying fluids.

Ethanol and acetone have been long used as the best fixatives for the enzymatic histochemistry, but not always considered as good fixatives for protein. Contrarily, formalin is known as one of the best fixatives for protein, while it inactivates enzymes severely, and therefore only cold neutral formalin is recognized to be useful for enzyme, provided the fixation time is short.

Greep's fluid,9,10,11) Lorch's fluid,11) and EDTA12) have been known as the excellent decalcifying fluids for ALP-ase. For example, Schajowicz et al12) reported that histochemical reactions, including ALP-ase, were very little altered by Greep's and Lorch's fluids, provided they were used for a few days.

![Fig. 1 Proximal end of rat tibia. Fixed in ethanol and decalcified with 5% EDTA-2Na salt in 10% neutral formalin, ALP-ase reaction. ×200. Hypertrophied cartilage cells and osteoblasts show a positive intense reaction.](image1)

![Fig. 2 Proximal end of rat tibia, fixed in ethanol and decalcified with Greep's fluid. ALP-ase reaction. ×200. Distribution of the reaction is similar to that in Fig. 1, while the reaction of hypertrophied cartilage cells is less intense and diffused.](image2)
Trott\textsuperscript{(23)} reported that EDTA was the most optimal decalcifying fluid for detection of a small amount of glycogen, and it was achieved by Balogh\textsuperscript{(24)} that decalcification of bone and teeth with EDTA preserved sufficient oxidative enzyme activity to permit histochemical demonstration. Recently, Cook et al\textsuperscript{(25)} have been described according to their analytical data that EDTA does not destroy the molecular structure of collagen and ground substance in bone, and fixation in formalin exerts a clear inhibitory effect upon the hydrolysis of the bone protein during decalcification. Therefore, it is assumed that EDTA is the best decalcifying fluid for enzymes and ground substances of hard tissues.

The results of the present study are coincided with the opinions of these authors, that is to say, deleterious effects of cold formalin fixation are as slight as those of ethanol and acetone, as seen in Table 1. Moreover, 5 % EDTA-2Na salt dissolved in 10 % neutral formalin solution show some suitable decalcifying effects in both enzymatic and structural histochemistry.

B) Sectioning Without Decalcification

To obtain the more sufficient sections to histochemical studies, sectioning methods of undecalcified hard tissues are investigated in the second series of this experiment. It is the first problem what ratio of butyl- and methyl-metacrylate in embedding medium is the most suitable for sectioning. In this regard, various ratios of these materials (90/10, 80/20, 70/30, 60/40, 50/50, and 40/60) were preliminarily tested. In consequence, as it was found out that the ratio 80/20 was the most satisfactory, and 70/30 was somewhat harder and brittler, and 90/10 a little tender, the ratio 80/20 was generally used in this study.

The second problem is how to remove away the hardened plastics medium from the sections before staining. Of course, as many dissolving agents of plastics have been known, the authors tried to test the effects of xylene, acetone, toluene and mixture of equal parts of acetone and toluene for 0.5, 1, and 2 hours, and the results obtained are shown in Table 2.

As can be deduced from the data, acetone and toluene exhibited a clear-cut result, and xylene was poor. As acetone is one of the best fixatives, acetone was applied as a removing agent of plastics throughout the experiment. In the sections obtained in this way, considerable good results of the various histochemical staining reactions were demonstrated (Figs. 3 and 4). Besides,

<table>
<thead>
<tr>
<th>Table 2. Effects of Three Agents on Removing Plastics from Sections.</th>
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<tbody>
<tr>
<td><strong>Agent</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>xylene</td>
</tr>
<tr>
<td>acetone</td>
</tr>
<tr>
<td>toluene</td>
</tr>
<tr>
<td>acetone-toluene</td>
</tr>
</tbody>
</table>

Notes; –: bad, +: fairly good, ++: good result.
an investigation on other embedding plastics more suitable for sectioning of the undecalcified hard tissues is under way.

**Summary**

Effects of 3 fixatives and 5 decalcifying fluids on the histochemistry of bone are comparatively investigated, using the tibia and the kidney of Wistar rats.

Of various decalcifying fluids, as far as tested on ALP-ase, ACP-ase, PMG stain, PAS reaction, 5 % EDTA-2Na salt in 10 % neutral formalin adjusted with NaOH at pH 7.4 gives the most satisfactory results. Five per cent EDTA aqueous solution and Greep’s fluid is the next, showing slightly reduced reactions, and two others (5 % EDTA-2Na salt in M/15 phosphate buffer and 5 % EDTA-4Na salt aqueous solution) are less useful, due to their deleterious effects on the reactions.

A new sectioning methods of the undecalcified hard tissues is successfully investigated. The tibia and the incisor of Wistar rats are fixed and dehydrated, followed by embedding in metacrylate (80/20 mixture of butyl- and methyl-metacrylate). The tissue sections can be cut at 8 μ without destruction of microscopical structures, and the plastics is removed with acetone for 2 hours. These sections can well reserve the stainabilities of some histochemical staining reactions.

**References**

1) Lillie, R. D. (1944) : Am. J. Path. 20 ; 291. 2) Dotti, L. B., Paparro, C. P. and Clarke,
Discussion

Dr. H. Takamatsu

I should like to add the results of our previous studies. I and Dr. Akahoshi have previously reported the method for decalcification which uses disodium salt and tetrasodium salt of EDTA. The mixture of equal volume of them shows about neutral pH. This mixture can be used for decalcification.

Dr. Fujita

How did you remove metacrylate from tissue sections, which have been previously embedded in methacrylate, to perform light microscopic stainings?

Dr. Yagi

Acetone, Xylene and toluene are said to give good results in removing embedding materials such as methacrylate. I have tested the effectiveness of them and found that acetone is most effective for 4~6 μ sections. Xylene and toluene are much less effective.

Histochemical Demonstration of Various Dehydrogenases in Developing Teeth.

Tsuneo Mizushima

Department of Oral Surgery (Prof. K. Kawakatsu) Osaka University Dental School, Joan-cho, Kita-ku, Osaka

Introduction

Histochemical studies of developing teeth have been made in detail especially for the distribution of hydrolytic enzymes. It is reported that alkaline phosphatase proved to be related with a matrix formation, mineralization and