10. The Hematoxylin-stainability of Decalcified Dentin and the Calcification

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It is quite certain from numerous experiments carried out in this laboratory that the striated pattern on decalcified section of rabbit and rat dentin produced by hematoxylin staining indicates the degree of calcification of tooth. It is, however, possible that there is some variation in the stainability of dentin to hematoxylin in accordance with the kind and concentration of acid used, temperature, and duration of decalcification of dentin. Since it is our method to determine the efficiency of tissue deposition of various calcium preparations by measuring the tone and width of layers dyed with hematoxylin appearing on decalcified section of dentin, the results of measurement will become incorrect if there were variations in the degree of hematoxylin staining according to decalcifying conditions. Accordingly, relationship between the degree of hematoxylin staining and variation in decalcification conditions (kind and concentration of acid, temperature, and period of decalcification) was examined using rabbit dentin to establish the limit of variation in hematoxylin staining. At the same time, relationship between the degree of hematoxylin staining and the amount of calcium remaining in the tissue was examined.

Methods. In the bio-assay of calcium preparations by the present dentin method, time recording in hard tissues is effected by injection of lead acetate. For decalcification of dentin, 0.2N (ca. 0.7 %) hydrochloric acid solution is used and hydrogen sulfide is bubbled through this solution at room temperature. With rabbit tooth of around 0.3 g in weight, decalcification is effected within 3–4 days and the procedure can be advanced to preparation of sections.

Dozen pieces of dentin sections (ca. 15 μ in thickness) prepared in such a manner were each placed in 50–200 cc of solutions of 0.7 %, 3 %, or 5 % hydrochloric acid, or 5 % nitric acid, either at room temperature or warmed to 30°C. The degree of hematoxylin staining of these sections was examined from 3 hours after immersion in the decalcifying solution until 5 days later. On the other hand, in order to find the manner of dissolution of calcium from hard tissues with progress of decalcification, rabbit incisors of 0.3–0.6 g in weight were placed in 200 cc of 0.7 % hydrochloric acid of room temperature and
5% hydrochloric acid warmed to 36°C, the teeth were transferred to a fresh solution every 24 hours, and the amount of calcium dissolved in the decalcifying solution was measured in 100 cc of the filtered solution by McCrudden’s gravimetry. For the measurement of residual calcium in decalcified tooth, a tooth sample of 0.2-0.3 g in weight was placed in 100 cc of 2% hydrochloric acid solution for 9 days, then in 100 cc of 5% hydrochloric acid solution for 5 days, and the decalcified tissue sample was analyzed for ash and amount of calcium in the ash.

The rabbit dentin sections decalcified to various degrees were examined by the spodogram method (620°C, 1 hour). The spodograms were then treated with carbon dioxide and distilled water, or submitted to Macallum’s calcium detection or detection of calcium by picrolonic acid for confirmation of calcium in the ash. Furthermore, historadiograms were taken of the decalcified sections of incisor dentin and ground section of dentin from the other incisor of the same rabbit, after administration of calcium preparation, with a soft X-ray (3000 volts, 10 minutes in the case of decalcified section; 3000 volts, 23 minutes in the case of ground section) in order to see if the hematoxylin-stained layers were radiopaque or not.

Results. (1) Degree of hematoxylin staining and decalcifying conditions: Staining of rabbit dentin by hematoxylin was invariably good for 5 days by decalcification either in 0.7% hydrochloric acid at room temperature or warmed to 30°C, and the tone of staining and width of stained layers in periodic pattern remained constant (Fig. 1). There was also no change in stainability when a tooth block was immersed in 0.7% hydrochloric acid solution for 20 days. With sections immersed in 3% and 5% hydrochloric acid, and 5% nitric acid solutions, all warmed to 30°C, the degree of staining became somewhat poor, and the staining of the piece immersed in 5% nitric acid solution warmed to 30°C became extremely poor after 5 days and the dark and light stripes became indistinct (Fig. 1, F). The sections disintegrated in 5% hydrochloric acid solution warmed to 30°C and staining became impossible.

From the foregoing experimental result, determination of the efficiency of tissue deposition of calcium preparations by the dentin method was found to give a constant result as long as 0.2 N (0.7%) hydrochloric acid solution is used at room temperature for decalcification and that the width and tone of hematoxylin-stained layers did not vary even if the period of decalcification extended over a longer period than was necessary.

(2) Degree of hematoxylin staining and calcium remaining in the tissue: Dissolution of calcium from dental hard tissues by the decalcifying solutions used was virtually completed with 24 hours and
Fig. 1. Degree of hematoxylin staining and decalcifying conditions (transverse section of rabbit incisor)

A and D: Ordinarily decalcified section

B: Ordinarily decalcified section was once more immersed in 0.7% HCl for 24 hours.

C: " " " in 3% HCl for 3 days.

E: " " " in 5% HNO₃ (30°C) for 3 days.

F: " " " in 5% HNO₃ (30°C) for 5 days.

B: Almost normally stained with hematoxylin, C and E: Somewhat poorly stained, F: Very poorly stained

Fig. 2

Fig. 3
the amount dissolving out in subsequent hours was extremely small. The amount of calcium dissolving out in weighable quantity was negligible after 10 days in 0.7% hydrochloric acid solution or after 3 days in 5% hydrochloric acid solution warmed to 36°C, by McCrudden method, as indicated in Fig. 2. The hourly change in the decrease of tissue weight by this decalcification was weighed by a torsion balance of 1-mg sensitivity in water and the result is indicated in Fig. 3. The amount of ash and calcium remaining in the decalcified tooth is shown in the following table. It is seen that only 2.9% of the ash

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<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
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<td>Mean:</td>
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A: Dry weight (g) of rabbit tooth before decalcification
B: Amount of calcium (g weighed as CaO) dissolved out from A in 100cc of 2% HCl after 9 days
C: Amount of calcium (g weighed as CaO) over again dissolved out from B in 100cc of 5% HCl after 5 days
D: Dry weight (g) of A after decalcification
E: Ash content (g) of D (1000°C, 3 hours)

originally present remains but even under such state of decalcification, degree of hematoxylin staining remained good and the same can be said of tooth block immersed in 0.7% hydrochloric acid solution for 20 days. Examination of these samples by the spodographic method (cf. Fig. 4) indicated that the layers stained well with hematoxylin in the striated pattern of dentin were rich in calcium or ash and

![Hematoxylin-staining and Spodogram](https://via.placeholder.com/150)

Fig. 4. Transverse section of rabbit incisor
the striated pattern appears distinctly in the ash picture. The section decalcified in 5% nitric acid solution warmed to 30°C, for 5 days, became poor to hematoxylin staining and the striated pattern of dentin was also indistinct in the spodogram.

Fig. 5 is the cross section of upper incisor of a rabbit given calcium preparations five times with 3-day intervals. The five layers deeply stained with hematoxylin produced by this administration were found to be rich in ash by the spodogram and these layers were more opaque to X-ray in the historadiogram. It is interesting that these layers in the other upper incisor, prepared into ground section without decalcification, were also opaque to X-ray, while the layers not stained by hematoxylin were more radiolucent.

Consideration. Judgement of the state of calcification by hematoxylin staining has been made in the past and this point was followed by the use of rabbit dentin. It was thereby clarified that the layers with affinity to hematoxylin staining invariably contained calcium and that this calcium did not dissolve out by the usual decalcification procedure, the calcium being removed by a more drastic process with consequent loss of hematoxylin stainability. It remains to be solved whether there is any difference in the state of bonding between this kind of calcium bonded to tissues and the calcium that dissolves out easily after a few hours in dilute acid solution, and the question of whether the hematoxylin staining is produced by lake formation with residual calcium after decalcification or whether the matrix of bones and teeth having affinity to calcium is stained.

Summary. Hematoxylin stainability and width of stained layers of rabbit dentin do not vary by the conditions of decalcification unless the decalcification is effected by a method so drastic as to destroy the matrix of the dentin. This hematoxylin-stained layer contains calcium
which does not dissolve out easily.

The striated pattern on decalcified dentin sections stained by hematoxylin is a delicate and sensitive indicator for tissue deposition of calcium in a living body.

References

7) Schour, I., and Ham, A. W.,: Arch. Pathol., 17, 22 (1934).