Studies on Histochemical Methodes for Hard Tissues (II) Fixation and Decalcification of Hard Tissues

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For the histochemistry of the hard tissues, selection of sectioning method is one of the most difficult problems. Recently, a study on sectioning of the undecalcified hard tissues has been developed, and was also reported by us (Sugimoto et al, 1962). Sectioning without decalcification, however, is not necessarily the best way for histochemistry, because of problems on stainability of the matrices of hard tissues, fixation of the soft tissues around them, and on diffusibility of the reagent solutions. On the other hand, in the previous study, we found out that some enzymatic activities such as alkaline phosphatase were better preserved by adding formalin into the decalcifying solution. So, in the present study, decalcification by EDTA solution containing some fixatives (formalin etc.) was examined. Histochemistry of the incisor and the femur of the normal rat will be reported here.

Table. 1 Decalcifying Solution

Solution	EDTA-2Na (%)	Formol (%)	Additional (%)	Duration (Days)		Decalcification	Preservation of the Structure
				at room temp.	in ice box		
A	5	10	—	7-10	10-14	Somewhat delayed	Good
B	5	10	Cetylpyridinium chl 0.5	7-10	10-14	Delayed	Good
C	5	10	Chloral hydrate 1.0	7-10	10-14	Similar to A	Good
D	5	10	Barbital sodium 1.0	7-10	10-14	Similar to A	Good
E	5	10	Picric acid 1.2	7-10	10-14	Fast	Destroyed
F	2Na 5) Equal 4Na 5) Parts	10	—	7-10	10-14	Similar to A	Good
G	10	10	—	5- 7	5-7	Good	Good
H	10	5	—	5- 7	5-7	Good	Fairly good
I	10	10	Cetylpyridinium chloride 0.5	5- 7	5-7	Somewhat delayed	Good
J	10	—	1.Unfixed 2.Fixed in 80% alcoh 3.Fixed in 10% formol	5- 7	5-7	Very fast Fast Control	Destroyed Shrunken Control

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Materials and Methods

Normal young adult rats, weighing about 120g., were used. The upper and lower jaws were sagittally cut through the median line. The incisors, from which the surrounding soft tissues were removed as possible, and the distal 1/3 parts of the femurs were treated in the various decalcifying solutions shown in Table 1 at room temperature or in ice box (ca. 4°C) for 5 to 14 days. The pH of the decalcifying solutions was adjusted at about 7.0 with N-NaOH. Control materials were (1) unfixed, (2) fixed in 80% alcohol or (3) fixed in 10% neutral formalin prior to decalcification by 10% EDTA (Solution J), while all the other materials were decalcified without previous fixation.

After decalcification the materials were dehydrated in alcohol, cleared in xylol and embedded in paraffin as usual. Paraffin sections were cut at 6 to 8 μ . The histochemical methods used were listed in Table 2. The enzymatic reactions were applied to only the materials treated in an ice box.

Table. 2 Histochemical Methods

For Hydrolytic Enzymes	
(1) Alkaline Phosphatase (pH 9.2)	<ol style="list-style-type: none"> 1. Gomori's Method : α-Naphthyl Phosphate, Fast Blue B. 2. Burstone's Method : Naphthol AS-MX Phosphate, Fast Blue RR.
(2) Acid Phosphatase (pH 5.0)	<ol style="list-style-type: none"> 1. Gomori's Method : α-Naphthyl Phosphate, Fast Garnet GBC. 2. Burstone's Method : Naphthol AS-MX Phosphate, Fast Blue RR.
(3) Non-specific Esterase	<ol style="list-style-type: none"> 1. Gomori's Method : α-Naphthyl Acetate, Fast Blue B. 2. Burstone's Method : Naphthol AS Acetate, Fast Blue RR.
For Polysaccharides	
(4) Periodic Acid-Schiff (PAS) Reaction (Hotchkiss, 1948)	
(5) Alcian Blue Method for Acid Mucopolysaccharides (Steedman, 1950)	
(6) Methylene Blue Extinction (MBE) at pH 4.5 and 2.5 (Dempsey & Singer, 1949)	
(7) Colloidal Iron Reaction (Müller, 1955; Modification of Hale's Method)	
For Proteins	
(8) DDD Reaction for Protein-bound SH and SS Groups (Barnett & Seligman, 1952)	
(9) Mercury-Bromphenol Blue (HgBPPB) Method for Proteins (Bonhang, 1955)	
For Histology	
(10) H-E Stain	
(11) Mallory-Heidenhain Stain (Cason, 1950)	

Observations and Discussion

I. Effects of the Decalcifying Solutions Used.

As is well known, decalcification with strong acids destroys the soft tissues and cellular elements, and should not be used for histochemistry. As the ability of organic acids to destroy the tissues is relatively weak, Greep et al (1947, 1948) and Lorch (1946) applied them for the demonstration of alkaline phosphatase in hard tissues. In these methods, however, the morphological structure and stainability of the tissues are still unsatisfactory.

Chelating agent, disodium or tetrasodium ethylenediaminetetraacetate (EDTA-2Na or-4Na), on the other hand, has been used successfully at neutral pH for the demineralization of hard tissues by Nikiforuk and Sreebny (1951, 1953). Even by this agent, shrinkage or destruction of the soft tissues surrounded by hard tissues such as in case of the incisor of rat can not be avoided during fixation, when they are fixed in alcohol or formalin prior to decalcification. To make up for this shortcoming, various mixtures of formalin and EDTA were examined in the present study and the results obtained are summarized as follows:

1) Preservation of the Structure of Soft Tissues (H-E stain and Mallory-Heidenhain stain). In the control group, decalcifying effect is most remarkable for the unfixed materials, and relatively high for the alcohol-fixed, but somewhat low for the formalin-fixed. Preservation of the structure and stainability, however, is better in the reverse order of description.

In the experimental groups, at first with regard to the concentration of EDTA, 10% solution is more suitable for decalcification at low temperature than 5% solution. When formalin is mixed in the EDTA solution, the decalcifying effect is somewhat decreased. Preservation of the structures is better in 10% formalin than in 5% formalin. Cetylpyridinium chloride (CPC) (0.5%) seems to give better preservation of soft tissues (Fig. 2), while decalcifying effect is considerably reduced. Picric acid (1%) may rather promote decalcification, but has no use for fixation of the tissues.

2) Hydrolytic Enzyme Reactions. In 5% EDTA-formol solutions, the phosphatase and esterase reactions are considerably decreased, because a prolonged decalcification is needed. When 10% EDTA-formol solution is used, the duration of decalcification is shortened and the enzyme reactions are better preserved. When chloral hydrate or barbital sodium are added to 5% EDTA solution, no preserving effect on the enzyme activities is observed. Although in picric acid-saturated solution no remarkable decrease of the reactions is seen, it can not be recommended, because of its strong destructive action on the soft tissues. As CPC has little destructive effect on both the reactions and structures of the tissues, it will be recommended.

3) Preservation of Polysaccharides (PAS reaction, Alcian blue stain, colloidal iron method, and MBE). All the staining reactions for polysaccharides are not disturbed by any decalcifying solutions composed of formalin and EDTA. Curran and Collins (1957) showed that EDTA behaved like Greep's solution and PAS-positive substances with exception of glycogen were well preserved. But, in the present study, even glycogen is well demonstrated by PAS reaction after decalcification in formol-EDTA solution, especially when CPC is added. CPC was recommended by Williams & Jackson (1956) to fix mucopolysaccharides, and its excellent effect was confirmed in our previous report (Sugimoto et al, 1963).

4) Stainability of Proteins (DDD reaction and HgBPB reaction). Little alterations of DDD reaction are seen after formol-EDTA treatment. With HgBPB reaction a greenish tint is prominent after treatment by CPC, while

bluish tint is marked in the alcohol fixed controls.

From the point of view of histochemistry, it may be concluded that the formol-EDTA solution is useful for decalcification of hard tissues. In these decalcified materials, most of the histochemical methods for polysaccharides and proteins can be applied without difficulties. Alkaline and acid phosphatases, especially the latter, are demonstrable in good localization if decalcification is finished in a short duration. Among the decalcifying solutions tested, Solution I (0.5% CPC-containing 10% EDTA-10% formol) seems to be the best, and Solution G and H are useful, but other solutions (5% EDTA-formol) are less useful because of mildness of their decalcifying effects.

II. Histology and Developmental Stages of Normal Rat Incisor (H-E and Mallory-Heidenhain Stains).

The incisor of rats, as those of the other rodents, continues to develop throughout the life, and therefore its histological structure is complicated and quite different from the other teeth. All stages of morpho- and histo-differentiation of the formative cells, matrix formation and apposition of minerals are seen at the same time in one incisor. For the convenient of description, at first, the incisor is divided into seven stages (a-g) according to its structural characteristics, as shown diagrammatically in Fig. 1.

Fig. 1 A Diagram of the Developmental Stages of the Rat Incisor

a	b	c	d	e	f	g	STAGES
Enamel organ. Undifferentiated dental epithelium.	Differentiation of lower ameloblast and odonto- blast.	Formation of dentin matrix.	Formation of enamel matrix. Calcification of dentin.	Papillary layer. Disappearance of Tomes' process.	Transitional enamel. Beginning of pigment- ation in ameloblast.	Reduced ameloblast. Dissolution of enamel. Pigmentation.	REMARKS

In the sections decalcified in formol-EDTA solution, some detailed structures of the incisor are shown even by the H-E and Mallory-Heidenhain stains. For examples, in the basal portion of the ameloblastic cytoplasm, some granules or droplets are seen which stain pink with eosin and brilliant red with Mallory stain. They appear just after differentiation of the ameloblast and are prominent in Stage d, but in Stage e these coarse granules or droplets become more fine, and they are less remarkable in Stage f-g, as the

(Figs. 2-11: Rat incisor. Figs. 12-19: Rat femur. Abbreviations: See Fig. 1.)
 Fig. 2 Solution I. H-E stain. Stage d. 200 $\times$. Fig. 3 Solution G. ALP-ase reaction (Burstone's method). Stage d. 200 $\times$. Fig. 4 The same as fig. 3. Stage e. 400 $\times$. Fig. 5 Solution i. PAS reaction. Stage d. 200 $\times$. Fig. 6 The same as Fig. 5 Stage f. 200 $\times$. Fig. 7 Solution I. Alcian blue stain, counter-stained with neutral red. Stage 4. 400 $\times$. Fig. 8 The same as Fig. 7. Fig. 9 Solution I. Colloidal iron reaction. Stage e. 200 $\times$. Fig. 10 The same as Fig. 9 Stage g. 400 $\times$. Fig. 11 Solution I H-E stain. Stage e. 400 $\times$. Fig. 12 Solution I. HgBPB reaction. Stage f. 200 $\times$.

pigments increase. Therefore these basal granules may have some relationship to the enamel formation or at least to the activity of the ameloblast, as reported by Nuckolls et al. (1947).

Corresponding to the site of terminal bar in the ameloblast, a bluish-purple membrane is often seen between the cytoplasm and Tomes' process in Stage d and this terminal membrane seems to become a limiting one between the cytoplasm and the fibrillar layer of the enamel in Stage e and thereafter (This membrane is seen in Figs. 5 and 6).

In Stage e and f, there appear some fine granules in the space around the capillaries in the papillary layer (Fig. 11), but the nature of these granules is still unknown.

III. Histochemistry of Normal Rat Incisor.

(1) Alkaline Phosphatase (ALP-ase)—The most remarkable reaction is observed in the stratum intermedium and in the pulp, especially in its peripheral zone adjacent to the odontoblast, throughout all the stages of development (Fig. 3). The odontoblasts exhibit moderate reaction in stage b and thereafter. The ameloblasts are generally negative with exception of the later stages (Stage f and g). Some moderate or weak reaction is seen in the papillary layer also in stage d-g (Fig. 4). The distribution of the enzyme is similar with either Gomori's or Burstone's method and generally agrees with those reported by Burstone (1960). Since Robison (1923, 1932) pointed out the close relationship between ALP-ase and calcification, numerous discussions and studies have been published. And now many authors (Harris, 1950; Burstone, 1960; Nuki & Bonting, 1961) seem to agree that the localization of the enzyme is not identical with the calcifying site in any developing hard tissues. A fact that the most intense reaction is localized in the tissues between the mineralizing area and the supplying vascular system, seems to support the Newman & Newman's (1958) hypothesis on this enzyme.

(2) Acid Phosphatase—In the rat incisor, no reaction is observed with either Gomori's or Burstone's method. The only element which exhibits a positive reaction is the alveolar bone (the osteoclasts and the adjacent matrix). The dentine matrix is negative, in spite of the positive reaction by Burstone (1959-a).

(3) Non-Specific Esterase—There is no remarkable reaction of this enzyme in the incisor under the experimental conditions applied. And only faint reaction is seen in the distal cytoplasm of ameloblast, while Burstone (1957, 1960) reported a positive reaction in the dentin adjacent to the pre-dentin.

(4) Polysaccharides (PAS, Alcian blue, MBE, colloidal iron reactions) Histochemical studies on polysaccharides and glycoproteins in the rat incisor have been reported by many authors: Engel (1948), Wislocki et al (1948), Wislocki & Sognnaes (1949, 1950), Bevelander & Johnson (1955), Fullmer & Alpher (1958). According to these authors, acid mucopolysaccharides and glycoproteins were demonstrated in the matrices of enamel and dentin and they decreased as the calcification proceeded.

In the present study, as mentioned above, polysaccharides are well preserved by Solution B and I. Glycogen is demonstrated by the PAS reaction and salivary digestion in the ameloblast, odontoblast, pulp and others. Glycogen granules are seen abundant in Stage a, and somewhat decrease in Stage b, but markedly increase again in Stage c and thereafter in both the ameloblast and odontoblast (Figs. 5 and 6).

With the Alcian blue stain, the predentin, dentinal canal, and the ground substance in the pulp are stained blue (Fig. 7 and 8). These structures stain metachromatically by MBE at both pH 4.5 and 2.5, and therefore they may contain much acid polysaccharides. With the Alcian blue and colloidal iron stains, the dentinal canals look like compacted lines in stage d, but in Stage e they become double lines running parallel along the canal, or small ring-shape in a cross section (Fig. 8). Therefore, as Wislocki et al (1948) has already stated, the dentinal canal seems to have something like a sheath surrounding the Tomes' fiber (odontoblastic process).

The matrix of enamel is quite colorless with the Alcian blue stain and MBE general, with exception of occasional blue stain of the transitional enamel in Stage f with MBE.

The matrix of dentin unstain with Alcian blue, and stain pinkish with MBE at pH 4.5 but purplish at pH 2.5 only in Stage e and thereafter. With colloidal iron reaction, the dentinal matrix stain blue intensely. Although the specificity of the colloidal iron method is said to be doubtful (Wiggleworth, 1952, Lison, 1953), this method generally shows a similar reaction to those of the Alcian blue and MBE, with exception of the intense staining of the dentinal matrix (Fig. 9). It is of interest that the pigment of ameloblast in Stage f and g stain dark blue with colloidal iron method (Fig. 10).

The cementum adjacent to the lingual dentin and the matrix of alveolar bone show definite positive reactions with the Alcian blue, MBE and Colloidal iron methods.

(5) Reactions for proteins (DDD reaction and HgBPB stain)

Basophilia and SH groups were studied by Wislocki & Sognnaes (1950), nucleic acids by Johnson & Bevelander (1954), and some protein reactions by Fullmer (1958). By these authors it has been shown that the enamel and dentinal matrices are positive for protein reactions, and that keratin and its associates are contained in the enamel matrix.

In the present study, the DDD reaction for protein-bound SH and SS groups in the ameloblast and odontoblast is less intense in early stage, but become intense after full differentiation (in Stage c and thereafter). The basal granules in the ameloblast show an intense DDD reaction. Both the predentin and dentin stain pale violet homogeneously with DDD reaction and no difference can be seen between them, while with HgBPB reaction the former stain far more intensely purple-blue than the dentin. The enamel matrix stains only sparsely or unstain with DDD reaction, but with HgBPB it stains purple to dark blue and shows a definite fibrous structure, especially in Stage e and f (Fig. 12).

According to these results and to the Mallory-Heidenhain stain, it will be emphasized that the protein reactions of the formative cells and of the matrices are closely associated with the cell differentiation and the mineralization of matrices, as shown by some authors cited above.

IV. Histochemistry of the Bone.

In young adult rats, the distal end of the femur shows a typical histology of the growing bone after decalcification by EDTA-formol solution even in an ice box. The cellular elements and the matrices of cartilage and bone are well preserved and show excellent histochemical reactions as follows:

(1) **Hydrolytic Enzyme Reactions (ALP-ase, ACP-ase and non-Specific esterase)** Although prolonged decalcification in EDTA-formol inhibits considerably the enzyme reactions tested, some intense ALP-ase reaction is found in the hypertrophic chondrocytes and in the adjacent matrices. The general result of ALP-ase reaction show little difference from that previously reported (Fig. 13).

On the other hand, ACP-ase is so labile and diffusible that it has been much difficult to demonstrate in the tissues demineralized with an usual method. In the present study, ACP-ase activity is well preserved in its intensity and localization. The Burstone's method gives some better results than the Gomori's.

According to previous reports by Burstone (1958, 1959-a, 1960) and others, ACP-ase reaction in hard tissues is found only in the osteoclasts and in the adjacent bone matrix. In the present study, however, besides high activity of osteoclasts there are some moderate reaction in the chondrocytes, osteoblasts, endothelia, and in the matrices of cartilage and bone (Figs. 14 and 15). And of interest is the fact that the reaction in the cytoplasm of these cells is seen as fine blue granules when the section is mounted in balsam after alcohol-dehydration and xylene-clearing, while it remains purple when mounted glycerin-jelly without dehydration. Although the difference of our result from the Burstone's is difficult to be explained, our positive result in many cells other than osteoclast may due to an excellent preservation of the enzyme activity and especially to a protecting effect on the diffusion of the enzyme. Moreover, when mounted in balsam, the cytoplasmic granular reaction changes into blue colour, while the reaction of nucleus and matrix remain diffuse and purple. This may be due to a difference of the materials in which the dye products precipitated.

It is another problem whether the diffuse purple staining of the nucleus and matrix means a true enzymatic activity. Burstone (1960) stated that "calcifying matrices from several species thus appear to exhibit a genuine acid phosphatase activity which is abolished or diminished by procedures known to inactivate or inhibit the enzyme." In the present study, the reaction of the alveolar bone matrix is light violet and distributes throughout the whole matrix, and the matrix of calcifying cartilage of the femur stains dark purple while the matrix of metaphyseal cartilaginous plate is almost negative. These findings seem to indicate that these reactions are not diffusion artifa-

Fig. 13 Solution H. ALP-ase reaction (Gomori's method). 200 $\times$. Fig. 14 Solution G. ACP-ase reaction (Burstone's method), mounted in balsam. 200 $\times$. Fig. 15 Higher magnification of Fig. 14. 400 $\times$. Fig. 16 Solution B. PAS reaction. 400 $\times$. Fig. 17 Solution B. Alcian blue stain. 400 $\times$. Fig. 18 Solution B. Colloidal iron reaction. 200 $\times$. Fig. 19 Solution B. MBE (pH 4.5). 200 $\times$. Fig. 20 Solution F. HgBPB reaction. 400 $\times$.

cts, giving support to the Burstone's opinion.

There is no definite esterase reaction in the femur tissue with only exception of few round cells scattered in the bone marrow. Sometimes a diffuse sparse reaction is seen in the ameloblast, peripheral zone of the pulp, and outer border of the alveolar bone, but none in the bone matrix as well as in the dentin. Savchuck & Burstone (1958) and Burstone (1960-b) reported an intense reaction in the calcifying matrices as well as in the dentin adjacent to predentin. They believe that this reaction is due to a genuine esterase activity, and emphasize that it delineates a zone in dentin which is associated with an early stage of calcification. According to our experiences, however, in the histochemistry of hard tissues, the specificity of staining reactions must be discussed with care, because the matrices of hard tissues seem to have a special reactivity or stainability different from those of the other soft tissues. Therefore a definite conclusion is held until further studies will be done.

(2) Polysaccharides—An intense PAS reaction is seen in the matrices of epiphyseal and enchondral bone tissue, but the cartilaginous matrix show a less intense reaction. The hypertrophic chondrocytes exhibit a large amount of PAS-positive granules which are removed by salivary digestion (Fig. 16). The chondrocytes have little or no glycogen in the proliferating zone and in the calcified cartilage. It must be emphasized that glycogen is well preserved after decalcification in EDTA-formol.

With Alcian blue stain, the most remarkable stain is found in the cartilage cavities (or lacunae) in the cartilage plate. As the flat chondrocytes grow into the hypertrophic stellate-shaped one, the blue-stained substance becomes rich and fills up the space between the lacunar wall and the chondrocyte, just like the so-called cartilage capsule (Fig. 17). In the cartilage matrix, and intense fibrous or flecked reaction is seen in the proliferating zone. The reaction decreases in the hypertrophic zone and much less intense in the calcified cartilage and in the trabeculae, indicating that Alcian blue-stained substance progressively decreases in volume as calcification proceeds.

With MBE at either pH level, the matrices of cartilage plate and the trabeculae stain blue to purple intensely. The matrices of the cell area (the territorial matrices) stain light or pinky violet metachromatically. The calcified trabeculae and cortical bone are unstained (Fig. 19).

With colloidal iron reaction, the so-called cartilage capsules and the territorial matrices in the plate stain dark blue as seen by Alcian blue stain (Fig. 18). But the interterritorial matrices stain pale greenish-blue and are rather similar to the PAS reaction.

(3) Proteins—The cartilage matrix is almost negative for DDD reaction, while the bone matrix shows a diffuse of fibrous reaction. The chondrocytes react intensely in the proliferating zone, but weakly in the hypertrophic zone. The osteoblast, osteocytes, osteoclast and some marrow cells generally show a moderate to intensive DDD reaction. By the HgBPB reaction, a similar result is obtainable; none in the cartilage matrix and blue stain in

the bone matrix. The reaction of chondrocytes is remarkable in the proliferating zone and less intense in the hypertrophic zone, but it increases again in the calcified zone. The osteoblasts and osteocytes stain dark blue (Fig. 20).

Summary

A method of fixing-decalcification (simultaneous) of hard tissues was studied from a histochemical point of view, and 10% DETA-10% formalin solution was found to be recommended for this purpose. Histochemical observations on alkaline and acid phosphatases, non-specific esterase, polysaccharides, and proteins in normal rat incisors and femurs treated with the fixing-decalcification method were reported with brief discussion.

References

- 1) Barnett, R.J. & Seligman, A.M. (1952) : *Science* 116 ; 323.
- 2) Bevelander, G. & Johnson, P.L. (1955) : *J. Dent. Res.* 34; 123.
- 3) Bonhag, P.F. (1955) : *J. Morphol.* 96; 381.
- 4) Burstone, M.S. (1957) : *A.M.A. Arch. Path.* 63; 164.
- 5) Burstone, M.S. (1958-a) : *J. Natl. Cancer Inst.* 21 : 523.
- 6) Burstone, M.S. (1958-b) : *J. Natl. Cancer Inst.* 20; 601.
- 7) Burstone, M.S. (1959-a) : *J. Histo. & Cytochem.* 7; 39.
- 8) Burstone, M.S. (1959-b) : *J. Histo. & Cytochem.* 7; 147.
- 9) Burstone, M.S. (1960-a) : *Ann. New York Acad. Sc.* 85; 431.
- 10) Burstone, M.S. (1960-b) : *R.F. Sognmaes, Am. Ass. for Advanc. Sc.* ; 217.
- 11) Cason, J.E. (1950) : *Stain Technol.* 25 ; 225.
- 12) Curran, R.C. & Collins, D.H. (1957) : *J. Pathol. Bacteriol.* 74 ; 207.
- 13) Dempsey, E.W. & Singer, M. (1946) : *Endocrinology* 38 ; 270.
- 14) Engel, M.B. (1948) : *J. Dent. Res.* 27 ; 681.
- 15) Fullmer, H. (1958) : *Lab. Invest.* 7; 48.
- 16) Fullmer, H.M. & Alpher, N. (1958) : *Lab. Invest.* 7; 163.
- 17) Gomori, G. (1952-a) : University of Chicago Press, Chicago.
- 18) Greep, R. O., Fischer, C.J. and Morse, A. (1947) : *Science*, 105 ; 666.
- 19) Greep, R.O., Fischer, C.J. and Morse, A. (1948) : *J. Am. Dent. Assoc.* 36; 427.
- 20) Harris, E.S. (1950) : *Anat. Rec.* 107; 105.
- 21) Hotchkiss, R.D. (1948) : *Arch. Biochem.* 16; 131., 1948.
- 22) Lison, L. (1953) : 2èd., Gauthier-Villares, Paris.
- 23) Lorch, I.J. (1946) : *Nature (London)*, 158; 269.
- 24) Müller, G. (1955-56) : *Acta Histochem.* 2, 68 & 73.
- 25) Newman, W.F. & Newman, M.W. (1958) : The chemical dynamics of bone mineral. Univ. Chicago Press.
- 26) Nikiforuk, G. (1951) : *J. Dent. Res.* 30 ; 472.
- 27) Nikiforuk, G. Sreebny, L. (1953) : *Ibid.* 32 ; 859.
- 28) Nuckolls, J., Leicester, H.M. & Dienststein, H. (1947) : *J. Am. Col. Dent.* 14 ; 118.
- 29) Nuki, K. & Bonting, S.T. (1961) : *J. Histo- & Cytochem.* 9; 117.
- 30) Robison, R. (1923) : *Biochem. J.* 17 ; 289.
- 31) Robison, R. (1932) : The significance of phosphoric esters in metabolism. New York ; New York University Press.
- 32) Savchuck, W. & Burstone, M.S. (1958) : *J. Den. Res.* 37; 1164.
- 33) Sreebny, L. & Nikiforuk, G. (1951) : *Science*, 114 ; 560.
- 34) Sugimoto, A., Yagi, T., Okamoto, K., Taguchi, F., Hiraoka, H., & Sakamoto, K. (1962) : *Proc. Jap. Histochem. Ass. Vol. 3* ;
- 35) Sugimoto, A. et al. (1963) : *Proc. Jap. Histochem. Ass.* 4. 41.
- 36) Steedman, H.F. (1950) : *Quart. J. micr. Sci.* 91; 477.
- 37) Wigglesworth, v. b. (1952) : *Quart. J. Microscop. Sci.* 93 ; 105.
- 38) Willams, G., & Jackson, D.S. (1956) : *Stain Tech.* 31; 189.
- 39) Wislocki, G.B., Singer, M. & Waldo, C.M. (1948) : *Anat. Rec.* 101; 487.
- 40) Wislocki, G.B. & Sognnaes, R.F. (1949) : *J. Dent. Res.* 28; 678.
- 41) Wislocki, G.B. & Sognnaes, R.F. (1950) : *Am. J. Anat.* 87; 239.