Scanning Microscopy

Volume 1 | Number 3 Article 26

5-4-1987

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Scanning Microscopy, Vol. 1, No. 3, 1987 (Pages 1109-1123)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/87$3.00+.00

THE USE OF SCANNING ELECTRON MICROSCOPY IN STUDYING ENAMEL CARIES

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(Received for publication February 23, 1987, and in revised form May 04, 1987)

Abstract

SEM studies related to carious change in dental enamel are reviewed, and their contribution to understanding the mechanism of formation of the early enamel lesion and of its repair evaluated. SEM has contributed significantly to understanding the mechanism of enamel dissolution at the level of the single crystal. Etching studies have yielded useful information on the effect of enamel structure on the pattern of acid dissolution at the microscopic level and have highlighted the importance of re-precipitation phenomena in modifying the pattern of mineral loss. High-resolution studies have provided interesting quantitative data on changes in crystal size, and also information on changes in crystal shape and orientation, during lesion formation and remineralization. However, further work is required in this area to clarify uncertainties about sampling bias and to relate the observed changes more precisely to the larger-scale structure of the tissue. Numerous observations on the surface morphology and internal structure of carious lesions have been made but preparation techniques used to date introduce artifact to a greater or lesser extent and interpretation of some of these results is therefore handicapped. We propose the use of a methacrylate replication technique as the method of choice for studying pore distribution in carious enamel and present preliminary results using this technique.

KEYWORDS: Scanning electron microscopy, teeth, dental enamel, caries, hydroxyapatite, acids, replicas, pores

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Introduction

The purpose of this review is to assess critically the contribution of scanning electron microscopy (SEM) to elucidating the patterns of mineral dissolution and precipitation during formation and repair of carious lesions of enamel. Discussion will be confined to the early (uncavitated) lesion. SEM has provided numerous important insights into the structure and formation of enamel. In the field of enamel caries, the technique is attractive because of the ability to examine structure at all levels between the whole tooth crown and the single enamel crystal. A further attraction is the ease of specimen preparation compared with the extreme difficulty of preparing ultrathin sections for transmission electron microscopy (TEM) of the tissue. However, sufficient allowance has not always been made for artifacts in the various preparation procedures. After summarising the main features of the early lesion, as known from polarising microscopy, microradiography and TEM, we shall review SEM studies under four headings: (1) dissolution at the crystal level; (2) etching of enamel; (3) crystal shape and size; (4) lesion structure and dissolution/precipitation patterns. Under the last heading, we present some new results of our own, using a replica technique which shows great promise in clarifying the pore structure of carious enamel.

The early lesion

Using polarising microscopy, four histological regions can be recognised in the early lesion [71]. Moving from the surface inwards, these are the surface zone, the lesion body, the dark (or positive) zone and the translucent zone.

The translucent zone represents the site of the earliest mineral loss, accompanied by opening up of micropores, both at the prism junctions and to a smaller extent within the prism bodies [19,43,49,59,86]. The initial changes involve selective loss of carbonate- and magnesium-rich mineral [33,34] which may be present as a separate phase, e.g., as dolomite [22]. The optical properties of the dark zone arise from the presence of minute pores. In this zone, more pronounced dissolution of the crystals and some

enlargement of micropores within the prism bodies is observed [49,59].

The lesion body accounts for the bulk of the mineral loss, the mineral content being as low as 20 vol % [7,30,54,79]. The structure of this zone tends to collapse on drying and the resulting shrinkage has been used to detect the lesion body, and measure its depth, by SEM of cut surfaces [23]. The crystals throughout the prism bodies show extensive dissolution but at the prism peripheries, next to the junctions, there are commonly found polyhedral, isodiametric crystals which are evidence of re-precipitation [43,49,52]. The existence of these crystals is often relevant to interpreting the SEM image, but their nature has not been firmly established. They are unlikely to be octacalcium phosphate [51] because this is rapidly converted to hydroxyapatite. There is evidence [85] in favour of their being whitlockite, like the so-called 'caries crystals' - large, rhombohedral crystals found in grossly carious enamel and dentine [52,56]. The Mg ions necessary for whitlockite formation [21] could be derived from dissolution of a Mg-rich phase of the enamel [33]. Brown et al. [16] suggested that dicalcium phosphate dihydrate (DCPD) might form during carious attack. Artificial lesions do show an increased HPO_4 content [2], but the evidence points to this being due either to surface modification of the enamel crystals [2] or to the presence of anhydrous dicalcium phosphate (monetite) [24,87], rather than DCPD. Neither monetite nor DCPD have been identified in natural lesions [58].

Except in very early lesions, which may show surface softening [1], the lesion is covered by a surface zone with a mineral content lower than sound surface enamel, but higher than the lesion body [7,30,79]. How the surface zone is produced and maintained is one of the central problems of caries research and numerous theories have been proposed (for recent review, see [1]).

Dissolution at the crystal level

A factor of possible importance in interpreting carious dissolution is that crystals of apatite appear to show differential dissolution rates along the different crystallographic axes. Although the evidence for enamel crystals [68] is indirect, one study indicated that large fluorapatite crystals dissolve more rapidly along the c-axis than in the a-axis direction [46]. In another study, however, the rates of calcium release from the different faces were approximately equal [83]. SEM illustrations in the latter study, which utilised windows isolated on each face by lacquer, indicate that acid might have 'tunnelled' beneath the lateral faces, leading to loss of calcium from beyond the defined window areas. Hydroxyapatite is usually assumed to show differential solubility like the isomorphous fluorapatite, but direct measurements have not been made.

During dissolution of large synthetic hydroxyapatite crystals containing more than 0.1% carbonate, a single hexagonal etch pit forms on the basal face [4,45], in contrast to multiple etch pits in fluorapatite [46,83]. The inner surface of the pit was shown by SEM to show a spiral pattern of ridges and a slight twist of the crystal as a whole was also detectable [4]. These features indicate the existence of a single screw dislocation running parallel with the c-axis, which promotes localised dissolution of the crystal centre. With time the hexagonal etch pit deepens and the crystal becomes hollowed out. Central dissolution does not occur in hydroxyapatite crystals with very low carbonate content and these are more perfect in form [4]. Thus lattice distortions due to carbonate inclusion appear to be responsible for the formation of the screw dislocation and thus for the central dissolution pattern [4]. Enamel crystals contain carbonate and show rapid dissolution of the central region when exposed to acid [43,48,49,59, 76,77]. The hollow crystals have been visualised by SEM in fractured specimens [48]. The hollowing-out process occurs rapidly, with little change in crystal diameter and eventually the crystals split, leaving fragments 10-20 nm thick [48,76,77].

Etching of enamel

Etching studies have value for caries research because they have stimulated ideas about the structural and compositional factors which might influence patterns of enamel dissolution. Etching agents have been applied to the natural surface [36,37,61] but in most cases to ground or polished facets, orientated usually tangential, but occasionally perpendicular [61], to the natural enamel surface.

Three main patterns are observed. In the Type 1, or 'honeycomb', pattern (Fig 1), the prism junction regions are elevated above the prism cores. In the Type 2 pattern, the prism junctions appear widened and are seen as clefts surrounding the prism cores (Fig 2). The Type 3 pattern is characterised by an irregular surface, with no features relating to the prism structure.

Most studies have employed unstirred etching solutions, with the following results. The Type 1 pattern was produced by 0.01-1.0M solutions of weak organic acids acting for periods between 15 min and 24 h [36,44,57,63], and by 0.01-1.0M HCl acting for more than about 30 min [44,77]. The Type 2 pattern was observed with EDTA at near-neutral pH [36,37,44,57,63,77]. Etching for short periods (2 min) with HCl at 0.01M or greater produced a pattern in which the prism cores had slightly elevated margins but were separated from each other by prism junction clefts [44]. With increasing exposure, the clefts progressively disappeared and the honeycomb pattern became predominant. A similar result was obtained after short exposure to HCl and to high concentration of weak acids (lactic, phosphoric) in a moderately stirred system [11]. The Type 3 pattern was produced especially by high concentrations of phosphoric acid but also by long exposure to lactic acid solutions [74]. With these latter treatments the etching pattern varied over the surface of each specimen.

Clearly, the Type 1 and Type 2 etching patterns are influenced by the prismatic structure of enamel. Of the possible controlling

factors, two have been most often invoked to account for the response to etching: (a) Differential porosity. TEM evidence [35,60] indicates that the largest pores in enamel are located at the prism junctions, where there is an abrupt change in crystal orientation. The 'tail' regions of the prisms may also be somewhat more porous than the prism bodies because of divergence of the crystals away from the parallel, closely packed arrangement in the bodies [35]. In more porous regions, the higher ratio of intercrystalline pore volume to crystal surface area will result in faster dissolution of mineral from the accessible surfaces. (b) Variations in crystal orientation between the prism bodies and the prism 'tails', combined with preferential dissolution along the c-axes (see above).

Differential porosity alone can account for the etching pattern produced by EDTA. Here the prism junctions are enlarged by dissolution to a considerable depth in the enamel and the prisms are thinned down by progressive dissolution of the peripheral crystals [77]. Variation in crystal orientation seems to have no influence on this pattern; combined SEM/TEM observations [77] indicate that at the advancing front prism bodies and tails are eroded at about the same rate.

Besides differential porosity and solubility, additional factors have been invoked to account for the honeycomb pattern. It has been suggested that the crystals adjacent to the prism junctions may be more acid resistant [77], either because of compositional differences, e.g., enhanced fluoride content [11], or because of protection by organic material [44,63]. However, an additional factor - re-precipitation - was demonstrated by Tyler [84], who showed that the ridges of the honeycomb pattern produced by brief (10 min) etching with unstirred organic acids were located at or even above the level of the original surface. Moreover, when vigorously stirred the same etching solutions preferentially attacked the prism junction regions to give a Type 2 pattern (Fig. 2). Boyde et al. [11] confirmed Tyler's findings by showing that crystals in the ridges of the honeycomb did not have the well-established pattern of orientation for enamel and that the ridges joined up with each other, whereas the true prism junctions do not. They also showed that the etching pattern observed is influenced by the collapse of porous masses of crystals on to the surface during drying.

The conclusion from these findings is that in unstirred solutions the concentrations of calcium and phosphate ions build up within the specimen when a steep concentration gradient, favouring inward diffusion of protons and outward diffusion of calcium and phosphate ions, is not maintained by efficient stirring of the bulk solution, to the point where calcium phosphate precipitation can occur at the prism peripheries. Ridges then form by preferential dissolution of intra-prismatic mineral.

Differential porosity combined with re-precipitation of dissolved mineral seem to be the dominant factors in producing the Type 1 etch pattern. The pattern cannot be explained by preferential dissolution along the c-axes, as this would result in at least some widening of the prism junctions through attack on the inclined crystals in the prism tails. The concept that the peripheral crystals are acid-resistant is contradicted by the results of etching with EDTA, where the dissolution pattern

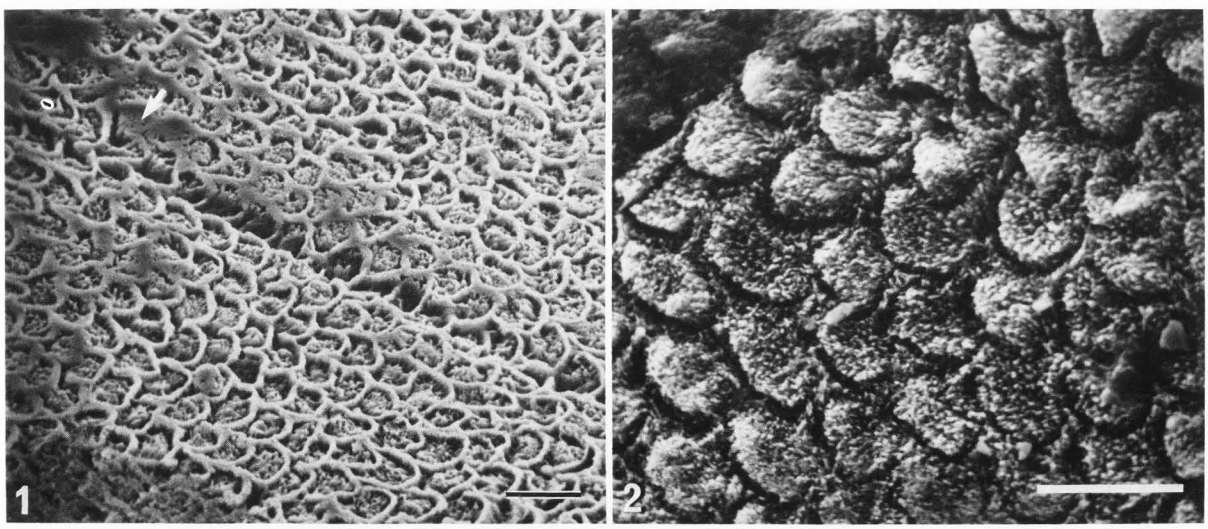

Fig. 1. Type 1 etch pattern. Specimen etched for 10 min with unstirred lactic acid (0.25M, pH 4.0). Note areas (arrowed), where the smear layer produced by cutting/polishing has been incompletely removed by etching. Bar = 10 μm

Fig. 2. Type 2 etch pattern, produced by a well-stirred solution of 0.25M lactic acid, pH 4.0 for 10 min. EDTA would produce deeper prism-junction clefts.
Bar = 10 μm

is not complicated by re-precipitation. TEM of HCl-etched enamel revealed the presence below the surface, between the prisms, of polyhedral crystals which did not show signs of partial dissolution like the intra-prismatic crystals [77]. These were not found in unaffected enamel so had formed during the etching. The ridges of a particularly well-developed honeycomb pattern developed in citric acid-etched fluorotic enamel [78] were composed of rounded, granular particles quite unlike the large, elongated apatite crystals characteristic of this tissue; further evidence for the importance of re-precipitation in formation of the pattern.

Re-precipitation would occur under conditions favouring a rise in the degree of supersaturation with respect to the precipitating calcium phosphate salt. Besides lack of stirring, these would include: relatively high pH; small volumes of etchant solution; prolonged etching times; low acid concentrations; the use of organic acids (which at a given molarity would produce a lower $[H^+]$ than mineral acids and would also tend to buffer the solution at pH 3.0-5.5). In general these conditions produce the honeycomb etch pattern. Phosphoric acid solutions, although having a low pH (pK_1 = 2.15), also supply phosphate anions which raise the calcium phosphate ion product and are thus likely to give a range of etching patterns.

It is extremely unlikely that solutions used in 'unstirred' etching experiments are truly static. Localised fluid movement, due both to thermal and mechanical disturbance and to CO_2 bubble formation (with acid etchants), are probably sufficient to account for variations from place to place observed in some experiments [74].

The etching of an enamel specimen differs from carious dissolution in several important ways. Material is removed directly from the surface as well as from beneath the surface, the etchant solution often has a much lower pH than occurs in plaque, and the etchant may be of a type (e.g., HCl, EDTA) or at a concentration which enamel is not exposed to in the mouth. Nevertheless, etching experiments contribute to the understanding of cariogenesis: (a) Dental plaque prevents fluid movement at the enamel surface, so that the attack of plaque acids on enamel corresponds to a completely unstirred etching experiment. On the basis of the duration of the Stephan pH curve, and the plaque acid profile [27], the conditions would approximate to etching with low concentrations of organic acids (0.05M or less) for short periods (approx. 10 min). Where the prism structure reaches the enamel surface, preferential dissolution at the prism junctions, accompanied by re-precipitation, would be expected. However, the porosity of the prism bodies and also of non-prismatic regions of the surface will be increased as well. In vivo, the enamel surface is exposed to fluctuating pH and to a medium containing calcium and phosphate ions. This is bound to modify the pore structure established by the initial exposure to acid. Thus SEM examination of the outer surface might yield relatively little information about the pore structure beneath the surface whether this presents an etch-like pattern or not. (b) The correlation between enamel structure and etch pattern indicates that the most obvious changes at the advancing front of a lesion would be at the prism junctions. (c) Although the crystals formed at the prism peripheries during acid etching [77] have not been characterised, they are strikingly similar in both morphology and location to crystals observed in early carious lesions [43,49,52]. This raises the possibility that the latter are formed during the initial dissolution of the enamel. Their occurrence only near the junctions may be due to differences in the nature of mineral in this region compared with that in intra-prismatic enamel (e.g., the presence of Mg which could promote whitlockite formation). Re-precipitated non-apatitic calcium phosphates would tend to re-crystallise at neutral pH into apatite but this process might be slow and in a lesion exposed repeatedly to acid conditions the crystals might persist for a considerable time. The occurrence of crystals at the prism peripheries has encouraged the belief that lesions may remineralize under natural conditions by precipitation of exogenous calcium and phosphate during periods when pH is near neutral [e.g., 43]. However, if the crystals are a relic of the initial dissolution process, then their presence does not signify partial reversal of mineral loss. Moreover, at the low supersaturation provided by calcium and phosphate concentrations in the oral fluids, new mineral is more likely to form by growth of pre-existent enamel crystals rather than by precipitation of new crystals. These considerations influence interpretation of SEM work on crystal size in carious enamel in relation to remineralization (next section).

An interesting footnote is provided by the effects of oxalic acid on enamel [65]. This acid produced a crystalline coating over the surface, together with numbers of hollow spheres attached to the tooth, both spheres and coating consisting of highly insoluble calcium oxalates. With time the spheres grew, often to a large size (2 mm diameter). Beneath the spheres, areas of subsurface damage, having some features of carious lesions, were produced, extending up to 500 μm into the enamel. A possible mechanism for these phenomena (GH Dibdin, personal communication) is as follows. Calcium ions released by the initial exposure to the acid precipitate with oxalate at the enamel surface but inside the layer there would be an excess of phosphate ions. Diffusion of protons from the bulk solution would compensate for the resulting charge imbalance and reduce the pH of the inner solution, which would thus begin to dissolve the enamel mineral and also the calcium oxalate 'membrane'. The integrity of the latter would be maintained by combination of calcium ions diffusing out through it with oxalate ions, resulting in precipitation of calcium oxalate at the outer surface. These phenomena have a certain similarity with some of those regarded by Brown [15] as important in the formation of carious lesions and the interactions of oxalic acid and enamel might provide a useful model system for investigating the chemistry of lesion formation.

Shape and size of crystals

SEM is a potentially useful method for evaluating changes in crystal size with demineralization and remineralization of enamel lesions because more specimens can be examined than with TEM and the ability to examine wider areas allows more extensive sampling of individual crystals. Whereas TEM can be used to measure thickness and width separately (assuming the crystals are sectioned more or less parallel with the basal plane), SEM yields an average diameter equivalent to the mean of thickness and width [3,5].

One qualitative study [18] employed polished surfaces etched with HCl (0.01M, 30 sec) but all other studies have used fractured surfaces, to avoid the artifacts induced by polishing. Usually, these surfaces have been washed with water only but sometimes have also been lightly etched (0.01M HCl, 10 sec) by Arends' group [3,5]. This procedure was used to improve image quality and was said not to alter crystal size significantly; comparative measurements indicated a reduction in diameter of about 2 nm. However, etching of any sort is probably best avoided. Because of the small crystal diameters, accurate knowledge of the thickness of the applied heavy metal coating is essential. Arends et al. [3,5] coated glass slides with gold and estimated coating thickness spectrophotometrically. They reported a thickness of 10 $\pm$ 0.5 nm but have not published details of how their calibration curve was constructed. Silverstone [72,73] coated standard latex spheres with platinum and reported a thickness of 5-7 nm. However, since the spheres had an uncoated diameter of 80 $\pm$ 5 nm, the error in his estimate of coating thickness is considerable.

Estimates for crystal diameters in non-carious enamel, both for normal [3,5,72,73] and fluorotic enamel [3,78], show good agreement with TEM measurements. Arends et al. [5] could demonstrate an increase in crystal size in surface enamel following eruption which agrees with observations of a post-eruptive reduction in porosity [20] and is relevant to caries in indicating that mineral from the oral environment can be incorporated into enamel, possibly as a result of both dissolution and reprecipitation.

The only published observations on crystal size in carious enamel are those of Silverstone et al. [72,73,75]. These workers used a technique in which ground sections were first characterised by light microscopy and then split carefully into slivers for SEM study. This procedure enables close correlation of optical and ultrastructural features and such an approach is to be commended for all SEM studies of carious enamel. They reported that crystal diameters in the translucent zone (25-30 nm) and in the body of the lesion (10-30 nm) were smaller than in sound enamel (35-40 nm). Crystals in the surface zone and, to a greater extent, the dark zone (40-80 nm and 50-100 nm, respectively) showed an increase in diameter compared with sound enamel. They interpreted these results as evidence that acid attack involved loss of mineral from crystal surfaces in the translucent zone and the lesion body, but that in the dark zone and the surface zone this was compensated or over-compensated by remineralization.

Several studies have dealt with the effects on crystal size of remineralization, whether by exposure to saliva *in vivo* or to calcium/phosphate solutions *in vitro*. The work of Arends et al. indicates that, following remineralization, crystals do not show central dissolution and tend to be less regularly orientated than in sound enamel [3,18]. Material scraped from the surfaces of lesions remineralized *in vitro* contained crystals up to 200 nm in diameter [17]. Crystals in lesions of bovine enamel observed *in situ* had mean diameters of 97 $\pm$ 11 nm after *in vitro* remineralization and 63 $\pm$ 7 nm following *in vivo* remineralization, compared with a value of 57 $\pm$ 7 nm for sound bovine enamel [3]. These results suggest that exposure to saliva promotes remineralization very slowly compared with laboratory treatments. Following *in vitro* treatment with a highly supersaturated solution (3 mM Ca, 1.84 mM P_i), Silverstone et al. [73,75] observed deposition of plate-like crystals on the outer surface of the lesion and crystal diameters of 50-75 nm within the lesion. After exposure to a less super-saturated solution (1 mM Ca, 0.61 mM P_i), surface deposition was not observed and crystal diameters within the lesion were in the range 50-150 nm, with some as large as 200 nm.

Interpretation of these results presents some problems. A difficulty common to all studies is that it is not clear what part of the enamel structure is being sampled. Sound enamel cleaves preferentially along the prism junctions [66], so that the exposed surface yields a selective sample of the crystal population. If carious enamel fractures like sound enamel, as a number of illustrations suggest it frequently does [8,14,42,67], there is a strong bias towards crystals which, because of their proximity to the prism junctions, the main diffusion pathways through the tissue, might be unrepresentative of the crystal population as a whole. On the other hand, if carious enamel fractures differently from sound enamel, both types of specimen should be sampled in such a way that similar populations of crystals are compared. Unfortunately, no details of the sampling procedure are reported in these studies. Therefore it is possible, for instance, that the larger crystals observed in remineralized lesions are confined to the prism junction regions. This applies also to the greater variation in crystal orientation noted in remineralized enamel.

In the work of Arends et al., it is in addition not clear whether remineralized lesions were sampled at a single depth or at all levels. Further, these workers have not given data for crystal sizes in the lesions *before* remineralization, so it is not possible to evaluate the full extent of crystal growth. Nevertheless, because they measured large numbers of crystals and presented details of size distributions, it is clear that crystal growth occurs after remineralization, resulting in crystals larger than usual *in vitro* and of about normal size *in vivo*. Ten Cate et al. observed [18] that in

remineralized lesions the crystals do not show central dissolution defects, even after brief etching. This suggests either that new crystals have been initiated or that partly-dissolved crystals have 'filled in' as well as expanded through growth. It also suggests that the crystals may be better crystallised and less acid-soluble than before the treatment. Combined SEM/TEM study of this material would be highly desirable.

While the data of Siverstone et al. on remineralized lesions agree with those of Arends' group, their results cannot be evaluated properly, since they report only the ranges of crystal sizes. They neither quote the mean value nor give any measure of the distribution of the values. Also, their results on crystals within lesions conflict in several ways with established knowledge of enamel structure: (a) TEM indicates dissolution of the original crystals in both the translucent and dark zones [49,59]. Short crystals possibly arising from re-precipitation tend to be found towards the body of the lesion [43,49,59]. It is difficult to reconcile this pattern with the report by Silverstone et al. that the size range for dark zone crystals was well above that for sound enamel and that the two ranges did not overlap. (b) In the body of the lesion, most crystals (within the prisms) show loss of material [43,49] but much of this is due to central dissolution and the outer dimensions of the hollow crystals range up to 120 X 60 nm [43]. Moreover, polyhedral crystals at prism peripheries are rather larger than this [43,49, 85]. These findings conflict with the narrow range of 10-30 nm diameter reported by Silverstone et al. A possible explanation is that the profiles measured were fragments remaining after central dissolution of the crystals. In a TEM study, Jongebloed et al. [48] found that acid-treated crystals with central defects had a size distribution little different from that for sound enamel, whereas acid-treated crystals without central defects showed increased numbers in the 10-20 nm range, because of inclusion in the sample of fragments of severely attacked crystals. (c) In view of the very close packing of crystals in sound enamel [35,49,60], increases in mean crystal diameter in the dark zone and surface zone, of the magnitude reported by Silverstone et al. [72,73,75], are extremely improbable, as they could be accommodated only by extensive re-crystallization to reduce the number of crystals per unit volume. The reduction in mean diameter of 10 nm (about 30 per cent) of translucent-zone crystals is also unlikely; it would imply an increase in pore volume greatly in excess of that actually observed (about 1 per cent [33]).

Boyde [8] illustrated a group of large rectangular crystals, identified in other work as whitlockite [52], on a fractured surface, presumably of grossly carious enamel. There exists no report of the polyhedral crystals frequently observed by TEM in uncavitated lesions. Whether this is a function of the types of lesion examined so far, or whether it indicates artifact in either the SEM or TEM techniques is not known.

Methods for studying lesion structure

Although the first published SEM images of carious enamel [12,13] employed back-scattered electrons, only secondary electron imaging has been used since. No studies exploiting the potential of back-scattered electron imaging [10] for studying density variations in carious enamel have been published.

Specimen preparation techniques have been adapted from those established in the study of sound enamel. The outer lesion surface has been examined both with and without removal of organic material using hypochlorite (sometimes combined with ultrasonication). Internal surfaces have been prepared by various methods, including fracturing and cutting, polishing and etching.

The outer surface of the lesion

Most SEM studies of early enamel caries have concentrated on following changes at the tooth surface. The surface of sound deciduous enamel appears relatively smooth and featureless when the outermost enamel is aprismatic [31]. However, this layer is often absent and the surface is then marked by shallow depressions marking the sites of the short Tomes processes of ameloblasts in the last stages of their activity. Sometimes the pits are deeper and the surface has a honeycomb appearance. Perikymata are not observed on deciduous enamel surfaces owing to the shallow slope of the Retzius lines but are usually pronounced on permanent enamel surfaces due to the much steeper inclination of the lines to the surface. Typically, Tomes process pits are found in the troughs of the perikymata while the perikyma crests are smooth, because of the presence of an aprismatic superficial layer [9,25,38,40,82]. This smooth surface may be punctuated by depressions a few μm deep, referred to as 'isolated deep pits' (IDP) [9] or 'focal holes' [25,38] and by small masses of mineral ('surface overlapping projections') [9]. The Tomes process pits, IDP and projections are phenomena of developmental origin, as they are all observable on unerupted teeth [9,25,38]. Usually the Tomes process pits and IDP are filled by organic material and become visible after treatment with urea [6,28] or hypochlorite [9,25,82]. It has been suggested that the IDP may result from the breaking away of surface projections [9].

Holmen and Thylstrup [39] observed, near fissures on partly erupted teeth, white patches of small-scale surface porosity which they interpreted as evidence of carious change occurring immediately on exposure to the oral environment. However, it is equally likely to reflect the inherent porosity of the last-formed enamel, which is reduced by post-eruptive maturation [20].

On surfaces overlying early carious lesions, changes occur in the features mentioned above. There is said to be an increase in the number of IDP [80], although this has not been systematically quantified. The Tomes process pits become accentuated, showing thickened peripheral walls often separated from the central depressions by

arcuate clefts [25,32,40,80]. Large areas of caries-affected deciduous enamel surfaces show this pattern where the aprismatic layer is absent [31]. Aprismatic surfaces, including the smooth crests of perikymata, assume an irregular, fissured appearance [25,31,32,82] and a network of fissures may also develop between the Tomes process pits [81]. Loss of material from the crests of the perikymata is observed, through fracture of layers weakened by partial dissolution [25,40,53,80]. This flaking of the outer, aprismatic enamel exposes underlying prismatic enamel.

These relatively large-scale changes suggested that there exist major points of entry in the surface layer, e.g., the prism junctions, through which plaque acid gains access to the subsurface enamel [31,32,80,82], although the IDP have been considered unimportant in this respect [32]. More recently, there has been increased recognition of the possible importance of a more widespread increase in porosity, partly through formation of small holes, about 0.5 μm in diameter [40], but mainly by generalised opening up of intercrystalline spaces by partial crystal dissolution [38,39,40]. In diphosphonate/lactic acid-induced artificial lesions, the latter appeared to be the main effect [41].

A generalised increase in porosity of the surface layer should not be a matter for surprise, since microradiographic estimates suggest mineral contents for this zone of only 75-85 vol % in natural lesions [7,79] and 48-68 vol % for artificial lesions [30]. The concept of a low surface-zone porosity (1-5 vol %) has been fostered mainly by polarised light studies [71], but recent work indicates that the theory on which these were based is defective [70].

Fejerskov et al. [25] pointed out that specimen preparation procedures, especially ultrasonication, appeared to increase artifactually the number of IDP on the surfaces of erupting teeth but insufficient attention has been paid to the possible effects of specimen preparation technique on the structure of porous surfaces overlying carious lesions. Such effects could include the flaking off of portions of the perikyma edges and the irregular fissuring of aprismatic surfaces.

Internal structure of the lesion

Ideas described above concerning the diffusion of acid into the tooth and the pattern of dissolution of the surface layer, being based largely on observations limited to the two dimensions of the enamel surface itself, must be tested by examining the internal structure of the lesion. While several such studies have been carried out, few useful results have emerged. This stems from the limited amount of information about porosity that can be extracted from the types of internal enamel surface examined. First, observation is restricted essentially to two dimensions, whether the surface is prepared by fracturing, diamond planing or polishing and etching, so that the true depth and form of pores which open at the surface cannot be determined. Secondly, each preparation method is prone to artifact. Diamond planing of carious enamel can pull out subsurface material. Voids at the surface of fractured specimens cannot always be interpreted as genuine pores because of the possibility that crystals and enamel fragments have been detached during fracturing. The worst artifacts are probably associated with cut/etched surfaces. The sectioning process inflicts damage for some depth into the section and creates an unusable 'smeared' surface [42], which can only be removed by etching. To minimise the amount of etching necessary, the smeared layer has to be reduced in thickness by polishing but this should employ a series of progressively finer abrasives. The use of fine abrasive alone may only polish the surface of the smeared layer without reducing its thickness sufficiently. The final etching process may introduce new artifacts. If not sufficiently thorough, it will leave some of the specimen covered by remains of the smear layer (see Fig. 1 for example), and in any case the acid will remove some undamaged tissue and can create artifactual voids. Finally, because of the porosity of carious enamel, critical point drying or freeze drying should be routinely employed to prevent collapse of the structure.

On surfaces planed smooth using a diamond knife in an ultramicrotome [50], the main SEM feature was loss of material in patches oriented in rows corresponding to Retzius lines, although TEM examination of sections cut from the block faces indicated significant mineral loss within prisms and widening of prism junctions. Fractured surfaces [14,67] indicate considerably increased small-scale porosity between the crystals at the surfaces of prisms separated longitudinally. A correlated polarised light/SEM study using fractured surfaces through artificial lesions [42] indicated that in the body of the lesion there appeared to be often pronounced loss of intra-prismatic mineral and that the inter-prismatic crystals were more rounded than those of sound enamel. The dark zone and translucent zone consisted of almost completely normal prisms.

Faces cut at right angles to the outer surface show, after etching, fissures or narrow pores, up to 1 μm wide, extending through the surface zone [29,31,32]. This finding agrees with TEM observations [26] but the SEM appearance of these pores must be interpreted with considerable caution because at a cut surface it may not be possible to distinguish pores from cracks reliably, and it is not known to what extent they are enlarged (or even created artifactually) by the etching and drying processes. This qualification applies especially to the report that the pores extend up to 100 μm into the enamel [29], as in this case the specimens were extensively etched (35 per cent phosphoric acid, 1 min). However, replicas prepared by infiltration with Epon followed by acid dissolution of mineral revealed pores extending through the surface zone [29]. Hence it can be concluded that major surface layer pores do exist, although almost certainly not to a great depth.

Internal surfaces of artificial lesions prepared by cutting, polishing with 0.25 μm

diamond powder and etching with 0.01M HCl for 10 sec showed a loss of detail in the prisms compared with the crystalline appearance of sound enamel and prominent gaps at the prism junctions [47]. The abnormally wide prism junctions of sound enamel depicted in this study indicate the presence of etching or shrinkage artifact. Haikel et al. [31,32] observed on cut surfaces etched with 0.02M HCl (apparently without prior polishing) broad prism sheaths but referred [32] both to 'complete loss' of prism cores and 'apparently intact' prism cores. The prism cores showed a rather inexplicable lack of detail. Despite uncertainties about the level of etching artifact, the observation of thickened prism sheaths agrees with findings on carious enamel exposed by chipping of the surface layer, either in vivo or during extraction [64,80]. These surfaces showed a honeycomb pattern of thickened ridges enclosing depressions corresponding to prism bodies, which seems to indicate consolidation of the sheath regions. The surface of the honeycomb appeared granular, lacking profiles of elongated crystals, and this perhaps represents re-precipitated material.

Studies of fractured specimens indicate that bacteria penetrate a high proportion of macroscopically uncavitated white-spot lesions [14] and even sound (fissure) enamel [67]. The bacteria were situated mainly between the prisms and were often reported to occupy cavities within the surface. Although bacterial forms have also been reported in carious enamel by TEM of replicas [52], these SEM results, especially those on sound enamel, are somewhat surprising and further work is needed to confirm them. Unfortunately, no details on precautions against microbiological contamination of the specimen surfaces were given. In addition, no stereo-pair photographs were provided to allow the three-dimensional relationship between the bacteria and the enamel to be studied.

Direct observations of pore structure

To avoid the artifacts associated with the methods used in the work described above, we have recently applied a replication technique to visualise the pores in carious enamel. Specimens are thoroughly infiltrated with methyl methacrylate monomer, which has a very low viscosity and a relatively small molecular size, after prolonged Soxhlet extraction with chloroform/methanol, to remove water, lipid and, most importantly, all imbibed air from the tissue spaces. Except for very small specimens, slow polymerisation (1-2 weeks) at 20-30°C is crucial to success of the method. First, it allows time for complete infiltration of all accessible pores. Secondly, it allows plastic flow of the partially polymerised resin [54], so that resin is continually drawn into the specimen and good replication is achieved despite the high bulk shrinkage (approx. 20%) due to polymerisation [54]. Some procedures have been published [33,54,69] and others will be described in full at a later date. The three-dimensional methacrylate replicas, exposed by dissolution of the enclosing mineral with 1-2M HCl, are mechanically stable but beam current must be minimised to avoid excessive heating of the replica, which causes shrinkage. Space replicas of both whole lesions and of particles micro-dissected from histological zones of sectioned lesions [33,34] have been examined. The following summary of our principal findings is illustrated mainly by replicas of lesions infiltrated in situ and exposed after preparing an internal surface by fracturing or by cutting and polishing. The lesions were either natural or were artificially produced by exposure to lactate/methylcellulose gel, pH 4.5 [69].

In all lesions, the inner border (translucent zone) was characterised by infiltration only of the prism junctions (Figs. 3,4). In the body of the lesion, there was extensive infiltration of the prisms themselves (Figs. 3,5-8), but there was an important difference between natural and artificial lesions. In artificial lesions the prism junctions remained heavily infiltrated and appeared as linear features between the spongy prism replicas (Figs. 3,6). In natural lesions, on the other hand, while prism junctions were often infiltrated in shallow lesions (Fig. 8), in deeper (more advanced) lesions the prism replicas were nearly always separated by clefts which tended to widen under the heating action of the beam (Figs. 7,8). We have not so far fully investigated pore structure in the dark zone. In lesions infiltrated in situ the transition between the inner zone of prism junction infiltration and the body of the lesion was marked by partial infiltration of the prisms (Figs. 3,8). Replicas of particles dissected from lesions showed complete infiltration of prism junctions in the inner translucent zone (Fig. 4) but in the outer part of this zone, towards the body of the lesion the junction replicas tended to be fenestrated (Fig. 9). Replicas of dark-zone particles examined so far have shown infiltration only of the prism junctions. These replicas were often very delicate, the prism-junction replicas being not only fenestrated but detached from each other (Fig. 10). Fenestration of the prism-junction replicas was also frequently observed in the inner region of natural lesions infiltrated in situ.

The surface zone was highly variable. In artificial lesions it showed extensive infiltration, the resin filling numerous fine, closely spaced pores lying perpendicular to the surface. In such lesions in deciduous teeth the surface layer was so porous that it could not be distinguished from the lesion body (Fig. 5). The surface layer of some artificial lesions in permanent teeth contained, as well as fine pores, more pronounced, laminar pores at the site of the prism junctions (Fig. 6). The surface layer of natural lesions varied from being almost as porous as in artificial lesions (Fig. 7) to a condition where it was traversed by much fewer, separate pores (Fig. 8). Porosity could be concentrated in the prism bodies (Fig. 7) or at the prism junctions, or could be apparently unrelated to prism structure (Fig. 8).

These results indicate that the principal event at the advancing front of the lesion is an

opening up of the prism junctions, which do not replicate in sound enamel except near the dentine in some teeth. This reinforces the concept that the junctions are more susceptible to the initial exposure to acid than the prisms (perhaps through the presence of more soluble mineral) and provide diffusion pathways into the enamel [19,33,34,49, 69,71,86]. It has been found [69] that deciduous enamel has a higher prism junction density, as estimated by planimetry of scanning electron micrographs, than permanent enamel and this was correlated with more rapid penetration of artificial lesions. In addition, the prism junction density will affect the rate at which acid gains access to the prisms and consequently the stage of lesion development observed in the body of the lesion, where mineral is lost from within the prisms. The mean surface area of the prism junctions is in deciduous enamel 0.78 m^2/cm^3 and in permanent enamel 0.64 m^2/cm^3. Combined with the larger volume fraction occupied by 'interprismatic' enamel (i.e., regions with less perfect crystal packing than in the prism cores [35]) in deciduous enamel (30 vol % versus

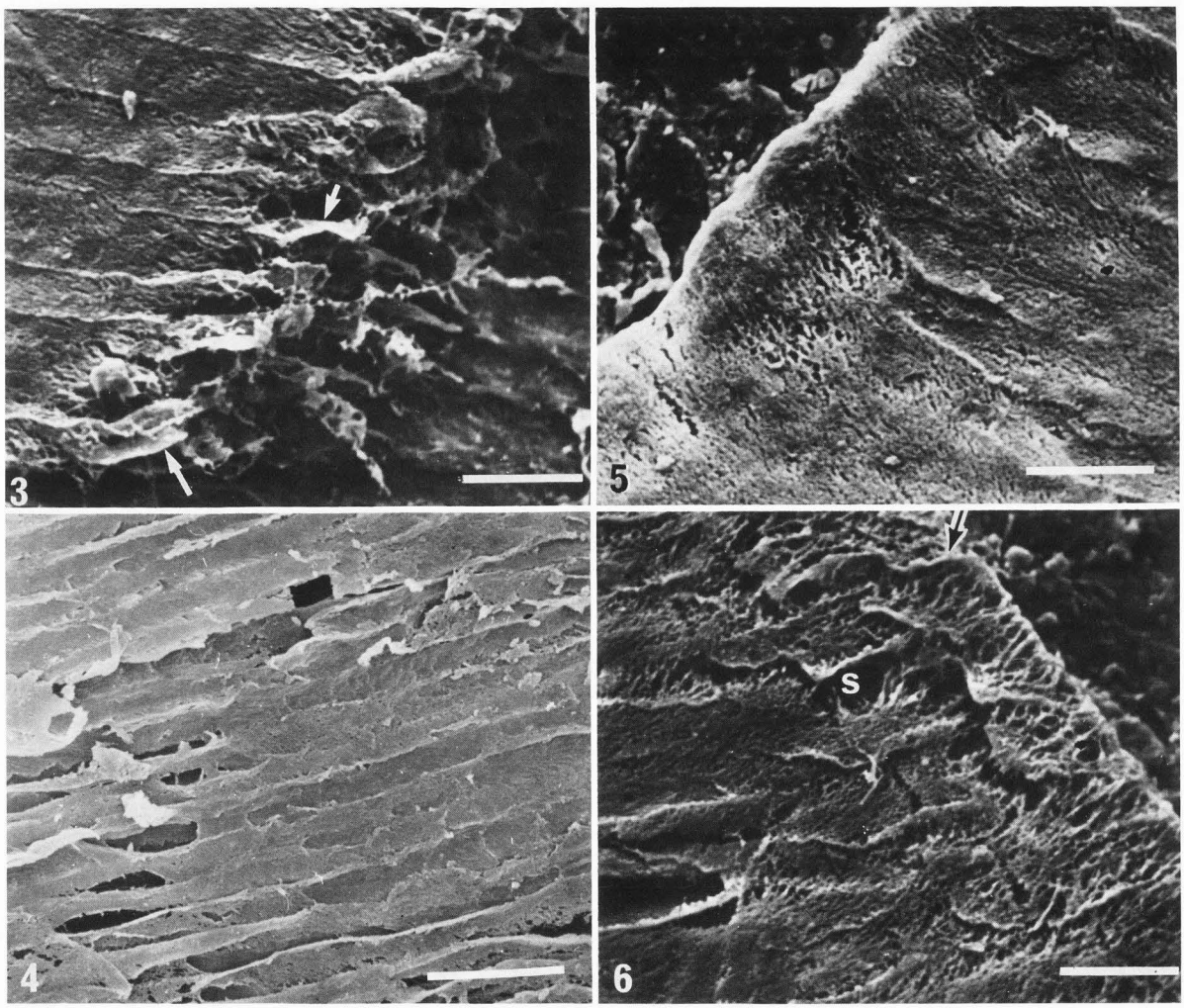

Fig. 3. Inner part of artificial lesion in deciduous molar. Narrow translucent zone running lower left to top right, with infiltration of prism junctions only (arrows). Body of lesion top left with infiltration of prism bodies and heavier infiltration of junctions. Between body and translucent zone a transitional zone showing partial infiltration of prism bodies. Polished surface. Bar = 10 μm.

Fig. 4. Particle micro-dissected from translucent zone of natural lesion in molar enamel. Inner region, showing complete infiltration of prism sheaths. Bar = 20 μm.

Fig. 5. Same lesion as in Fig. 3. Both surface zone (centre) and body of lesion (below) are extensively infiltrated and to similar levels. Polished surface. Bar = 10 μm.

Fig. 6. Artificial lesion in permanent premolar. Surface layer (upper right) is less completely infiltrated than the body of the lesion (lower left) and is traversed by replicas of micro-pores and by laminar replicas of prism junction pores (arrow). s = artifactual gap opened by beam-induced shrinkage. Polished surface. Bar = 10 μm.

23 vol % [69]), it would be predicted that the body of the lesion would lose mineral faster in deciduous than in permanent enamel. Qualitative comparison with polarised light [69] suggests that this is so and very low mineral contents (26-33 vol %) have been measured microradiographically in natural lesions of deciduous enamel [55].

Artificial and natural lesions show clear differences in the body of the lesion and in the surface zone. With respect to the lesion body, whereas in artificial lesions the prism junctions retain their porosity, they usually appear to be less porous in established natural lesions. The latter agrees with microradiographic observations [76,86] and we assume that the loss of porosity is due to re-precipitation, which is prevented in artificial lesions by continuous exposure to

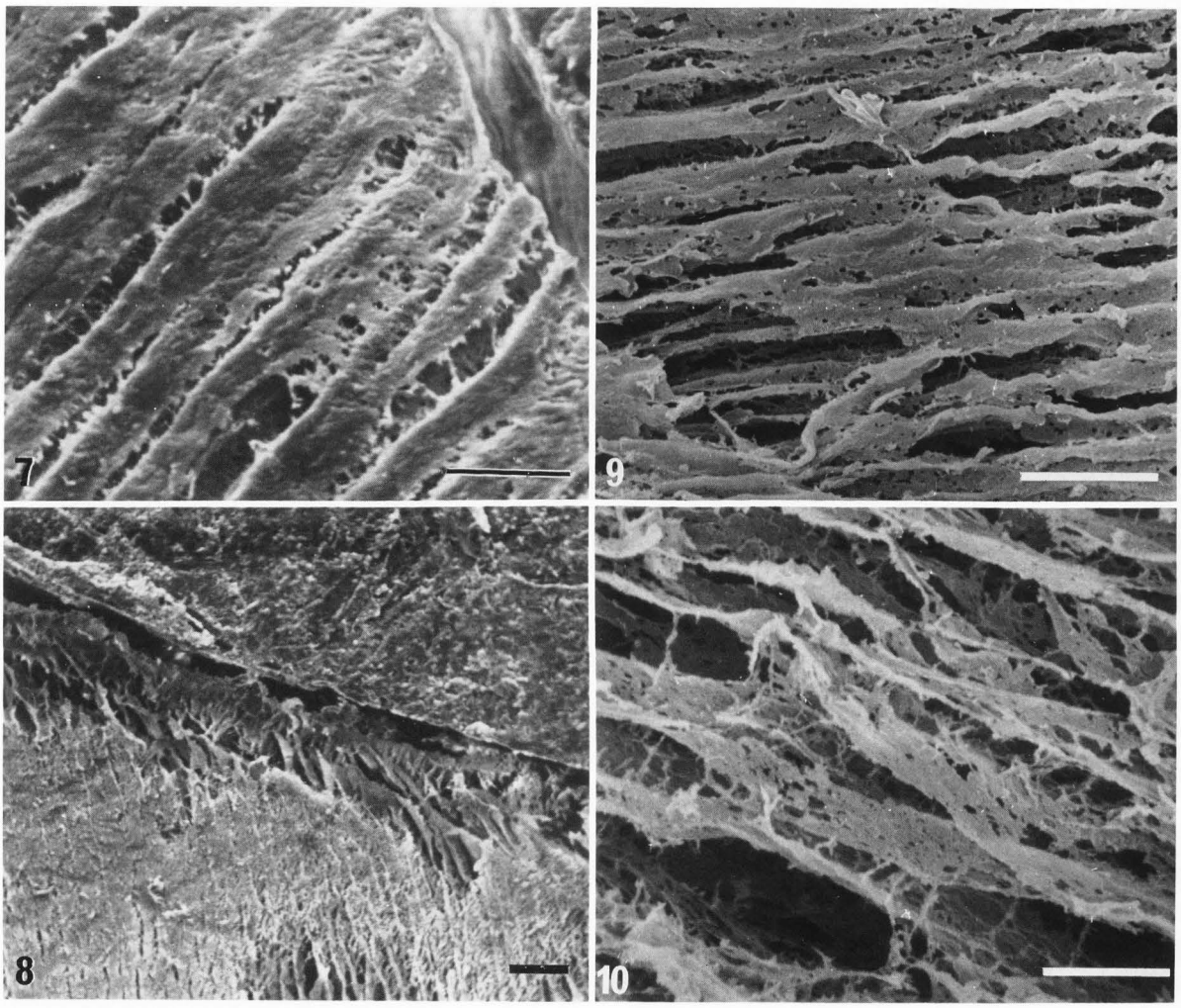

Fig. 7. Natural lesion in permanent premolar. Prism bodies in the surface layer (upper right) show about the same level of infiltration as those in the underlying body of the lesion. Clefts at prism junctions extending to surface. Polished surface. Bar = 10 μm. Note: specimen shrinkage has artifactually widened the prism junction clefts and caused some loss of focus.

Fig. 8. Natural lesion in permanent molar. Surface layer running diagonally across centre shows less complete infiltration than lesion body and is traversed by markings like Retzius lines. In the shallow marginal part of the lesion (right), clear replicas of prism junctions and partly infiltrated prism bodies, whereas in the deeper, central part (left), prism bodies heavily infiltrated and separated by clefts. Polished surface. Bar = 20 μm.

Fig. 9. Replica of same translucent-zone particle as in Fig. 4, but showing the outer region, towards the dark zone. Here the prism junction replicas are fenestrated, indicating partial occlusion of the pores, and are prone to shrink and separate. Bar = 20 μm.

Fig. 10. Replica of particle micro-dissected from dark zone of natural lesion. Only prism junctions are infiltrated but the replicas are very delicate (cf. Figs. 4,9), extensively fenestrated and have detached from each other during preparation for SEM. Bar = 5 μm.

acid. This structural difference presumably implies that different diffusion pathways exist in the two types of lesion.

As to the surface layer, our results confirm the existence of micro-pores [29,31,32] in many lesions but these are finer and usually much more abundant than suggested by replication with Epon [29], a highly viscous resin which would be prevented by surface tension effects from entering any but the largest pores. In artificial lesions the surface layer develops micro-pores over its whole area, in agreement with the generalised dissolution observed at the outer surface of diphosphonate lesions [41], but these may be augmented by the formation of laminar pores at prism junctions in permanent teeth. In natural lesions, the pattern is highly variable. Micro-pores, often with no obvious relationship to prism structure, were common and appear to correspond with voids seen by TEM [26,62] and with small holes detected by SEM at the outer surface [40]. The localisation of porosity, in different specimens, to either the prism junctions or the prism cores, corresponds with the variability observed in the appearance of the outer surface. Clearly, many more specimens need to be examined to establish the range of variation and the frequency of each type of structure, and to correlate the morphology of the outer surface with the underlying pore structure.

Our observations to date on the dark zone do not conclusively establish how the micro-porosity responsible for the optical behaviour of this zone is produced. Incipient dissolution of mineral within the prism bodies would produce micro-pores [49,59] and is a necessary transitional stage between the opening-up of the prism junctions in the translucent zone and the advanced demineralization in the lesion body. However, we cannot assign the zone of partial prism body replication in our lesions infiltrated in situ to the dark zone with certainty. Replicas of particles dissected from histologically identified dark zones showed only infiltration of prism junctions and suggested that partial occlusion of these laminar pores, presumably by re-precipitation of mineral, is a factor in producing micro-pores, in agreement with Silverstone [71]. However, as the micro-pores produce their optical effect by exclusion of media such as quinoline [71], which has approximately the same molecular size as the methyl methacrylate monomer, there must be a strong possibility that micro-pores too small to be infiltrated exist within the prism bodies.

Conclusion

SEM has contributed significantly to knowledge of the early carious lesion of enamel at all levels of structure. Although specimen preparation techniques have sometimes been applied without full consideration of their effects with respect to the information desired, the ease of specimen preparation compared with TEM has undoubtedly resulted in many more studies of lesion ultrastructure being undertaken than would have been the case if SEM were not widely available. In several instances, the instrument has provided information not accessible by other methods, or only with extreme difficulty. It is to be hoped that future work will continue to add to detailed knowledge of the morphological changes in carious enamel and thereby act as a corrective to over-simplified views of the caries process. In this respect, more studies combining SEM with other methods, chemical as well as microscopical, would be especially valuable.

We advocate the methacrylate replication technique for objective evaluation of the development of the pore system in carious lesions and for following changes resulting from experimental treatments such as remineralization procedures. It can be combined with optical microscopy and microradiography and, in conjunction with micro-dissection and gravimetric measurements [33,34], can yield quantitative information about porosity in fairly well defined parts of the lesion.

Acknowledgements

We thank Drs GH Dibdin, DFG Poole, C Robinson, JE Tyler and JA Weatherell for helpful discussions, Dr Tyler for the micrographs in Figs. 1 and 2 and Mrs MK Coomber for preparing the illustrations.

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Discussion with Reviewers

S.J.Jones: Using their replica technique, did the authors find that cross-striations were more easily demonstrated in natural or artificial caries compared with normal enamel?
Authors: Prism bodies do not replicate at all in sound enamel and we have only one (not very convincing) micrograph suggesting variation of porosity along the prisms in carious enamel. Small variations in porosity will probably be obscured in polished specimens and all our fractured specimens to date are of artificial lesions. Further work with fractured natural lesions might supply this information. Retzius lines are commonly seen as regions of increased porosity in natural lesions.

S.J.Jones: Was any evidence for lateral spread of the lesion seen at the periphery of natural or artificial caries, so that prisms unaffected at the surface were involved in deeper zones?
Authors: No. The only location where prisms were affected deep in the enamel but not at the surface was in the inner enamel adjacent to lesions which had reached the dentine. This would not be due to lateral spread but to acid reaching the inner ends of the prisms by way of the carious dentine.

S.J.Jones: How can one be sure that the fenestrations present in the prism boundary zones - replicas in, for example, Figure 9, represent real features?
Authors: We believe they are real because they can occur in some parts of a specimen and not in others, e.g., in translucent zone particles towards the dark zone but not near the sound enamel. They do not appear to be beam-damage artifacts; over-exposure to the beam tends to cause shrinkage of the replica as a whole.

A.Boyde: Your usage of prism periphery and prism junction is not too clear. What do you mean by these terms?
Authors: By prism junctions we mean the laminar pores formed by the planes of discontinuity of crystal orientation partly surrounding the prism bodies. The prism peripheries are the immediately adjacent layers of crystals.

L.Holmen: How come that the authors find it equally likely that white opaque areas on partly erupted occlusal surfaces should reflect lack of maturation rather than caries demineralization, when these surfaces were covered with bacterial deposits for a significant period of time, as clearly stated in Ref. 38?
Authors: During eruption, the crown of the emerging tooth will become colonised by bacteria. Therefore, post-eruptive maturation presumably proceeds despite exposure to bacterial activity.

J.D.B.Featherstone: What does the imbibition technique reported by the authors tell us about porosity that cannot be learned by other techniques, and what does the information tell us about the interpretation of other techniques?
Authors: The main advantage of the technique is that it provides a 3-dimensional image of the pore structure and gives qualitative information about relative porosities, pore sizes and pore shapes. Microradiography quantifies the average porosity throughout the thickness of a section. Polarising microscopy is very sensitive to small pores but only if they are orientated appropriately and a substantial fraction of the pores in enamel do not contribute to the image (ref. 70). TEM gives very detailed information but can sample only small volumes, gives only 2-dimensional information and is subject to severe artifact unless the sections are prepared by ion-beam thinning. The replication technique will therefore complement other methods, especially if used in conjunction with them on the same material.

J.D.B.Featherstone: The most important channels in the caries process may be the molecular sized openings between the crystals. Is this technique able to tell us anything about these smaller diffusion channels, since they may be the rate-determining, and hence the most important, diffusion pathways during the caries process?
Authors: The pores detectable by replication are those which: (a) can be penetrated by the monomer, (b) allow room for polymerisation, and (c) provide a replica substantial enough to be self-supporting. The molecular diameter of the monomer (estimated from the molar refraction) is about 0.45 nm but probably only pores several times larger than this can be visualised. In sound enamel the only pores replicated are: superficial porosity in newly-erupted teeth (ref. 20); stress cracks, especially in cuspal enamel; prism junctions in inner permanent enamel, where decussation is marked; tufts and lamellae. Narrow intercrystalline pores are thus not replicated. It seems likely that the rate of penetration of a lesion into enamel will be controlled by the larger pores (prism junctions) rather than by the small pores. See ref. 69.

J.D.B.Featherstone: How does the imbibition technique contribute to our knowledge about the transport of molecules through the organic matrix of enamel or even dentin? The organic matrix is removed before incorporation of the methacrylate and it may be that the protein/lipid is the primary controlling matrix in terms of diffusion of ions or molecules through enamel, carious enamel or dentin.
Authors: We remove only lipid (or part of it) in the preparation procedure, not the protein (although this could be done) and the replicas must consist of methacrylate + some protein, although

fine pores filled with protein will not be penetrated by the resin and not made visible. The effects of organic constituents on diffusion will have to be studied by other methods, e.g., tracer diffusion.

J.Arends: Is the etch pattern of the enamel related to the fluoride content of sound and carious enamel?
Authors: This point has not been studied. Most studies use polished facets in the enamel and are thus concerned with regions with relatively low fluoride compared with the surface.

J.Arends: Can porosity be observed by SEM of carious enamel or do the proteins precipitate under high vacuum and is the porosity 'apparent'?
Authors: Bearing in mind the small fraction of organic material in enamel (about 2 vol %) and the large surface area of the crystals, it seems possible that in sound enamel the protein may be present as a condensed film adsorbed to the crystals which would change little on drying. Carious enamel contains additional, exogenous protein which could be in a more extended state and an increase in the apparent porosity due to collapse of this protein could be a source of artifact in, e.g., SEM study of fractured carious enamel.