From: The Norwegian Institute of Dental Research. (Director: Dr. philos. B. Nygaard-Östby)

EFFECT OF LACTATE BUFFERS ON DENTAL ENAMEL IN VITRO

as observed in polarizing microscope

by

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INTRODUCTION

A carious defect in the enamel may result either from removal of the minerals or from complete destruction of the organic matter. It is well known from histological studies that it is difficult to preserve the organic stroma when the enamel is decalcified, and if the organic stroma is destroyed the enamel loses the tissue connecting the prisms and becomes brittle. The usual appearance of caries at an advanced stage is a macroscopic defect — a loss of both inorganic and organic substance. According to *Miller* (1890) both components are destroyed by the carious process, the inorganic elements through acid dissolution, the organic elements through proteolysis.

However, a study of the macroscopic defect tells us nothing about the initial lesion (the first changes in the normal enamel surface). *Miller* believed that the primary lesion was an effect of acid. For a long time his theory has been uncontested, and even today it has the largest number of supporters. Recently, however, there has been considerable discussion concerning the primary stage. A group of investigators maintains that the organic component is attacked first (*Pincus,* 1940, *Gottlieb,* 1944, *Nuckolls & Frisbie,* 1946). Supporters of this theory agree that acid disso-

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lution of the inorganic elements takes place, but they consider this process to be secondary, made possible only by the preceding attack on the organic elements. Thus, in both theories the effect of acid is regarded as an important factor.

Since no definite proof of either of these theories has been produced, a study of the effect of acid on enamel seems justified.

The importance of the effect of acid in the primary stage can best be studied *in vivo*, i.e. on the living tooth in the mouth. Investigations of this kind present many difficulties. On the other hand, many of the problems involved may be studied by experiments *in vitro*. This is true, for instance, of such problems as the significance of the concentration of the acid, its acidity, and the duration of its action on the enamel. Such experiments *in vitro* will form the basis for later studies *in vivo* and will be a great help in interpreting the results of the latter. Valuable information may be gained by comparing the results obtained by the two methods. It should also be mentioned that material for *in vitro* experiments is more easily obtainable.

REVIEW OF LITERATURE

Summaries on the subject have been given by *Rosebury* (1938), *Fosdick & Starke* (1939), and *Bagnall* (1950).

Earlier laboratory experiments with the effect of acid on the enamel surface have primarily been undertaken in order to induce caries *in vitro*. Other experiments have attempted to establish the pH range in which the enamel can be decalcified and the time of exposure necessary for decalcification.

The earliest experiments were handicapped by the fact that the acid used was quickly neutralized by salts from the tooth. *Ehrensberger* (1930) improved the method by using buffer solutions. According to her a slow process of decalcification (often lasting several months) resulted in an opaque spot with no surface break of the enamel. In these cases the organic matter was preserved. If the decalcification proceeded more rapidly a cavity was formed. Characteristic opaque spots appeared in experiments with lactic acid — Na-lactate buffer, pH 4.6—5.0. Decalcification also occurred at higher pH-values with a correspondingly longer time of exposure, and even with distilled water. The

hydrogen ion concentration was not the decisive factor, as different solutions with the same pH-value gave widely varying degrees of decalcification.

More recently also *Enright, Friesell,* and *Trescher* (1932) have employed lactate buffers (pH 4.0 8.0), which all caused dissolution of the enamel. They found that buffers presumably saturated with calcium and phosphate had less decalcifying power, and they maintain that lactate and citrate buffers, previously saturated with calcium phosphate, above pH 5.0 only caused a barely discernible decalcification. This pH-value appears in later literature as the so-called critical pH-value, above which enamel *in vivo* cannot be decalcified. But *Besic* (1953) argues that the buffers used by *Enright* and his collaborators were not saturated with calcium and phosphate ions. He claims to have produced saturated buffers and finds that these do not dissolve the enamel even at pH-values down to 3.54.

The amount of dissolved enamel increases with the concentration of H-ions. *Rosebury* (1938) has shown that five times as much enamel is dissolved at pH 3.5 as at pH 4.5. According to *Fosdick & Starke* (1939), it is impossible to determine accurately the solubility product for enamel. In order to get a definite solubility product for a compound, it must be pure and must have a definite chemical formula. *Dobbs* (1932) points out that extracted teeth, immersed in a 0.75 % lactic acid solution, are first decalcified on the smooth, worn surfaces. Unerupted teeth do not have a surface resisting property similar to that of erupted teeth. Several investigators claim to have found great individual variations in the ability of enamel to resist the effects of acid (*McIntosh et al.,* 1925, *Enright et al.,* 1932).

In the earliest decalcification experiments the whole tooth was immersed in the acid. Recently the use of isolated experimental areas has been preferred (*Brudevold,* 1948, *Keil,* 1949, *Besic,* 1953). The latter method offers several advantages.

Enright et al. (1932) state that the microscopic investigation "is the most delicate method for detecting action of acids on enamel". *Keil* (1949) strongly emphasizes the advantages of polarized light. Polarized light was also employed by *Syrrist* (1949) for studies of changes in the enamel surface layer following application of fluorides. It may be mentioned that *Gustafson*

et al. (1947) used this method for investigation of natural hypocalcifications of the enamel.

While *Ehrensberger* (1930), *Enright et al.* (1932), and *Besic* (1953) claim that changes in the enamel effected by means of pure acids and buffers correspond to real caries, others, e.g. *Fasoli & Manicardi* (1926), and *Cox* (1950) maintain that these changes represent unspecific decalcification.

In short, it appears that the problem of the effect of acid on enamel has not yet been fully clarified. Since different investigators have employed different methods without always describing their techniques adequately, it is difficult to evaluate earlier findings.

In this paper a series of experiments will be described, in which clearly defined methods were used.

MATERIAL AND METHODS

The material in the present study consisted of newly erupted premolars extracted in the course of orthodontic treatment of school children, aged 10—14 years. Immediately after extraction the teeth were placed in physiological saline solution and used within a few days. By means of a dissecting microscope a selection was made of teeth that appeared to have a normal and intact enamel surface. A number of teeth with early stages of caries were selected for the purpose of comparison.

A small area of the enamel was isolated, using a modification of Brudevold's "window" technique (1948). The tooth was quickly dried with filter paper, and a circular, macroscopically intact enamel area, about 3 mm in diameter, was walled off with blue inlay wax, while the rest of the tooth was covered with Tenax wax. The experimental areas were mainly chosen on the buccal and lingual surfaces of the teeth. Over the isolated area, and at an angle of 90°, a wax cylinder with an internal diameter equal to the diameter of the experimental area was mounted. The length of the cylindrical tube was about 12 mm. During the mounting of the tube, the enamel surface was kept moist with physiological saline solution.

The solutions used for decalcification were placed in the tubes which were sealed with wax. Some of the experiments were performed at room temperature, others in a thermostat at 37°C.

After preliminary experiments lactate buffers, pH 3.51 to 5.70 were chosen for the final series. The lactate buffers were prepared by mixing 0.5 M lactic acid and 0.5 M Na-lactate. Thus, all the buffers had the same ionic strength. The pH-values were controlled by means of a glass electrode. The composition and pH-values of the buffers are shown in Table 1.

In experiments lasting more than two days the buffer solution in the wax tube was changed every 48 hours. 119 experiments were included in the final series.

80 preliminary experiments were conducted with lacate buffers (pH 2.0—4.75), which had a different composition and varying ionic strength. For this reason the preliminary experiments were not included in the present study, and will only be briefly mentioned in the following discussion.

Table 1
Composition of the lacate buffer solutions and their pH-values.

Lactic acid	Sodium lactate	pH
12	8	3.51
8	12	3.91
7	13	4.00
6	14	4.12
5	15	4.22
4	16	4.31
3	17	4.48
1	19	4.93
1	34	5.12
1	50	5.25
1	90	5.43
0	20	5.70

After varying time of exposure all wax was mechanically removed from the tooth surface, which was then studied under a dissecting microscope. Without embedding, ground sections of the teeth (through the centers of the experimental areas) were prepared. The main emphasis was put on the microscopic investigation in polarized light, since this method proved superior to examination in ordinary light. Fig. 1 presents a ground section imbibed in Canada balsam, as seen in an ordinary microscope. Fig. 2 shows the same specimen in polarized light. Fig. 3 shows

the same specimen in polarized light, but imbibed in water. It is obvious that polarized light yields a far more differentiated picture than ordinary light. Furthermore it is evident that by imbibition in water more delicate details are disclosed than by imbibition in Canada balsam. On the basis of these comparisons the present work was carried through as an investigation of water-imbibed specimens in polarized light.

According to the pioneer studies by *Schmidt* (1923, 1938) the birefringence of the enamel is the result of interaction between the negative intrinsic birefringence of the crystallites, (micells), and the positive form birefringence of the intermicellary areas. In the early stages of the development of the tooth, when the crystallization has just begun in the newly formed enamel, this enamel will exhibit positive birefringence when studied in a polarizing microscope. (It is assumed that the specimen has been imbibed in distilled water and that the prisms are oriented with their longitudinal axes diagonal through the positive quadrants). Due to the scarcity of crystallites at this early stage, the positive form birefringence of the intermicellary areas will dominate. As the mineralization progresses the amount of crystallites will increase at the expence of the intermicellary areas. Thus, the positive birefringence in the enamel will gradually decrease until it reaches a point where the positive birefringence of the intermicellary areas and the negative birefringence of the crystallites are equally strong and balance each other. At this point the enamel will appear isotropic (pseudo-isotropic). When the amount of crystallites increases further the enamel will show negative birefringence, most strongly in the fully mineralized part of the enamel. Incompletely mineralized enamel can be imbibed in fluids of different refractive indices, whereby the intermicellary areas will be filled. The birefringence will then change from positive, through isotropic, to negative — or in the opposite direction, depending on circumstances. *Gustafson et al.* (1947) ascribes the form birefringence to preformed spaces in the enamel matrix. For details on the method of polarization microscopy the reader is referred to *Schmidt* (1938).

Since the process of decalcification may be considered the reversed process of mineralization, the same stages can be observed, but in reversed order, i.e. the birefringence will change from negative through isotropic to positive. This behaviour has been utilized in the present investigations.

It is generally recognized that the enamel cuticle cannot with certainty be observed in ground sections. In view of this fact we have preferred to describe our enamel specimens without any special regard to the cuticle. That a cuticle is present in our specimens is, however, quite likely, since we are dealing with teeth from young persons and with tooth areas that have not been in contact with opposing teeth.

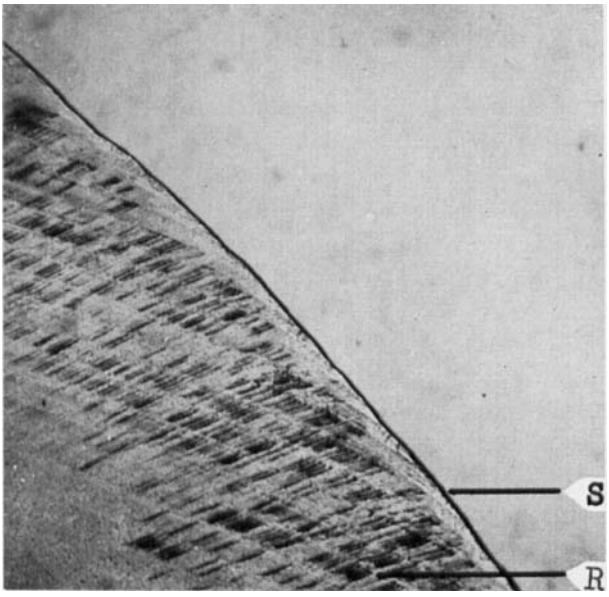


Fig. 1. Canada balsam. Ordinary light. Defect: Outer spot (Ia). Not to be clearly distinguished.[1]

RESULTS

In our experiments two types of defects appear. Fig. 4 a—c presents a schematic view of the enamel in an unstained ground section of a tooth viewed in polarized light with crossed nicols and with application of a compensator (gypsum, red, 1st order). The prism boundaries are indicated by fine lines. When a compen-

[1]All the pictures represent ground sections. Fig. 1 is derived from investigation in an ordinary microscope. Figs. 4, 5, and 18 are derived from investigations in a polarizing microscope with the use of a gypsum compensator. The rest of the figures are from investigations in a polarizing microscope without this compensator.

Abbreviations:

S = surface of the enamel.
R = striae of Retzius.
I. s. = inner spot.
O. s. = outer spot.
D = dentine.
+ = positive birefringence.
−+− = negative birefringence.
o = isotropy.

sator is used, areas with negative birefringence appear yellow. Blue color indicates areas with positive birefringence, i.e. areas with strong dissolution of the submicroscopic crystallites. Finally, red color indicates isotropy (pseudo-isotropy), i.e. areas which represent an intermediate stage between the two other stages with regard to degree of decalcification (negative and positive birefringence neutralize one another). In Fig. 4a the polarization colors show that a strong dissolution of the mineral component (blue color) has taken place from the surface. A less decalcified, narrow zone (red color) separates the affected area from the interior, apparently intact, enamel (yellow color). The superficial layer of the enamel is preserved in spite of decalcification of the deeper layer. Under the dissecting microscope this defect will appear as a distinct white area. Fig. 4b shows a condition representing a further development of the defect just described. A cavity has been formed, bordered by a narrow positive zone, which in turn is separated from the deeper, negative areas of the enamel by an isotropic zone. The loss of substance will normally be detectable in the dissecting microscope, and the perikymata will no longer be seen. In the following these types of defect will be referred to as types Ia and Ib, respectively.

The second main type, type II, is seen in the drawing in Fig. 4c. In this case the defect does not seem to develop in the outer zone of the enamel but just below and parallel to it. The defect has a positive central area, surrounded by an isotropic border zone. A narrow zone of negative enamel separates the defect from the enamel surface. Examinations both with the fluorescence microscope and with incident light also indicate that the degree of mineralization in this zone is about the same as that of the rest of the negative birefringent intact enamel.

The prisms proper in this outer zone appear to be intact, but the effect of acid can be observed in the prism sheaths and in the interprismatic substance. These changes can also be observed on the other side of the defect, towards the inner part of the enamel. Type II shows great similarities to hypocalcifications of developmental origin in the enamel. In the dissecting microscope this type appears as a faint white spot with a shiny surface.

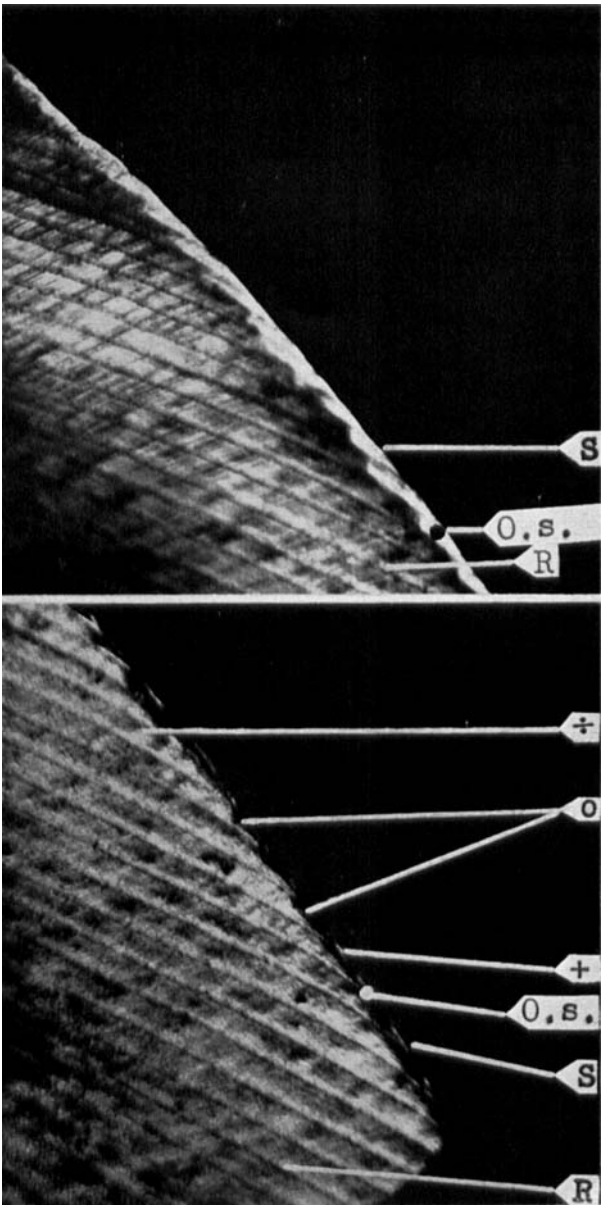


Fig. 2. Same section as in Fig. 1. Canada balsam. Polarized light. Outer spot to be distinguished.

Fig. 3. Same section as in Fig. 1. Distilled water. Polarized light. Defect shows differentiation (outer zone +, border zone o, intact enamel ÷).

Macroscopically both types of defect resemble the opaque spot indicative of initial caries (German: Kreidefleck). "Spots" must therefore be considered an appropriate term, since it also describes their appearance in the polarizing microscope (see Figs. 7, 11a and 18a). Consequently, the types I and II will be referred to as 'outer spots' and 'inner spots', respectively. The two main types of defects have here been described in their most characteristic appearance. But, if we consider different stages in the development of the main types, a series of 'subtypes' may be recognized.

The following stages of the two main types are conceivable and have, in fact, all been observed in the material under study:—

Type I — Outer spots:

Type	Surface zone	Intermediate zone	Inner zone
Ia	isotropic		negative
Ia	positive	isotropic	negative
Ib	cavity	positive isotropic	negative

Type II — Inner spots:

Surface zone	Intermediate zone	Inner zone
negative	isotropic	negative
negative	isotropic — positive — isotropic	negative

We are in a position to present cases where an inner defect becomes an outer one. Both types can be imbibed. Experiments with imbibition (Figs. 11a and b) show that the positive form birefringence is present as well in the isotropic as in the positive zones. This seems to show that the structure of the organic stroma is preserved in these zones. The results of the experiments with respect to the appearance of outer or inner spots are given in Table 2.

Table 2. Type of decalcification in relation to pH and time

pH	Time			No. of exper.	Type I	Type II
	min.	hours	days			
3.51	30			1	1	
3.51		16		1	1	
3.51			2	1	1	
3.91	15			1	1	
3.91	30			2	2	
3.91	40			2	2	
3.91		3		2	2	
3.91		24		2	2	
3.91			2	1	1	
4.00	15			1	1	
4.00	30			1	1	
4.12	10			1	1	
4.12	15			3	2	1
4.12	20			6	4	2
4.12	30			4	4	
4.12	45			2	2	
4.12	50			2	2	
4.12		12		2	2	
4.12			2	3	3	
4.22	30			1		1
4.22	45			2	1	1
4.22		2		1	1	
4.31	40			1	1	
4.31	45			4	3	1
4.31		4		5	2	3
4.31			2	5	5	
4.48		2		2		2
4.48			1	2	1	1
4.48			2	2	2	
4.48			5	4	3	1
4.93		8		1		1
4.93		19		1		1
4.93			1	2	1	1
4.93			2	3	1	2
4.93			5	1	1	
4.93			6	2	2	
4.93			12	1	1	
5.12			1	1		1
5.12			2	13	1	12
5.12			3	3		3
5.12			5	2		2
5.12			7	2	2	
5.25			2	1		1
5.25			4	1		1
5.43		12		1	1	
5.43		22		1	1	
5.43			1	1	1	
5.43			2	7	7	
5.43			3	3	3	
5.43			6	2	2	
5.70			2	1	1	
5.70			3	2	2	
			Total	119	81	38

Table 2 shows that three different pH-ranges can be distinguished with regard to the relation between pH and type of decalcification:

1. At pH 3.51—4.00 only outer spots appear.
2. At pH 4.12—5.25 both inner and outer spots appear. The inner spots were seen in relatively short experiments only. This will be discussed later.
3. At pH 5.43—5.70 once again only outer spots appear.

Ad 1. Three experiments were carried through at pH 3.51, with the time of exposure varying from thirty minutes to two days. All the experiments resulted in the appearance of an outer spot with cavity in the surface (Ib. See Fig. 6). Ten experiments were made at pH 3.91, with approximately the same times of exposure as at pH 3.51. When the time of exposure was less than two hours an outer spot without cavity in the surface was produced (Ia, Fig. 7). When the time of exposure exceeded two hours, type Ib appeared. At pH 4.0 only two experiments were carried out, both of brief duration and both resulting in an outer spot without a cavity. An outer spot at a very early stage is presented in the drawing in Fig. 4d. In the preliminary experiments the same result was obtained at pH 3.0 with a 2 minutes exposure. The affected area contains small, positive double-refractive fields, triangular in form, with the apex pointing inwards, and separated from the negative enamel by a narrow isotropic zone. These fields have developed in the areas between the striae of Retzius, which apparently have resisted destruction. It appears that the course of the striae of Retzius determines the shape of these fields. To the left the striae of Retzius run steeply towards the surface. To the right their course to the surface is more oblique, with the result that the distance between the striae is greater here than to the left. The defects, too, are broader here, since they develop in the enamel between the striae. When the time of exposure is prolonged, the defects coalesce, but a somewhat wavy line of separation from the intact enamel remains. This wavy line is also seen in inner spots (Fig. 8) and in outer spots obtained at higher pH-values (Fig. 9).

Ad 2. An inner spot appeared only within the pH-range 4.12—5.25. It was noticed that this type of spot was produced at each

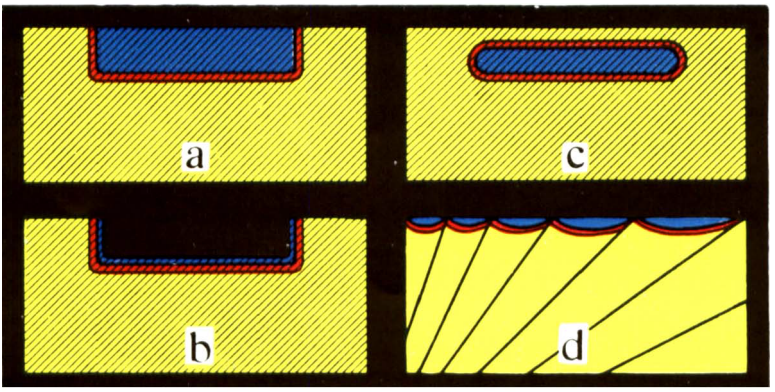


Fig 4

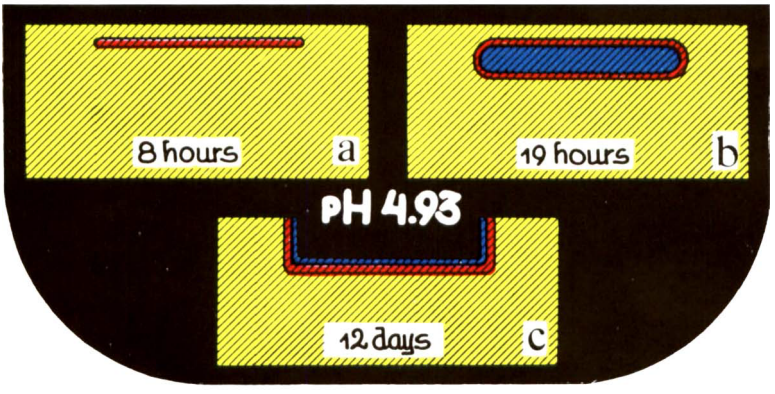


Fig. 5

Fig. 4. Schematic drawing. Types of defect. Polarized light (compensator). Distilled water. The prisms oriented with their longitudinal axes diagonal through the positive quadrants. Yellow = ÷, blue = +, red = o, a: outer spot (Ia), b: outer spot (Ib), c: inner spot (II). Prisms in a, b, and c drawn as faint lines. d: outer spot at very early stage. Relations to striae of Retzius. Prisms not drawn.

Fig. 5. Schematic drawing. Development of an inner spot (a and b). Change to an outer spot (c).

of the pH-values in this range only when the time of exposure was below a certain time limit dependent on the pH-value.[1] When this limit was exceeded an outer spot appeared, i.e. the original inner spot became an outer spot. The time limit is determined by the pH-value of the buffer: the higher the pH, the longer the time of exposure needed before the spot changes from an inner to an outer one. The fact that sometimes inner, sometimes outer spots appear before this limit is reached, probably depends on individual variations in the composition of the enamel.

Table 3 shows how the time limit varies with the pH-vaiue, as well as the relationship between the number of inner and outer spots within this time limit. We notice that the time limit increases from pH 4.12 to 5.25. It should be mentioned that one of the five experiments at pH 4.48 gave a deviating result, inasmuch as an inner spot appeared (see Table 2). Outer spots predominated, however, in the five day experiments at this pH-value (3: 1). In experiments lasting two days or more only outer spots appeared; the ratio in the one day experiments was 1: 1.

Table 3

pH	Duration of experiments resulting in both kind of spots	No. of experiments	Inner spots	Outer spots	Duration of experiments resulting in outer spots only	No. of experiments	Time limit for change from inner to outer spot
4.12	10—20 min.	10	3	7	30 min.—2 days	13	30 min.
4.22	30—45 min.	3	1	2	2 hrs.	1	2 hrs.
4.31	45 min.—4 hrs.	8	4	4	2 days	5	2 days
4.48	2 hrs.—5 days	8	3	5	2—5 days	6	2 days[1]
4.93	8 hrs.—2 days	7	5	2	5—12 days	4	5 days
5.12	2—5 days	12	11	1	7 days	2	7 days
5.25	2—4 days	2	2	0			

[1] One case deviates (5 days).

[1] At pH 5.25 the time of exposure was apparently not sufficient to produce an outer spot.

The experiments at pH 4.93 particularly demonstrate how an inner spot develops and changes into an outer spot as a result of exposure (Fig. 5). Fig. 5a shows the result of an 8 hour exposure: a narrow isotropic zone is separated from the enamel surface by a negative double-refractive zone (inner spot, first stage). In the 19 hour experiment (Fig. 5b) the isotropic zone widened, and a positive central area with isotropic border zones developed. After an exposure of 12 days (Fig. 5c) the outer negative zone was decalcified and an outer spot with a cavity developed.

At pH 5.12 the inner spot always appeared only as an isotropic zone. (Fig. 10). Inner spots at maximum development, i.e. with maximum width of the central, positive area, appeared at pH 4.31—4.48. (Figs. 11a and 18 b). The fact that pH 4.12 is the lowest value at which an inner spot has appeared in our experiments, does not exclude the possibility that decalcification of the enamel at even lower pH-values starts as this type of defect. But with such high concentration of H-ions the time of exposure must be quite short. This, in turn, means that the affected enamel zone becomes so narrow that a histological determination of this type of defect is impossible. It has been found that the negative zone peripheral to the inner spot becomes gradually narrower as the pH-values decrease from 5.12 towards 4.12.

3. One surprising result of the experiments was that only outer spots appeared at pH-values above 5.43. The destruction caused by the buffers at these pH-values was very slight (Fig. 9) and in many cases only a narrow isotropic zone in the surface of the enamel was evident.

A three day experiment at pH 5.70 resulted in a defect that was partly an outer, partly an inner spot (not included in Table 2). The peripheral intact zone was, however, extremely narrow, thus differing distinctly from the corresponding zone at pH 5.25, the nearest lower value at which an inner spot was observed.

It is known from earlier publications that enamel is decalcified also at pH-values above 5.70. We have not, however, extended our experiments above this value, since higher pH-values could only be obtained by changing the concentration of the components of the buffer, which would mean that the results would not have been directly comparable with those reported here.

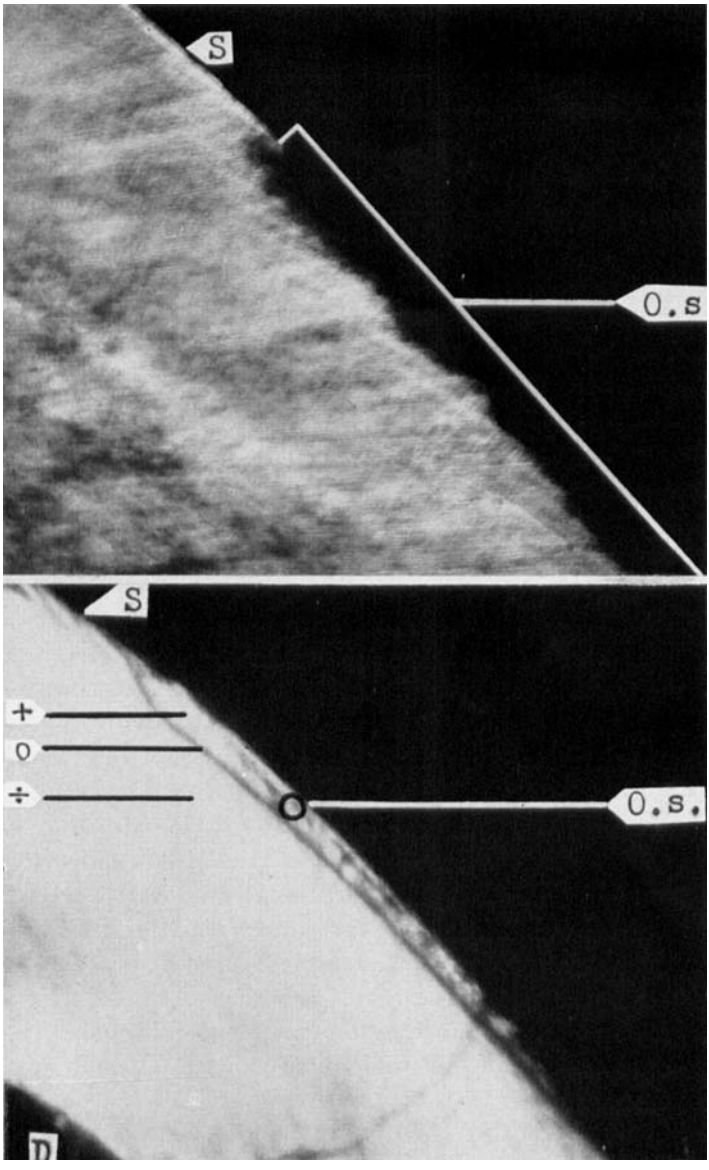


Fig. 6. Distilled water. Polarized light. Part of outer spot (Ib. See Fig. 4 b).

Fig. 7. Distilled water. Polarized light. pH 3.91. Time of exposure: less than 2 hours. Outer spot (Ia).

DISCUSSION

Ehrensberger (1930) in experiments similar to ours obtained changes in the enamel. She classified these as two types of defect:

1. Defects with cavity in the surface.
2. Defects with intact surface.

Gore (1940) makes the same distinction. Their classification is based on examination in ordinary light.

The classification of the two types of defect, as inner and outer spots, is based on our findings in polarized light. Our division is made possible by the fact that the polarizing microscope gives a more differentiated picture of ground sections than an ordinary microscope. The inner spot, as we have described it, does not correspond to any of the conditions found by earlier investigators in similar experiments.[1] Both *Ehrensberger* and *Gore* used a long time of exposure. This probably explains why they did not observe an inner spot. It seems that it is not the outline of the enamel surface — whether it is intact or not — that is the determining criterion, but solely the dissolution of the calcium salts, as it manifests itself in polarized light. Apparently intact surfaces occur not only with inner spots but also with one of the types of outer spots (Ia). Our findings show that the inner spot represents a certain phase of the decalcification process, a phase which occurs only under certain conditions, viz. within a certain pH range and within a relatively brief period of exposure. *Gore* states that in the cases where he found an intact surface, the decalcification was limited to the interprismatic substance. In our inner spots we have been able to observe also a dissolution of the calcium salts in the prisms proper.

It is not easy to explain how an inner spot develops. The decalcification of enamel is undoubtedly a complicated process, influenced by several factors. *Ehrensberger* explains her results by adopting the view of *Gysi* (1921) who assumed that hypercalcified zones occurring in caries resulted from remineralization. According to this view the acids which penetrate into the enamel become saturated with calcium salts which may be precipitated

[1] *Keil* (1949) presents a picture of a defect similar to our inner spots. This is, however, produced by bread-saliva mixture.

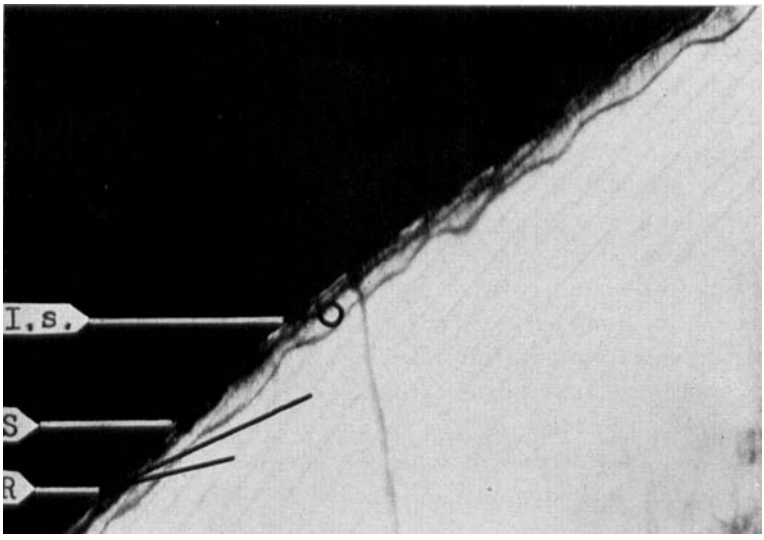


Fig. 8. Distilled water. Polarized light. Inner spot with a wavy line of separation from the intact enamel.

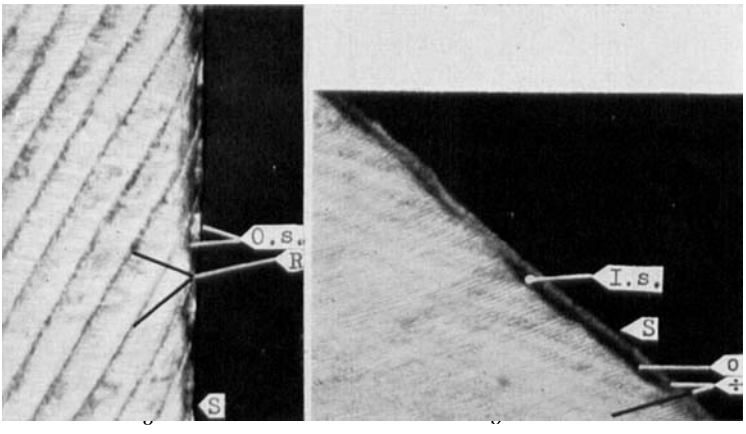


Fig. 9. Distilled water. Polarized light. pH 5.43. Time of exposure: 2 days. Narrow outer spot with relation to striae of Retzius.

Fig. 10. Distilled water. Polarized light. pH 5.12. Time of exposure: 2 days. Inner spot only as an isotropic zone.

in other areas of the enamel, for instance in the previously decalcified enamel surface. This may be valid for real caries and also for *in vitro* experiments with intermittent exposure to the acid. But in experiments with continuous exposure the conditions necessary for remineralization are not present. Even if we assume that the penetrating buffer becomes saturated with calcium salts in a certain layer inside the enamel, we must also assume that the dissolved salts diffuse inwards as well as out of the enamel. The salts diffusing outward meet the entering buffer and move towards an increasing concentration of H-ions. This means that the degree of saturation decreases towards the surface, and it is therefore inconceivable that precipitation of the dissolved salts can take place in the enamel surface. Consequently, the negative double-refractive zone between the inner spot and the surface of the enamel can hardly be due to continuous remineralization.

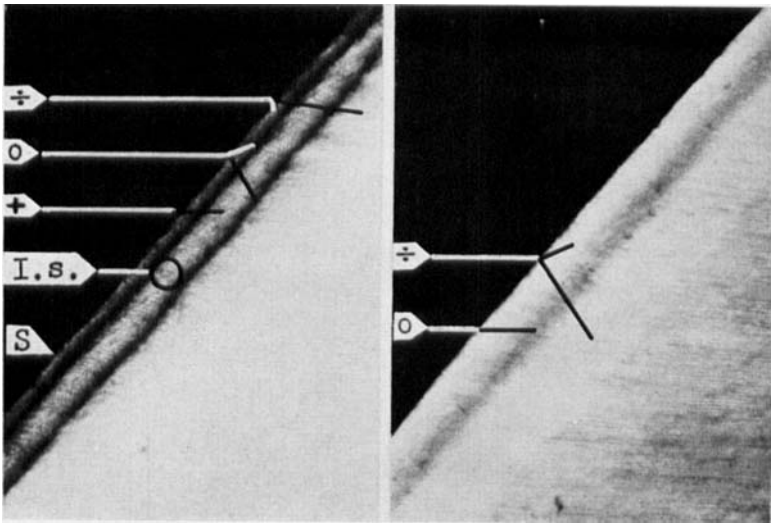
We have tried to explain the development of the inner spots by simplifying the problem, considering only the factors that are assumed to be most important in the process of decalcification: —

As Table 2 shows, the so-called inner spots appeared in our experiments only within a certain pH range (4.12—5.25). Outside this range only outer spots were observed. It is further evident from Table 2 that within this pH range the type of defect depended on the time of exposure. But even under experimental conditions generally resulting in development of an inner spot, an outer spot did occasionally appear. As mentioned earlier this must be assumed to be due to variations in the composition of the enamel, i.e. normal variations which always may occur in biological material.

The type of spot is thus primarily determined by the following factors:

1. The pH-value of the buffer.
2. The time of exposure.
3. The composition of the enamel.

Conditions quite similar to the inner spots we have observed in our experiments, are found in developmental hypocalcification of the enamel, as well as in early stages of caries. In our experiments an inner spot appears at pH-values corresponding to those



a. b.

Fig. 11. Inner spot. pH 4.31. Time of exposure: 45 min.
a. Distilled water. Polarized light. Maximal width of central positive area. See Fig. 4 c.

b. The same specimen in anilin. Polarized light.

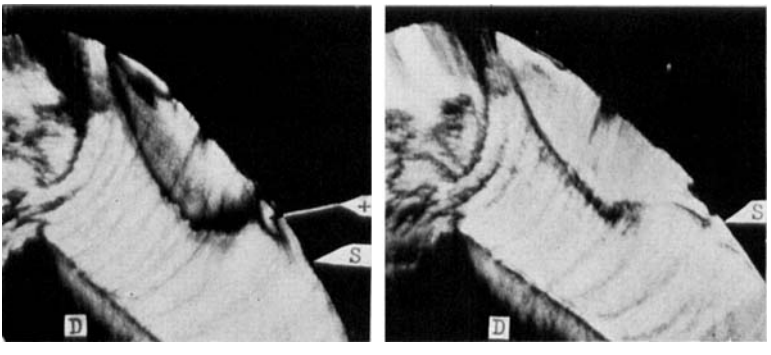


Fig. 12. Fig. 13.

Fig. 12. Distilled water. Polarized light. Caries in the enamel.

Fig. 13. The same specimen as in Fig. 12. Cinnamon oil. Polarized light. Positive areas partly disappeared.

found in plaque (*Stephan,* 1940, 1944, *Strålfors,* 1948). Both the early stages of caries and the inner spots we have induced are imbibable.[1] There are, in other words, several points of similarity between our inner spots and caries. Since an inner spot does not only appear under the conditions of our experiments, we may assume that it is due to conditions in the enamel itself.

In order to act in the depth an acid must penetrate into the enamel. In principle penetration and free diffusion are processes so closely related that they can be subject to the same mathematical treatment.

The formula for free diffusion is:

$$C = k \cdot C_0 \left(1 - \frac{2}{\sqrt{\pi}} \int_0^y e - y^2 \, dy\right)$$

$$\text{where } y = \frac{x}{2\sqrt{D\,t}}$$

Adair (1920) has shown that the formula also is valid when the diffusion takes place in a gel, and we may assume that it also is valid for penetration of the enamel. In the formula C is the concentration of the diffusing substance, at a certain distance, x, from the boundary, after the time t. The constant k depends on the conditions of the experiment. If the diffusion occurs from a solution into an equally large volume of the pure solvent, the concentration of the dissolved substance will, when equilibrium has been established, be the same throughout the whole system, viz. $\frac{C_0}{2}$. In this case $k = \frac{1}{2}$. In our experiments the volume of the buffer has been large in relation to the amount which penetrated the enamel. The concentration outside the enamel may therefore be considered constant $= C_0$, and k is consequently $= 1$.

The decalcification is caused by the H-ions only. It must be

[1] *Keil* (1937) mentions that natural caries, contrary to hypocalcifications, cannot, or only with great difficulty, be imbibed. We have found that this distinction cannot be maintained, since all ground section specimens of enamel caries are imbibable. (Figs. 12—13). Recently *Keil* (1954), too, has found natural caries to be imbibable.

assumed that H-ions and lactate-ions penetrate with equal speed. Therefore, C may represent the concentration of H-ions.

If certain values for x are chosen, and t is varied, a calculation of C according to the formula above will give a set of curves. Each curve represents a given value of x and shows how the concentration of H-ions at a given distance from the boundary

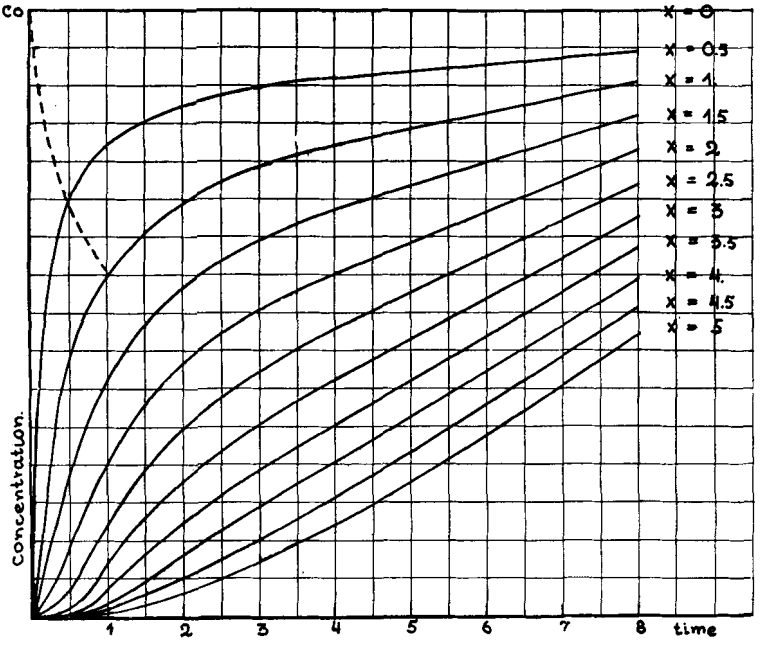


Fig. 14.

(enamel surface) varies with the time of exposure. Fig. 14 shows the result of such a calculation. The value of D in our experiments is not known. Therefore, an arbitrary value for D has been chosen. Whatever value is assigned to D the curves will have the same form. Different values for D will merely cause different slopes of the curves.

Thus, if we consider an area of the enamel at a certain distance from the surface, C will increase with the time. Therefore, the reaction between the H-ions and the apatite may be regarded as a reaction of zero order. Consequently, in a certain area of the enamel the amount of minerals dissolved per time unit will be proportional to the concentration of H-ions in this area. In the

period $t = 0$ to $t = 1$ the amount dissolved in the outermost layer of the enamel, from the surface to the distance $x = 1$, will be proportional to the area delimited by the ordinates $t = 0$ and $t = 1$, and by the curve connecting the points (t_0, x_0), $(t_{0,5}, x_{0,5})$, (t_1, x_1). The amount dissolved in the same layer in the period

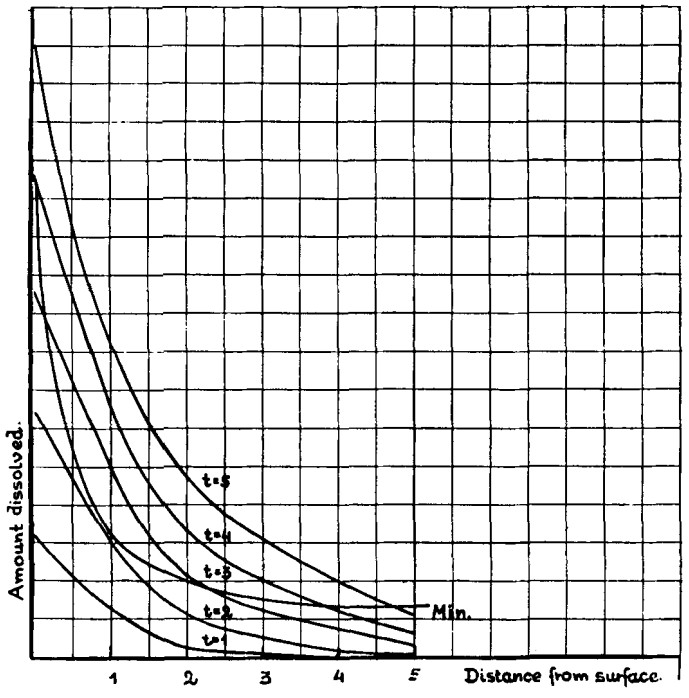


Fig. 15.

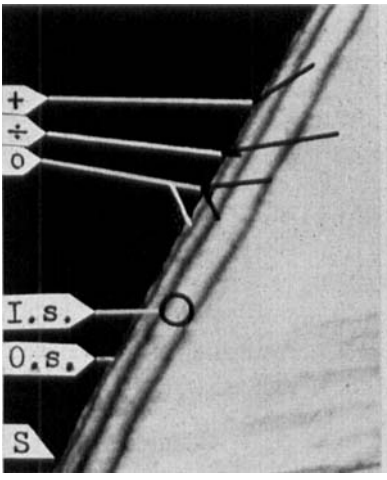
between $t = 1$ and $t = 2$ may be calculated in the same way. In the course of two time units the amount dissolved is proportional to the sum of the two areas. The amount dissolved in each layer after different lengths of time may be calculated in the same way. The result of such a calculation is shown in Fig. 15.

The curves t_1, t_2, t_3, etc. represent the amount dissolved after 1, 2, 3 etc. time units.

The graph also includes a curve marked 'min.'. As mentioned above, the organic stroma of the enamel is positively double-refractive, while the mineral component is negatively double-

refractive. The curve marked 'min.' on the graph represents the excess amount of minerals, i.e. the amount over and above the amount needed to compensate the positive form birefringence.

This curve shows that we assume the enamel to be more strongly mineralized in the surface and the amount of minerals to decrease inwards. This assumption agrees with the findings of *Thewlis* (1934, 1937) and *Sullivan* (1954). It appears from Fig. 15 that in the time $t = 1$ so small quantities of the enamel are dissolved that an excess of minerals remains in all layers, giving negative birefringence. With a short time of exposure, therefore, the enamel appears unaffected. At $t = 2$ we see that the dissolution curve touches the mineral curve at the point $x = 1$. At this point, in other words, the amount dissolved is exactly the same as the excess of minerals -- that is, at the distance $x = 1$ from the surface we will find an isotropic line. Both closer to the surface and further inside, the enamel will exhibit negative birefringence. At $t = 3$ we see that the dissolution curve twice crosses the mineral curve. Again, the points of intersection represent isotropic zones, one at $x = 0.2$, the other at $x = 2.2$ on the graph. Between these two points the amount dissolved has exceeded the excess of minerals needed for negative birefringence. This range represents a zone with positive birefringence. A small excess remains in the areas outside and inside the two points of intersection. The specimen, then, will present the following sequence of zones from the exterior to the interior: a narrow negative zone (apparently intact surface), then a narrow isotropic zone, a broader positive zone, a narrow isotropic zone, and finally, the rest of the enamel, again negative. At $t = 4$ it is noticed that the most exterior negative zone has disappeared, the outer isotropic zone has extended to the surface, the positive zone has widened and is bordered on the interior side by a narrow isotropic zone, which is next to the deeper, still negative, enamel. At $t = 5$ the isotropic surface zone has disappeared, the positive zone has extended to the surface and is on the interior side separated by an isotropic zone from the deeper enamel, which still remains negative. At this stage, in other words, the inner spot has become an outer spot. From the diagram Fig. 15 different conditions may be deduced, which all have been observed in our experiments. Under the given



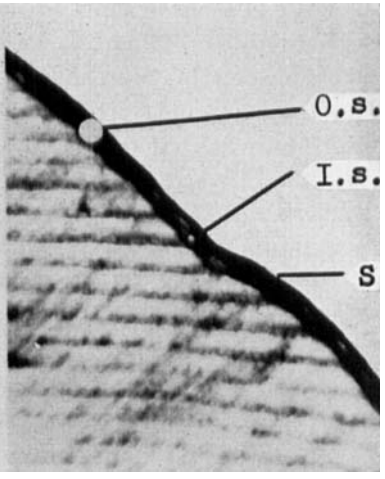
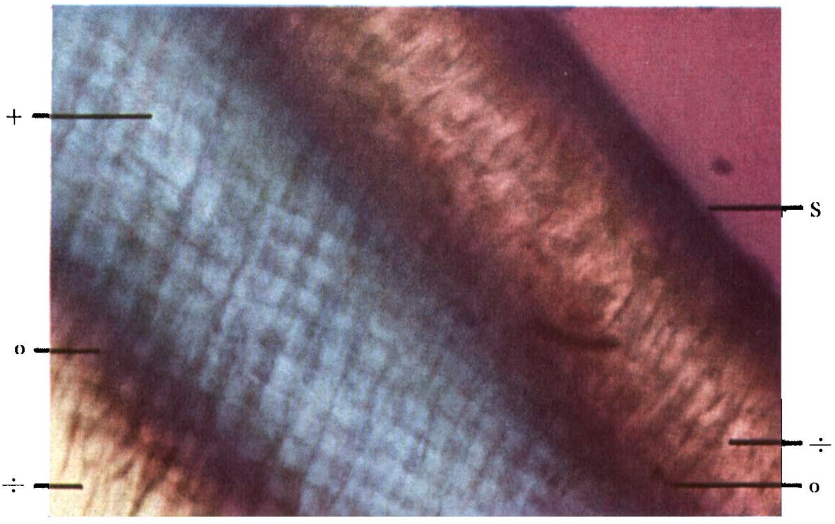


Fig. 16. Fig. 17.

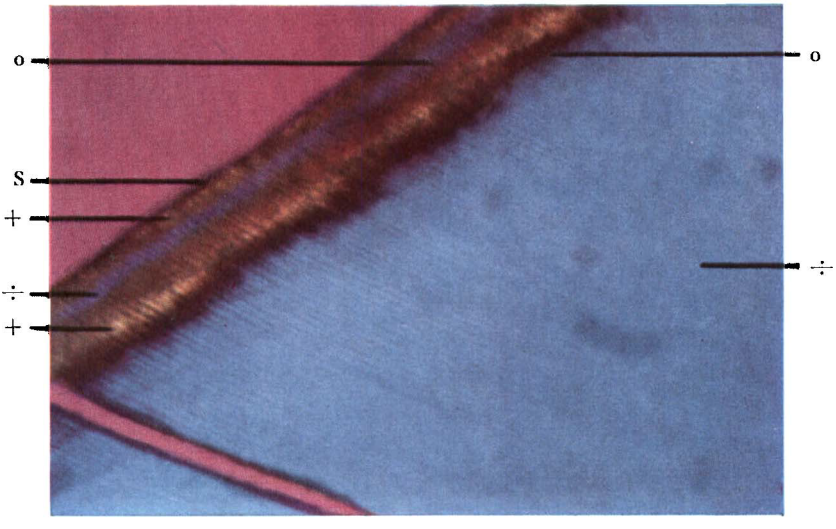
Fig. 16. Distilled water. Polarized light. pH 4.31. Inner spot and outer spot in the same experiment.

Fig. 17. Distilled water. Polarized light. Outer spot (Ia). Rest of inner spot is seen. Note: Background made white for better contrast.

conditions the type of decalcification is determined by the length of the time of exposure. With the use of a buffer with a suitable pH-value, an inner spot will appear only within a certain length of time. At shorter exposure there will be no apparent effect. At longer exposure an outer spot will appear. The graph also shows that an inner spot appears only when the dissolution curves rise in such a manner that they intersect the mineral curve at two points. If the slopes of the dissolution curves are steeper than the slope of the mineral curve, only an outer spot will appear. In our experiments only outer spots were produced at pH-values below ca. 4.10 and above ca. 5.30. It is evident that the dissolution proceeds more rapidly at lower pH-values. At a low pH-value the concentrations of H-ions in the surface is very high; we may, therefore, get a strong dissolution in the surface before the H-ions have time to penetrate the enamel. It is possible that an inner spot may occur at very short exposure, but we have not succeeded in observing an inner spot at such a low pH-value.



a



b

Fig. 18. Distilled water. Polarized light (compensator).
a: regular inner spot at high magnification (compare Fig. 4 c). In the central area of the defect cross striations of the prisms are visible.

b: both inner and outer spot in the same experiment. Because the prisms in this specimen are oriented through the negative quadrants the polarization colours are complementary to those shown in a and also to those shown in Figs. 4 and 5.

Blue = ÷, red = o, yellow = +.

It is more difficult to explain why only outer spots appear at higher pH-value. One possibility is that the electric charge of the enamel proteins is a factor in this pH range. The diffusion of ions will take place most easily at the iso-electric point. According to *Klein* (1932), the iso-electric point of enamel lies at about pH 4.3. At pH-values above the iso-electric point the proteins will be negatively charged and thus capable of binding H-ions. In buffers with a high pH the concentration of H-ions is low already at the start, but it will suffice to cause dissolution of minerals in the surface. A considerable part of the H-ions penetrating into the enamel may be bound to the negatively charged proteins. The concentration of H-ions will thus decrease quite rapidly with the distance from the surface. This decrease will manifest itself as a very steep slope of the dissolution curve. This means, as already mentioned, that the conditions for the development of an outer spot are present. We cannot, at the present moment, tell whether this theory is correct, but we have been unable to find any other plausible explanation.

In making our calculations we have simplified the problem, and we want it to be understood that the diagrams represent a simplified model only.

As mentioned, the decalcification is a very complicated process. As the H-ions penetrate into the enamel, part of them react with the apatite. This will cause a drop in concentration in addition to the decrease calculated from the diffusion formula. Since we have used buffer solution, this effect may, however, be small.

Apparently, the prism sheaths are more permeable than the prisms proper. The penetration will, therefore, first occur in the prism sheaths. The penetration into the prisms will, thus, occur both from the prism sheaths and from the surface of the enamel.

The penetration probably takes place in the organic material, while the crystallites present obstacles to the penetrating ions. As the crystallites are dissolved, it may be assumed that the enamel as a whole becomes increasingly permeable. Thus, the penetration coefficient D, in the formula, will increase with time.

When the calcium salts are dissolved, the buffer inside the enamel will become more or less saturated, and, therefore, the action of the H-ions is diminished.

The calcium salts dissolved in a certain layer of the enamel will diffuse inwards as well as out of the enamel. Thus, the action of the acid will depend not only on the penetration of the H-ions, but also on the diffusion of calcium salts out of the enamel.

All these factors have not been considered in our calculations, as this would have made a mathematical treatment impossible. However, since all the possibilities that may be deduced from the diagram agree with those observed, it may be assumed that our model does give a fair picture of the decalcification under certain conditions.

From the diagram we have deduced that an inner spot occurs in a certain time interval. When the time of exposure is longer, the inner spot changes to an outer one. This transformation depends on a widening of the inner spot until it reaches the surface. It is also possible, under certain conditions, that the decalcification in the superficial layer of the enamel reaches such a degree that this layer shows isotropy or positive birefringence before the inner spot reaches the enamel surface. In other words, both an inner and an outer spot may be formed in the same experiment. This has in fact been observed and such a condition is shown in Figs. 16 and 18b.

In Fig. 17 is shown a defect which must be classified as an outer spot without cavity. Here the outlines of a previous inner spot can be clearly seen. In this case, however, it is impossible to tell how the transformation from an inner to an outer spot has taken place.

Our explanation concerning the development of an inner spot is based on the assumption of a higher degree of calcification in the outer layer of the enamel. If this were not true, our explanation would not hold. We have tried to test this experimentally. On four teeth the superficial enamel was ground off and the ground surface polished. As in the other experiments an area of the enamel was then exposed to buffer, under conditions which in the other experiments most consistently resulted in inner spots (pH 5.12, 2 days). In all ground teeth exclusively outer spots appeared.

CONCLUSIONS

It was found that polarized light is superior for microscopic examination of defects caused by acid decalcification of the enamel.

By action of the described lactate buffers two main types of defects were observed in polarized light, with imbibition in distilled water. These two types were classified as 'outer spots' and 'inner spots'.

In the main type of outer spots the originally negative birefringence of the enamel has changed to positive in the superficial layer of the defect. The outer spots appear with, as well as without the formation of a cavity in the surface. The inner spots never show a break of the surface. In the main type the negative birefringence persists in the superficial layer of the enamel. Inside this layer appears a positive area bordered by isotropic zones.

The type of spot depends primarily on the pH of the buffer. In the pH range 4.12—5.25 either type may develop, while at pH-values above 5.25 and below 4.12 only outer spots appear. In the pH interval 4.12—5.25 the type of spot depends on the time of exposure. An inner spot develops at a relatively short time of exposure. When the time of exposure is prolonged the inner spot gradually changes to an outer one. The time limit for the change from an inner to an outer spot depends on the pH. The higher the pH, the longer is the time needed for this change. Furthermore, the type of spot occuring in this pH range seems to depend on the composition of the enamel. It is believed that the condition necessary for the formation of an inner spot is a higher concentration of calcium salts in the surface than in the deeper zones of the enamel.

The inner spot and hypomineralized zones of developmental origin as well as early stages of natural caries show several similarities.

An attempt has been made to explain the development of an inner spot by means of the diffusion formula, considering the factors which are assumed to be most important. The course of the decalcification has been illustrated by a diagram. From this diagram different stages in the development of an inner spot

have been deducted. These stages were actually observed in the experiments. The iso-electric point of the enamel (*Klein,* 1932) falls within the pH range in which inner spots appear. The occurrence of outer spots above pH 5.25 may depend on the electric charge of the proteins. At a pH above the iso-electric point the proteins will be negatively charged and therefore able to bind H-ions.

A relation exists between the inner boundary of the defects and the striae of Retzius, as the areas between the striae are most strongly attacked. In an outer spot this is observed even in the earliest stage of decalcification.

From experiments with imbibition may be concluded that even in highly decalcified zones, for instance in the positive zone of an inner spot, the structure of the organic matter is preserved.

SUMMARY

The present investigation was undertaken in order to study the very first stages of the decalcification of human dental enamel.

For the experiments newly erupted premolars were selected and used immediately after extraction. On these teeth experimental surface areas were isolated by the *Brudevold* technique. Over the experimental areas wax-tubes were fitted which were filled with lactate buffer solutions, the ionic strength of which was constant. The pH range was 3.51—5.70. The time of exposure was varied from 15 min. to 12 days. A series of 119 experiments is reported. The main emphasis was laid on examination of ground sections in polarized light.

On examination in polarized light with imbibition in distilled water two main types of defects were observed which could be characterized as inner spots and outer spots. Both principal types could be further subdivided. The outer spots manifested themselves macroscopically as dull whitish areas, with or without a loss of substance. On the microscopic examination an outer spot without loss of substance showed positive birefringence in the external zone of the enamel, separated by a narrow isotropic zone from the intact negative double-refractive enamel lying deeper. With short exposure to the lactate buffers the

alteration of the enamel in some cases was only a narrow isotropic zone in the surface layer of the enamel. In the case of an outer spot with loss of substance it was limited by a narrow positive double-refractive zone with an isotropic border zone. The inner spots manifested themselves macroscopically as slightly opaque spots with a shiny, apparently intact surface. On microscopic examination of inner spots the enamel showed a surface layer of varying width where the negative birefringence was retained. Inside this layer the real defect was found which in its characteristic form consisted of a positive central area with isotropic border zones.

It appeared that the type of spot was dependent on the pH of the buffer and the time of action. Below pH 4.12 and above pH 5.25 only outer spots appeared, independent of the time of exposure. Inner spots appeared only when buffers with pH from 4.12 to 5.25 were used, and then only with short time of exposure. A longer time of exposure produced outer spots also within this pH range. The time necessary for the development of an outer spot increased with rising pH.

The inner spots were in some respects similar to hypomineralized zones in the enamel and to early stages of caries.

The formation of the inner spots is explained by means of the diffusion formula. The main factors which are supposed to influence the course of decalcification have been taken into consideration. A graph of the course of such decalcification showed that it was possible to deduce all the various phases which were observed in the development of the inner spot.

RÉSUMÉ

L'EFFET DE SOLUTIONS TAMPONNÉES DE LACTATE SUR L'ÉMAIL DENTAIRE IN VITRO

observé au microscope polarisant

Les recherches effectuées par les auteurs ont pour but d'élucider le problème des premiers stades de la décalcification de l'émail dentaire humain.

Des prémolaires, en place sur l'arcade dentaire depuis peu et soigneusement sélectionnées, sont utilisées immédiatement après extraction. Des tubes en cire sont adaptés sur des plages d'essais

choisies à la surface des dents, préalablement préparées selon la technique de *Brudevold*. Ces tubes sont remplis de solutions tamponnées de lactate à pH constant. Le pH des solutions utilisées s'étend de 3,51 à 5,70. Les surfaces traitées furent soumises à l'action des solutions pendant une période variant de 15 minutes à 12 jours. 119 essais ont ainsi été réalisés. L'examen des coupes minces minéralogiques, usées à la meule, en lumière polarisée, a fait l'objet d'un soin tout particulier.

Deux types principaux d'altérations ont été observées au microscope polarisant sur des coupes imbibées d'eau distillée: d'une part des taches internes et d'autre part des taches externes.

Les taches externes se présentent macroscopiquement comme des zones opaques blanchatres, avec ou sans perte de substance. L'examen au microscope polarisant d'une tache externe sans perte de substance montre une biréfringence positive dans la couche externe de l'émail qui est séparée de l'émail indemne sous-jacent, à biréfringence négative, par une zone isotropique. Dans certains cas, où le contact avec des solutions de lactate a été de courte durée, on observe qu'une étroite zone isotropique à la surface de l'émail. L'éxamen d'une tache externe avec perte de substance montre une étroite zone à biréfringence positive bordée en dedans par une couche isotropique.

Les taches internes se présentent macroscopiquement comme des zones faiblement opaques à surface brillante et apparemment intacte. A l'examen microscopique d'une telle tache, l'émail présente une couche superficielle, d'épaisseur variable, à biréfringence négative. C'est à l'intérieur de cette couche que l'on rencontre l'altération réelle dont l'aspect caractéristique consiste en une zone centrale positive entourée par des zones isotropiques.

Il semble que le type de tache obtenue dépend du pH de la solution tampon et de son temps d'action. Les taches externes apparaissent au-dessous d'un pH de 4,12 et au-dessus d'un pH de 5,25, indépendemment de la durée de l'épreuve. Par contre les taches internes n'apparaissent qu'à des pH variant entre 4,12 et 5,25 et après une attaque de courte durée. En effet à ces derniers pH, si l'on prolonge la période d'action de la solution tampon, on obtient des taches externes. Le temps nécessaire pour produire une tache externe augmente avec l'accroissement du pH.

Les taches internes présentent une similitude avec les zones

hypo-calcifiées de l'émail et les premiers stades de la carie dentaire.

Les auteurs expliquent l'apparition des taches internes par les lois de la diffusion. Les principaux facteurs intervenant au cours de la décalcification ont été analysés. On peut déduire les différentes phases observées au cours du développement des taches internes à partir d'un graphique représentant les différents phénomènes se succédant dans une telle décalcification.

ZUSAMMENFASSUNG

EINWIRKUNG VON LAKTATPUFFERN AUF DEN ZAHNSCHMELZ IN VITRO

im Polarisationsmikroskope untersucht

Diese Untersuchungen bezwecken eine Abklärung der Verhältnisse in den allerersten Stadien des Dekalzinierens des menschlichen Zahnschmelzes.

In den Versuchen wurden neulich hervorgebrochene Prämolare gleich nach der Extraktion angewandt. An diesen Zähnen wurden Probeflächen nach *Brudevolds* Technik isoliert. Über den Probeflächen wurden Wachstuben montiert, die dann mit den Säure-Probelösungen gefüllt wurden. Als Probelösungen wurden Laktatpuffer mit konstanter Ionenstärke benutzt. Das pH-Gebiet der Puffer war 3,51—5,70. Die Einwirkungszeit wurde von 15 Min. bis auf 12 Tagen variiert. Der Bericht umfasst eine Serie von 119 Versuchen. Das Hauptgewicht ist auf die Untersuchung von Schleifschnitten in polarisiertem Lichte gelegt worden.

Es traten zwei Haupttypen von Defekten auf, die bei Untersuchung in polarisiertem Licht als innere und äussere Flecke bezeichnet werden können. Die Schnitte wurden in destilliertem Wasser imbibiert. Beide Haupttypen können in Unterabteilungen eingeteilt werden. Ein äusserer Fleck erscheint makroskopisch als ein mattes, weisses Gebiet, mit oder ohne Substanzverlust. Bei der mikroskopischen Untersuchung zeigte ein äusserer Fleck ohne Substanzverlust positive Doppeltbrechung in der äusseren Zone des Schmelzes, von dem weiter nach innen gelegenen unversehrten (negativ doppeltbrechenden) Schmelz durch eine schmale isotrope Zone abgegrenzt. Bei einer Einwirkung von kurzer Dauer bestand die Veränderung in einigen Fällen nur in

einer schmalen isotropen Zone in der Oberflächenschicht des Schmelzes.

Beim äusseren Fleck mit Substanzverlust wird die Begrenzung der Kavität von einer schmalen, positiven Zone mit isotoper Übergangszone ausgemacht.

Die inneren Flecke zeigten sich makroskopisch als schwächere, undurchsichtige Stellen mit schimmernder, anscheinend unversehrter Oberfläche.

Bei mikroskopischer Untersuchung innerer Flecke zeigte der Schmelz eine Oberflächenschicht wechselnder Breite, wo die negative Doppeltbrechung beibehalten war. Innerhalb dieser Zone wurde der eigentliche Defekt gefunden, der in seiner meist charakteristischen Form aus einem positiven Zentralgebiet mit isotropen Randzonen bestand.

Es stellte sich heraus, dass der Flecktypus von dem pH des Buffers und der Einwirkungszeit abhängig war. Unter pH 4,12 und über 5,25 traten nur äussere Flecke, unabhängig von der Einwirkungszeit, auf.

Im pH-Gebiet 4,12—5,25 traten innere Flecke bei kurzer Einwirkungszeit auf. Bei langer Einwirkungszeit traten auch in diesem Gebiet äussere Flecke auf. Die notwendige Zeit, um den äusseren Fleck hervorzurufen, nahm mit steigendem pH zu.

Der innere Fleck zeigte verschiedene Ähnlichkeiten mit hypomineralisierten Zonen in dem Schmelz und mit frühen Stadien von wirklicher Karies.

Die Bildung des inneren Fleckes wird aus den Gesetzen für die Diffusion heraus erklärt. Dadurch wurde Rücksicht auf die Faktoren genommen von denen angenommen werden muss, dass sie sich für den Verlauf des Dekalzinierens im Schmelz am meisten geltend machen. Eine graphische Darstellung des Verlaufs zeigte, dass es möglich war alle die verschiedenen Phasen, die bei der Entwicklung des inneren Fleckes beobachtet wurden, aus dem Diagram herzuleiten.

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