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ENAMEL LESIONS PRODUCED IN VITRO BY SUGAR-SALIVA MIXTURES

by

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In an early report on experiments *in vitro* with lactate buffer solutions well defined enamel defects called outer and inner spots were described (1). In a few cases these two types of defects appeared simultaneously. In a subsequent study (2) such a combination was frequently observed in extracted teeth which had been incubated in sugar-saliva mixtures. The writers recognized similar defects in carious dental enamel (Fig. 1) and also as a result of experimental caries produced by the gold plate method (3) (Fig. 10). These experiments, however, did not demonstrate the gradual development of the early stages of the defects in question. Therefore, it was decided to carry out a thorough study of the development of the defects which develop when dental enamel is exposed to sugar-saliva mixtures.

MATERIAL AND METHOD

Newly erupted premolars from individuals 10–12 years of age were selected. On each macroscopically normal and intact buccal surface an experimental area was encircled by means of blue inlay wax. The rest of the tooth was covered with Tenax wax. The experimental area was divided into two halves by a shallow longitudinal groove filled with wax. Each experimental area was exposed at 37° C to a sugar-saliva mixture (10 % sucrose w/v).

This mixture contained large amounts of microorganisms as a toothpicking was made during the collection of the saliva samples. Usually one half of the experimental area was covered with wax before the end of the incubation period and accordingly was exposed to the sugar-saliva mixture for a shorter period of time than the other half (Table 1).

Table 1.

Exposure time in hours	Number of exp. surf.	Exposure time relation in hours	
		Experimental half	
		Left	Right
0	10	0	12
		0	18
12—14	30	0	24
		0	48
18	8	12	12
		12	18
24	17	12	24
		18	18
48	29	18	24
		24	48
72	12	48	48
		48	72
96	13	48	96
		48	120
120	5	72	72
		96	96
144	6	120	120
		144	144
168	2	168	168
		216	216
216	10		

In supplementary experiments the areas were exposed to sugar-saliva mixtures, the acidity of which had been stabilized at defined pH-values (5.5, 4.5, or 3.8) by sterilizing the mixture with phenylmercuriacetate or chloroform, thus stopping further acid production.

If sterilization of this kind was not carried out during the incubation period the sugar-saliva mixtures usually reached the pH-value 5.0 after 12 hours, 4.0—4.4 after 24 hours, and 3.7—3.8 after 3 days. After 5 days the pH-values tended to increase.

At the end of the experiment the wax was removed from the tooth and the tooth was rapidly rinsed with water. The macroscopic appearance of the experimental area was assessed and described and a ground section was prepared. This section was cut in a direction perpendicular to the long axis of the tooth and some longitudinal sections were prepared as well. The sections included the typical regions of destruction on both halves.

Imbibed in media of different refractive indices (4) the ground sections were then examined in polarized light. The picture thus visualized was controlled in transmitted ordinary light, incident ordinary light and by dark field microscopy. Control specimens were also studied with the aid of fluorescence microscopy (5). Representative sections were studied by means of microradiographic techniques.

OBSERVATIONS

The developmental stages in the formation of the lesion, as observed in polarized light, are represented in Fig. 2. Photomicrographs of the corresponding stages are shown in Fig. 3.

The earliest discernible alteration is an accentuation of the prism sheaths in a narrow superficial *zone 1* of the enamel (Figs. 2—3, a). This picture may occasionally be observed after 12 hours of exposure. More often, however, after 12 hours, the surface layer reveals a narrow outer spot, *zone 4*, demarcated inwards by zone 1 (Figs. 2—3, b). The justification of the designation of zones is explained below.

The outer spot turned out to be isotropic in distilled water and negatively double-refractive in anilin ($n = 1.58$) and quinolin ($n = 1.62$). In the latter media the lesion was barely discernible. In these early stages sometimes V-shaped figures extended from zone 4 into the subjacent enamel layer. These figures were not visible in sections imbibed in anilin or quinolin.

Occasionally, a defect was seen which gave the impression of being an extremely narrow inner spot, located immediately below the enamel surface (Fig. 4). The area outside of this defect showed negative birefringence. It had a thickness of about 1 micron and revealed no structural detail. It was assumed to be the primary enamel cuticle (possibly with secondary coverings),

because lines of similar appearance were observed on intact enamel areas. Enamel cuticles were not considered in the original definition of outer and inner spots (1). Therefore, the defect shown in Fig. 4 should be regarded as a very narrow outer spot.

After 18 hours of exposure four zones could be observed in polarized light in sections imbibed in water (Figs. 2—3, c). As was the case after 12 hours of exposure an isotropic outer spot was visible in the superficial layer of the enamel. Below this zone 4, a negatively birefringent *zone 3* was observed. It separated zone 4 from a narrow isotropic *zone 2* which thus had the characteristics of an inner spot. Zone 1 is now broader than in the preceding stages. Isotropic cones extended from zone 4 into zone 3 which disclosed accentuated prism sheaths as well. However, on imbibition in media, the refractive index of which was 1.58—1.62, these details could not be seen. By further increase of the exposure time the outer and inner spots tended to confluesce, owing to a broadening of the outer spot and of the cones extending from it. Simultaneously, the inner spot expanded inwards and now turned out a positive central area with an isotropic border zone (Figs. 2—3, d). Thus, after 48 hours of exposure (Figs. 2—3, e), the total area corresponding to the three zones, 2, 3, and 4, often gave the appearance of a broad positive outer spot still bordered inwards by a zone 1. After imbibition in media, the refractive index of which was 1.55—1.62, three separate zones (2, 3, and 4) were clearly visible, now bordered by a transparent zone 1, all of them displaying varying degrees of negative birefringence, zone 2 showing the lowest degree (Figs. 5—6). Similar zones were visible in these media even in stages preceding cavitation, the latter usually appearing after 7—9 days of exposure (Figs. 2—3, f). In the stages preceding cavitation a narrow zone separated an outer spot from a broad inner spot.

The positive zone in regular caries, occurring in sections mounted in Canada balsam (Fig. 7), is by *Darling* (6) shown to be caused by the influence of alcoholic media used for dehydration and is not visible in watery media. It is produced by this author in a case of "artificial caries" as well. If existing in the present material, it should be localized to the inner part of zone 2. However, a positive zone like that was never observed in our sections (Fig. 8).

The developmental stages described above were checked by the control methods.

Fig. 9 presents a microradiograph picturing a stage similar to that in Figs. 2—3 d. The demineralization of the outer spot with cones (zone 4) and of the inner spot (zone 2) is obvious. The fluorescence examinations support these findings. Zone 3 as a non-fluorescent area separates the fluorescent zones 4 and 2. In zone 1 the fluorescent prism sheaths are conspicuous.

In the supplementary experiments with stabilized pH-values only outer spots were produced at pH 5.5, while a simultaneous appearance of outer and inner spots were always found after incubation at pH 4.5 and 3.8.

COMMENTS TO THE DESCRIPTION OF THE ZONES

Imbibition in watery medium, $n = 1.62$, will completely eliminate the form birefringence, thus displaying the negative birefringence of possibly remaining crystallites (4). In the present study the entire defect, by such imbibition, turned out with negative birefringence – of varying degree in different developmental stages. This means that nowhere in the lesion had the demineralization been complete. The subsurface layer always displayed less destruction than the other zones. Zone 1, on such imbibition, gets a translucent appearance, caused by the attacked prism sheaths being filled by an imbibition medium, the refractive index of which is equal to that of the crystallites.

The various stages described indicate a development of the lesion in the following manner: From the surface the agents penetrate into the enamel through the prism sheaths, and during the further development of the lesion the agents always follow these pathways. This is the reason why a zone of accentuated prism sheaths always represents the advancing edge of the lesion, and these alterations are actually the first to be detected. When this advancing edge has penetrated about 10 microns into the enamel the prisms proper in the surface layer are also altered (b). Still the lesion only represents a slight degree of demineralization. Gradually the prisms proper will be demineralized in the subjacent layers as well, but the subsurface layer (zone 3) reveals a remarkable resistance (c). Next, the demineralization of this

layer takes place from the surface and possibly, to a slight degree, from within (d—e). This process goes on concomitantly with a further progress of the whole lesion into the enamel. At last cavitation takes place (f).

The scheme (Fig. 2) and the photomicrographs (Fig. 3) aim at demonstrating the gradual development of early stages in "artificial enamel caries". However, due to variations in the resistance of the enamel and in the acid-producing capacity of the salivary samples, the lesion obtained at, for instance, 48 hours of exposure may in one case be similar to a defect obtained at an exposure time of 24 hours in another case and *vice versa*. It should also be mentioned that two stages which in the scheme succeed each other may be observed in the same experimental area, rendering further support to the correctness of the described developmental course.

DISCUSSION

Pictures similar to the lesions described in the present paper occasionally were found by the writers in cases of incidentally encountered caries (Fig. 1) and were also observed in caries produced experimentally by the gold plate method (Fig. 10). These observations together with the findings of *Darling* (6, 7) intimate the histologic identity of regular caries and the type of "artificial" caries described in this paper. The present material, however, is to small for a definite statement, especially as it seems necessary to consider possible variations in the histologic picture of regular caries. Thus, the gradual development of early stages in the enamel lesions described above differs from that of regular caries described by *Gustafson* (8).

No adequate explanation can be given to the question why a positive zone of the kind presented in Fig. 7 could not be observed when our sections were treated with alcohol and imbibed in balsam or quinolin. The features of zone 1 may represent a further difference from regular caries. As mentioned above this zone in water-imbibed sections reveals accentuated prism sheaths (Figs. 2 and 3). These details were not seen in a few water-imbibed sections of regular caries, prepared for comparison

(Fig. 11). Possibly, the differences mentioned above are due to the relatively short time of exposure to the sugar-saliva mixture.

Further, the present observations seem to give additional evidence of the prevailing role of easily fermentable carbohydrates in dental caries initiation.

In the main part of the experiments outer spots preceded inner spots as a rule, thus forming a complicated type of defect. A similar development could only be occasionally observed in a previous study where lactate buffers were employed. In the experiments described in the present study no buffer solutions with a constant pH were used, as the pH of the sucrose-saliva mixtures gradually dropped from neutrality to pH 3.7—4.0. If a similar drop had occurred in pure lactate buffer solutions [compare the theory advanced in the study mentioned above (1)] the following effect on the enamel could be expected: During the time taken for the pH to reach the value of 5.3 a slight dissolution with the characteristics of a beginning outer spot might be observed (actually, in the present study, after 12—14 hours of incubation, a single outer spot was frequently found). The consequence of this would be a less favourable condition for the formation of an inner spot during the time required for the subsequent drop in pH to 4.0. In contrast to these theoretical considerations, inner spots subjacent to the outer spots were easily produced in the sucrose-saliva mixtures. It was also surprising that inner spots were observed even at pH-values as low as 3.7, because our experiments with lactate buffers always showed outer spots at pH-values lower than 4.12.

The explanation of the phenomena discussed above is probably found in the difference between the composition of the lactate buffer solutions and the sucrose-saliva mixtures, viz. the latter contain calcium and phosphate ions, while the former do not. This contention is supported by the findings of *Coolidge et al.* (9) who, using lactate buffer solutions containing calcium and phosphate ions and having a pH of 3.5, produced enamel lesions similar to those which the present writers designate "inner spots".

The main part of the enamel consists of calcium phosphate. Therefore, the rate of dissolution of the surface layers ought to be lower in sucrose-saliva than in corresponding lactate buffers. As the hydrogen ions are much more mobile than calcium

or phosphate ions great differences cannot be expected in the deeper layers. Hence, the presence of calcium and phosphate ions in the saliva will facilitate the formation of inner spots by the sucrose-saliva mixture. The validity of this assumption was checked by placing enamel specimens in saliva acidified with lactic acid, or in sugar-saliva acidified with hydrochloric acid. Inner spots were produced in solutions with a pH as low as 3.0.

It must be emphasized that certain components of the sugar-saliva mixture probably can speed up the diffusion of particles into the enamel by dissolving certain minor constituents blocking the pathways of penetration. The variations in effect on different teeth produced by the same sucrose-saliva mixture indicate that this possibility must be considered.

SUMMARY AND CONCLUSIONS

Enamel lesions ("artificial caries") have been produced *in vitro* by exposing the surface of the enamel of newly erupted teeth to sugar-saliva mixtures. By varying the exposure time from 12 hours to 9 days the gradual development of early stages of the lesions could be recorded in the subsequent examination of ground sections by means of polarized light. The agents penetrate from the surface into the enamel through the prism sheaths, which thereby show up more distinctly, and later on, in the advancing edge of the lesion, the agents always follow these pathways. Following the penetration of the enamel surface via the prism sheaths a superficial demineralization takes place, involving the prisms proper as well ("outer spot"). In the further development a demineralized zone occurs, subjacent to the outer spot and separated from it by a relatively intact subsurface layer. This zone ("inner spot") will mainly expand centripetally. Next follows confluescence of outer and inner spots, and eventually cavitation of the surface takes place.

The similarity between these artificial enamel lesions and the lesions produced experimentally on living teeth by the gold plate method (3) has been demonstrated. Incidentally observed lesions of regular caries in some respects revealed the same features, but differed in certain details.

The course of development of early enamel lesions described in the present paper forms the basis for the registration of the preventive effect of topically applied solutions previously presented (2).

RÉSUMÉ ET CONCLUSIONS

LÉSIONS DE L'ÉMAIL PRODUITES IN VITRO PAR DES MÉLANGES SUCRE-SALIVE

Des lésions de l'émail ("carie artificielle") ont été produites *in vitro* en exposant la surface de l'émail des dents ayant fait récemment leur éruption à des mélanges sucre-salive. En faisant varier la durée de l'exposition de 12 heures à 9 jours, on a pu, lors de l'examen consécutif de coupes longitudinales en lumière polarisée, enregistrer de développement progressif des premiers stades de ces lésions. Venant de la surface, l'agent pénètre dans l'émail en passant par les gaînes des prismes qui apparaissent en conséquence plus distinctement, et ensuite, sur le bord en progression de la lésion, c'est toujours ces chemins que l'agent suit. A la suite de la pénétration de la surface de l'émail via les gaînes des prismes, une déminéralisation superficielle se produit, intéressant aussi le prisme lui-même ("tache externe"). Au cours du développement ultérieur, il se produit une zone déminéralisée, sousjacente à la tache externe et séparée d'elle par une couche relativement intacte. Cette zone ("tache interne") va s'étendre surtout d'une manière centripète. On assistera ensuite à la confluence des taches externe et interne, et éventuellement à la formation d'une cavité à la surface.

Il a été démontré que ces lésions artificielles de l'émail et les lésions produites expérimentalement sur des dents vivantes par la méthode de la plaque d'or (3) sont similaires. Des lésions de carie véritable observées fortuitement ont présenté dans une certaine mesure les mêmes caractères, mais en différaient par certains détails. Le mode d'évolution des lésions précoces de l'émail décrit dans cet article forme la base de l'étude des effets préventifs de solutions appliquées localement qui a été présentée précédemment.

ZUSAMMENFASSUNG

SCHMELZVERÄNDERUNGEN HERVORGERUFEN IN VITRO MITTELS ZUCKER-SALIVA-LÖSUNGEN

Zucker-Saliva-Lösungen wurden an die Schmelzoberfläche intakter Zähne *in vitro* appliziert, und Schmelzdefekte ("artifizielle Karies") wurden erzeugt. Durch Variationen in der Exponierungszeit von 12 Stunden bis 9 Tagen konnte die allmähliche Entwicklung der Frühstadien der Veränderungen registriert werden. Dünnschliffe durch die veränderten Bezirke wurden im Polarisationsmikroskop untersucht.

Es wurde festgestellt, dass die wirksame Stoffe von der Oberfläche den Prismenscheiden entlang in den Schmelz eindringen, und dass die hierdurch breiter gewordenen Prismenscheiden nun deutlicher hervortreten. In der weiteren Entwicklung folgen die Stoffe stets diesen Eindringungswegen an der vorderen Frontlinie des Defektes. Wenn die Oberfläche des Schmelzes in dieser Weise penetriert ist, folgt eine oberflächliche Demineralization der Schmelzprismen. Dadurch wird ein sogenannter äusserer Fleck gebildet. In den weiteren Entwicklungsstufen treten eine demineralizierte Zone im Inneren des Schmelzes auf (innerer Fleck) die durch eine verhältnismässig intakte Zone von dem äusseren Fleck separiert ist, und die sich hauptsächlich nach innen verbreitert. Durch Verbreiterung des äusseren Fleckes folgt später eine Zusammenschmelzung von dem äusseren und dem inneren Fleck. Schliesslich tritt eine oberflächliche Schmelzkavität auf.

Ferner konnte eine Übereinstimmung festgestellt werden, zwischen den *in vitro* hervorgerufenen Veränderungen und denen welchen an lebenden Zähnen experimentell hervorgerufen wurden. Reguläre Kariesangriffe wiesen mehrere derselben Zügen auf.

Die beschriebene Befunde bezüglich der Entwicklung der Schmelzkaries dienten als Grundlage der Registrierung der kariesvorbeugenden Wirkung von lokal applizierten Lösungen, die die Verfasser früher veröffentlicht haben (2).

REFERENCES

1. *Hals, E., T. Mörch & H. F. Sand:* Effect of lactate buffers on dental enamel in vitro — as observed in polarizing microscope. Acta odont. scand. *13*: 85, 1955.
2. *Mörch, T., P. Torell & E. Hals:* Effect of topically applied agents on enamel. I. Methods for experiments in vitro. Acta odont. scand. *14:* 335, 1956.
3. *Nygaard Östby, B., T. Mörch & E. Hals:* A method for caries production on selected tooth surfaces in vivo — employed in a preliminary study of the caries-inhibiting effect of topically applied agents. Acta odont. scand. *15*: 357, 1957.
4. *Schmidt, W. J.:* Polarisationsoptische Analyse des submikroskopischen Baues von Zellen und Geweben in Abderhaldens Handb. d. biol. Arbeitsmethoden. Abt. V, Teil 10, p. 435, 1938.
5. *Hals, E.:* Fluorescence microscopy of developing and adult teeth. Odont. Tidskr. *61*: 1, 1953.
6. *Darling, A. I.:* Studies of the early lesion of enamel caries with transmitted light, polarized light and radiography. Brit. dent. J. *101:* 289 and 329 1956.
7. *Darling, A. I.:* Studies of the early lesion of enamel caries. Brit. dent. J. *105*: 119, 1958.
8. *Gustafson, G.:* The histopathology of caries of human dental enamel — with special reference to the division of the carious lesion into zones. Acta odont. scand. *15:* 13, 1957.
9. *Coolidge, T. B., F. C. Besic & N. H. Jacobs:* A microscopic comparison of clinically and artificially produced changes in enamel. Oral Surg., Oral Med. and Oral Path. *8*: 1204, 1955.

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PLATES

With the exception of Fig. 9, all pictures are based on the examination of ground sections in polarized light by crossed nicols. Colour photomicrographs present cases in which a gypsum compensator, red 1. order has been used.

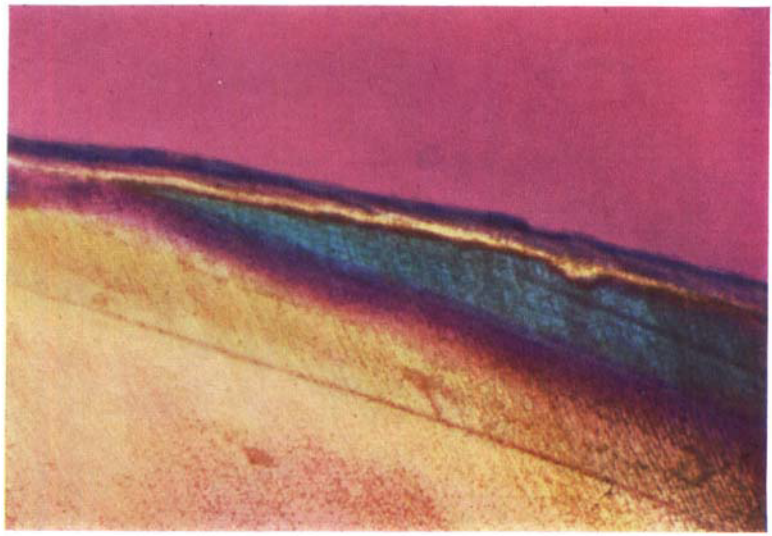

FIG. 1.

Regular caries, peripheral area of defect. Simultaneous appearance of inner and outer spot. Longitudinal section imbibed in distilled water.

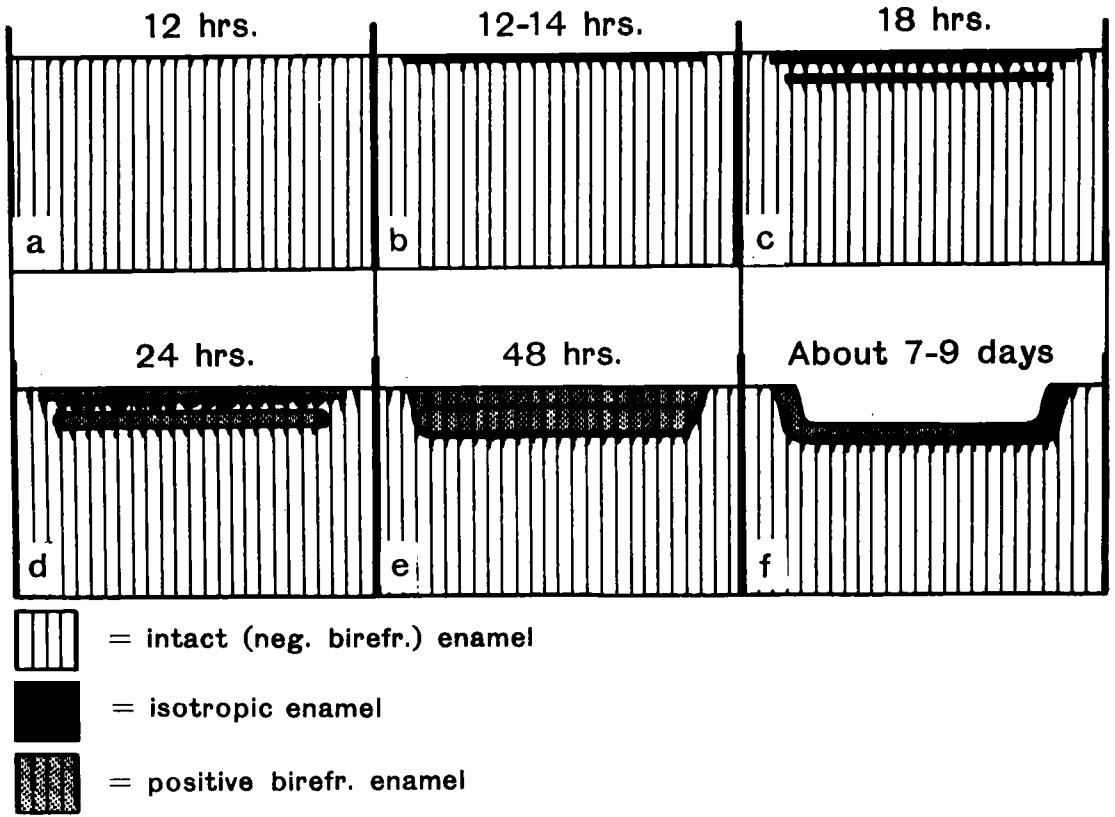

FIG. 2.

Schematic drawing of developmental stages in the formation of enamel lesions. Time of exposure varying from 12 hrs. to 9 days. Sections perpendicular to the long axis of the teeth. Prisms indicated as vertical columns. No distinction made between prism sheaths and interprismatic substance. Sections imbibed in distilled water.

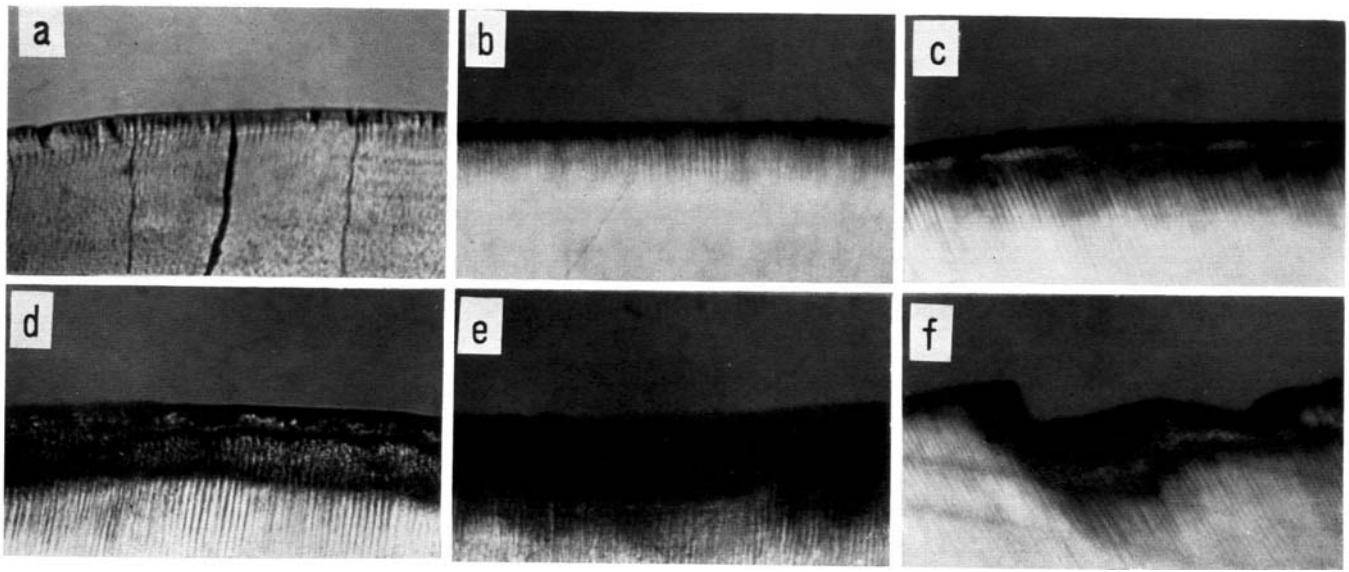

FIG. 3.
Photomicrographs of the stages presented in Fig. 2. Imbibition: distilled water.

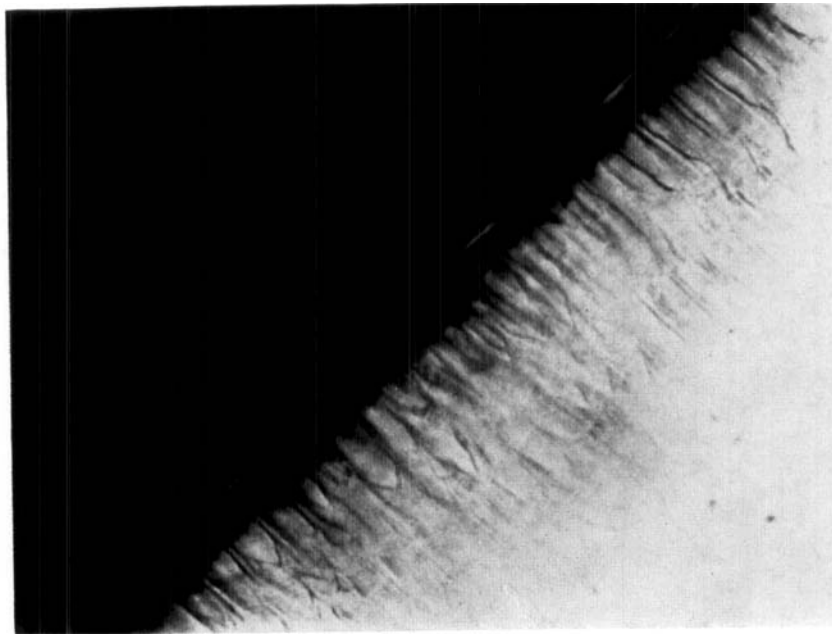

FIG. 4.

Narrow outer spot (zone 4) assumed to be covered by primary enamel cuticle, possibly with secondary adhesion. Transverse section. Distilled water. (High magnification).

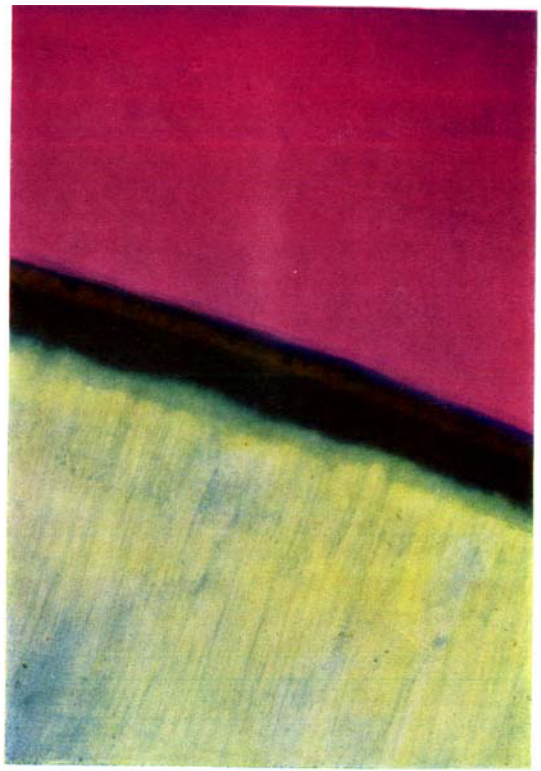

FIG. 5.

Defect, corresponding to the stage presented in Figs. 2–3 (d). Almost complete confluescence of zones 4 and 2. Note zone 1, revealing accentuated prism sheaths. Transverse section. Distilled water.

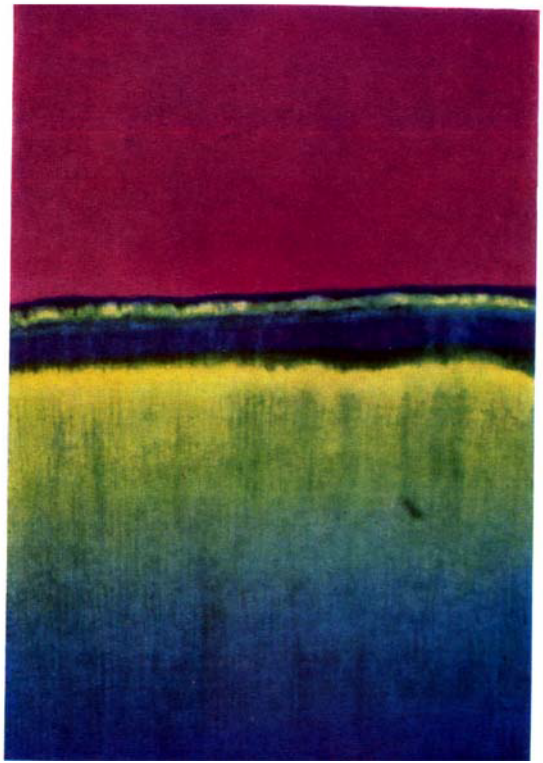

FIG. 6.

The same defect as shown in Fig. 5 following imbibition in Canada balsam. Zones 2, 3 and 4 visible as distinctly separate zones. Zone 1 now appears as a transparent zone. All zones reveal negative birefringence, which, however, is very weak in zones 4 and 2.

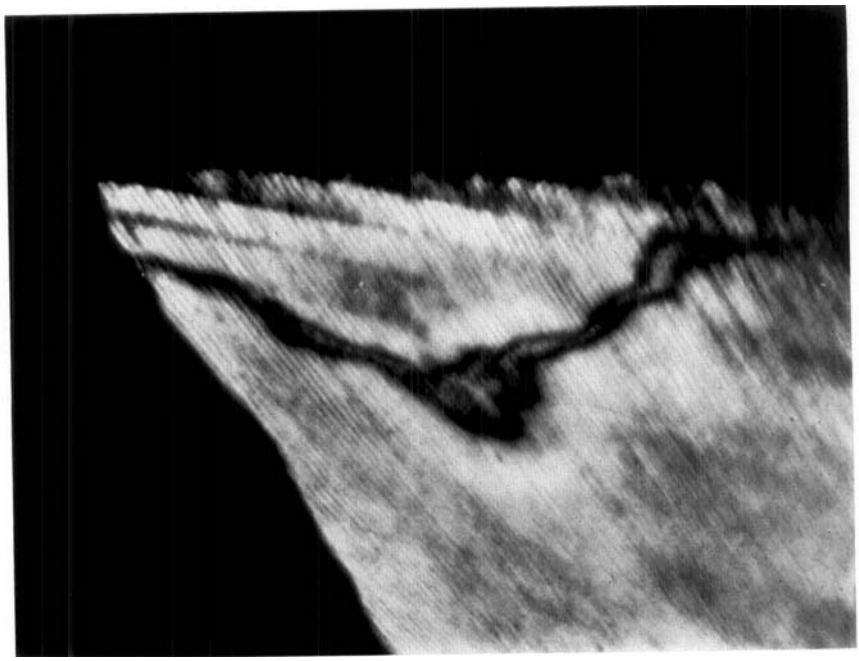

FIG. 7.

Regular caries. Positive zone remained after treatment with alcohol and imbibition in quinoline. Longitudinal section.

FIG. 8.

Artificial lesion. a. After imbibition in distilled water. Note zone 1–accentuated prism sheaths. b. The same lesion after imbibition in quinoline. Zone 1 now appears as a transparent band. The whole lesion presents negative birefringence. Fig. 8b to be compared with the regular carious defect in Fig. 7.

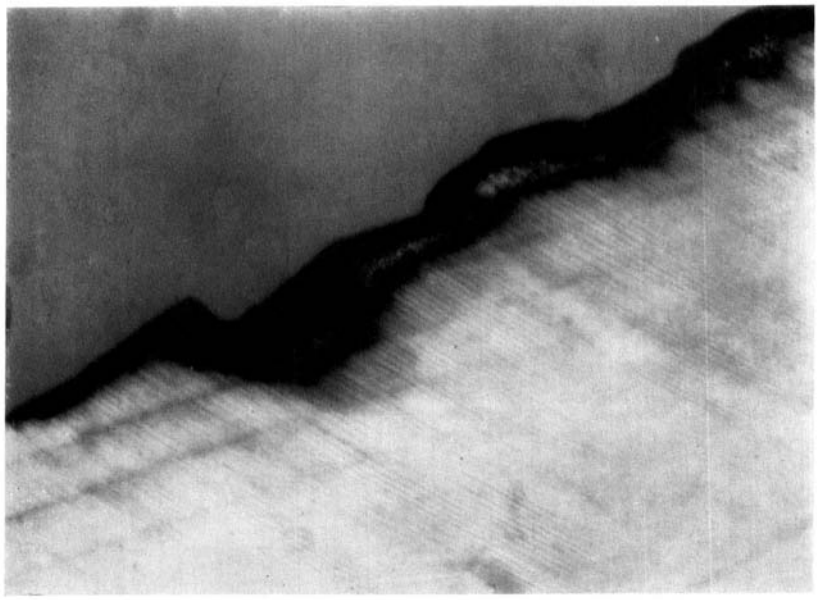

FIG. 8a.

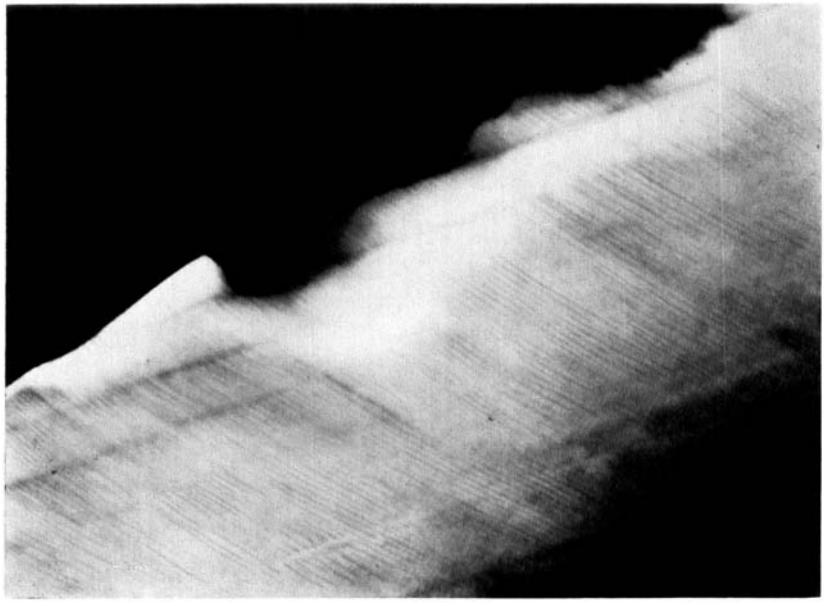

FIG. 8b.

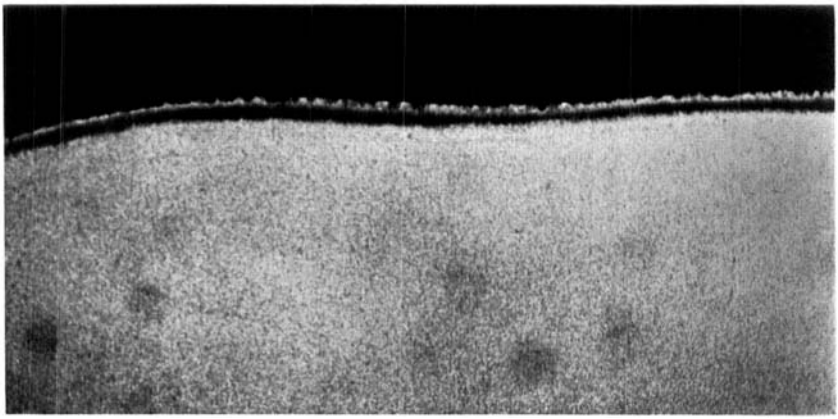

FIG. 9.

Microradiograph presenting a stage corresponding to that illustrated in Fig. 3d (polarized light). Ground section.

FIG. 10.

Experimentally produced caries in a premolar *in situ*. Experimental period four weeks. The same four zones as seen in Figs. 2–3 (c–d). Due to the long experimental period in this case, the inner spot (zone 2) broadened. Note accentuated prism sheaths in zones 3 and 1. Transverse section.

FIG. 11.

Regular caries. Longitudinal section, distilled water. Accentuated prism sheaths corresponding to those of zone 1 (Figs. 3c–f and 8a) are not seen.

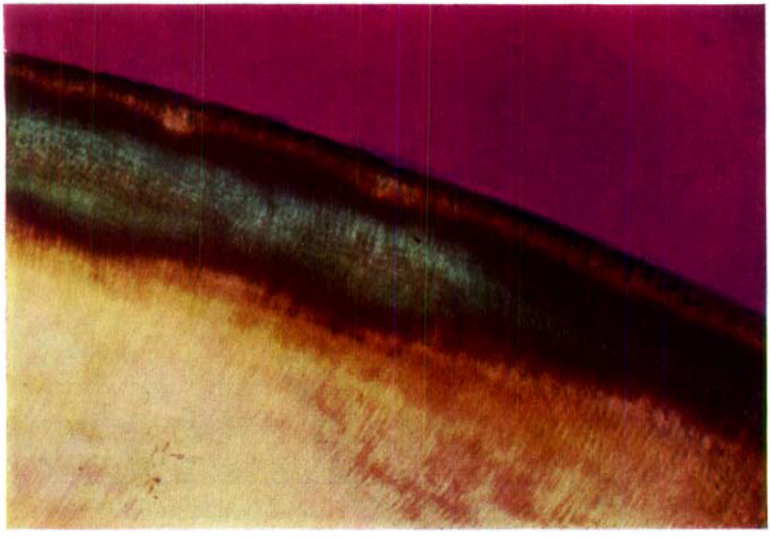

FIG. 10.

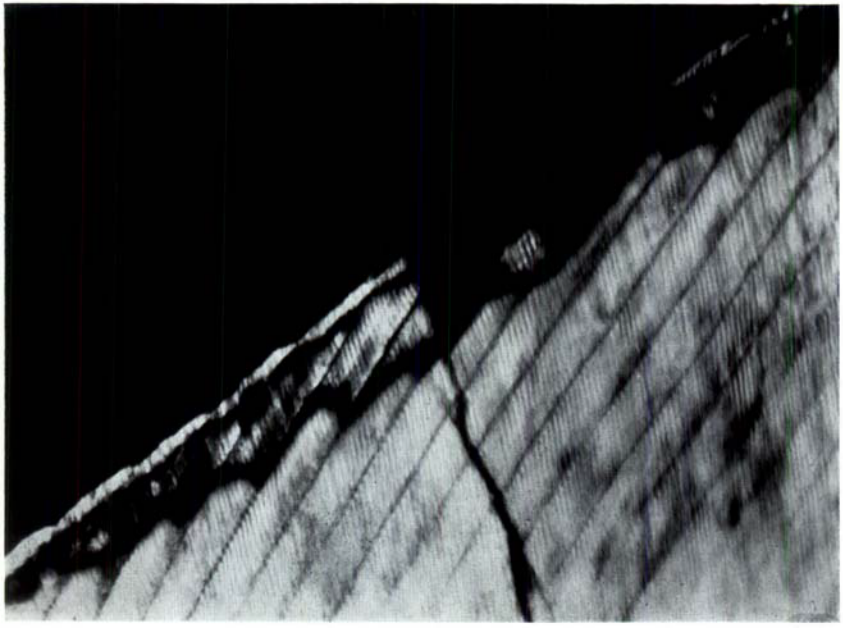

FIG. 11.