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Some Preliminary Observations on Caries ("Remineralization") Crystals in Enamel and Dentine by Surface Electron Microscopy

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Beobachtungen zur Remineralisation von Zahnhartsubstanzkristallen, Oberflächen-Elektronenmikroskopie

Zusammenfassung. Beschreibung verschiedener Kristallmodelle in cariösen Zähnen als Ausdruck eines Remineralisationsprozesses. Es können 2 Kristalltypen im cariösen Schmelz nachgewiesen werden: a) ein flach rhomboider Kristall mit 2 rhomboiden Breitseiten und b) ein prismatischer Kristall mit charakteristischem hexagonalem Querschnitt und typisch abgeflachten Enden. An der Schmelzoberfläche cariöser Zähne werden ferner bakterienähnliche Körper gefunden mit kristallähnlichen Oberflächen. In den Dentinröhren cariöser Zähne finden sich zusätzlich 3 Typen kristalliner Einschlüsse. Der größte Anteil kristalliner Verunreinigungen kann mit der Röntgenstrahlbrechung als β-Tricalciumphosphat identifiziert werden. Es ist wahrscheinlich, daß dieser Verbindung die rhombischen Kristalliten entsprechen.

Summary. The aim of this paper is to demonstrate a variety of crystalline forms that occur in the enamel and dentine of teeth with lesions undoubtedly due to caries and which are presumably the result of a "remineralization" process. Two definite crystal types were found in carious enamel; a flattened rhombohedral (monoclinic) crystal with two large rhomboidal faces, and a prismatic crystal with a hexagonal cross-section and typically flat end-faces. Also found on carious enamel surfaces were bodies strongly resembling bacterial forms and with crystal faces apparent at their surfaces. In carious dentine, three different morphological types of crystalline inclusion were found in tubule lumens in addition both to the rhombohedral crystals usually described and to the accumulation of peritubular (intratubular) dentine. The most abundant crystalline species found as a contaminant was identified as β-tricalcium phosphate by X-ray diffraction, and we presume this to correspond to the rhombohedral crystallites.

Introduction

There have been a number of reports of the occurrence in both carious enamel and dentine of crystals which are larger than, and of a different form to, those generally regarded as constituting the sound tissue (for example, HELMCKE, 1955, 1962; LENZ, 1955; TORELL, 1957; HÖHLING, 1961a, 1961b, 1966; TAKUMA and KURAHASHI, 1962; HERTING, 1966). Other workers have described structures similar to these "caries crystals" in teeth apparently unaffected by caries, although affected by processes such as masticatory wear, erosion, or osteoclastic resorption (see for example, SOGNNAES, 1963; KATTERBACH et al., 1965; BOYDE and LESTER, 1967). Although caries crystals, as generally conceived, represent the "crystallization by-products" of the caries process, they have received scant attention in their own right. It seemed important therefore, to examine human teeth with lesions undoubtedly due to caries to see if some basis could be established for comparison of the morphology of the caries crystals with those of apparently similar crystals occurring in other situations.

Materials and Methods

25 deciduous and 25 permanent human teeth with gross caries were fixed upon extraction in neutral formol saline and stored in 70% ethanol. 6 specimens were fixed and stored in acetone to eliminate the possibility of some demineralization occurring as a result of formic acid formation in the formalin solution. The carious cavity in each of these teeth involved extensive loss of enamel and dentine and the pulp chamber was "exposed" in most instances. Excess food debris (if present) was removed from the carious cavity with a suitable hand instrument. The specimens were placed in glass extraction thimbles in a Soxhlet condenser and subjected to 1:2-diamino-ethane ($NH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2$ and commonly known as "ethylene diamine") at a temperature of 116° C, the organic solvent being distilled through this apparatus continually for a period of at least 14 days. At the end of this period, 3 changes of absolute ethanol were distilled through in place of the diamino-ethane in order to wash the specimens, which were then stored in a desiccator until required. Specimens were broken open (by closing the jaws of a small vice in which they were held) so as to expose surfaces of enamel and dentine adjacent to the lesion. These prepared surfaces were coated prior to examination by one of two methods as described below.

The specimens for scanning electron microscopy (Cambridge Instrument Co. *Stereoscan*, operated at 10 kV; 100 seconds recording time) were given an electrically conducting coat of ca. 200° A of carbon and then ca. 300° A of gold, which were evaporated *in vacuo* onto the specimens whilst they were rotating and facing at an angle of approximately 45° to the evaporation sources.

Some details of the method for preparing material for examination via the replica method for transmission electron microscopy have been given by BOYDE (1967). However, the method used in the present study differs in that the specimens were extracted with 1:2-diamino-ethane before the carbon replica coating was applied. Thus, to release the replica it was only necessary to dissolve the resulting substrate in N/10 HCl. Some preselection of areas of interest was exercised by destroying the carbon replica coat on the tooth in the region which we did not wish to examine. The stereo-pair transmission electron micrographs were prepared using the standard tilt cartridge for the Siemens *Elmiskop I* (operated at 80 kV; 100 μ condenser aperture). The tilt device was calibrated by the method described by BOYDE and WILLIS (1966). The methods of stereophotogrammetric analysis applied to the resulting images have been detailed previously (BOYDE, 1967).

We would emphasize that the images as reproduced here will be of little value and may be confusing *unless* examined stereoscopically when they will be easily interpreted for what they are, namely, an indication of the three-dimensional shape of the surface in question. The easiest method of viewing these paired images stereoscopically is by means of a simple stereoviewer, which consists of two lenses held at their focal distance from the images. The distance between the lenses should correspond to the inter-ocular width of the observer. The use of these lenses relaxes the focus of the eyes to infinity when the viewing axes of the eyes also become parallel. With some practice, it is possible to separate the functions of focus and of convergence of the eyes so that the pairs may be seen stereoscopically without the aid of a viewer.

Observations

Enamel. The carbon replica method provides a clear illustration of three morphologically distinct types of "caries crystal" within carious enamel. These crystals are found both within the bulk of the enamel surrounding the cavity, where surfaces were exposed for study by deliberate fracture, and on the surface of the enamel forming the walls of the caries cavity itself.

One type of caries crystal is typically flattened or tabular, with its two largest faces (up to 0.75 μ across) rhomboidal in outline (Fig. 1 and HELMCKE, 1955, 1960, 1962; TORELL, 1957; HÖHLING, 1961 b). These crystals occur typically in extensive clusters over the surface of recognizable well-ordered enamel, there being an abrupt transition from the one to the other. The individual caries crystal elements,

Fig. 1

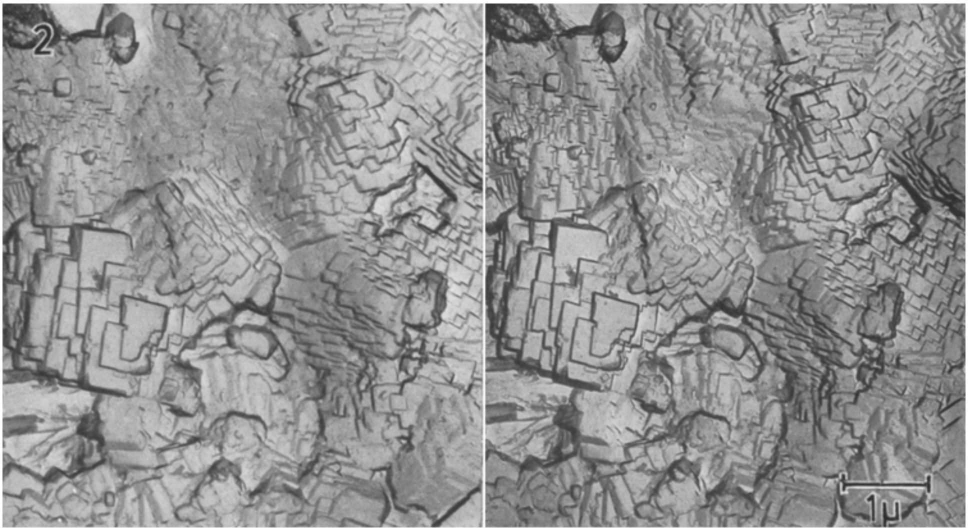


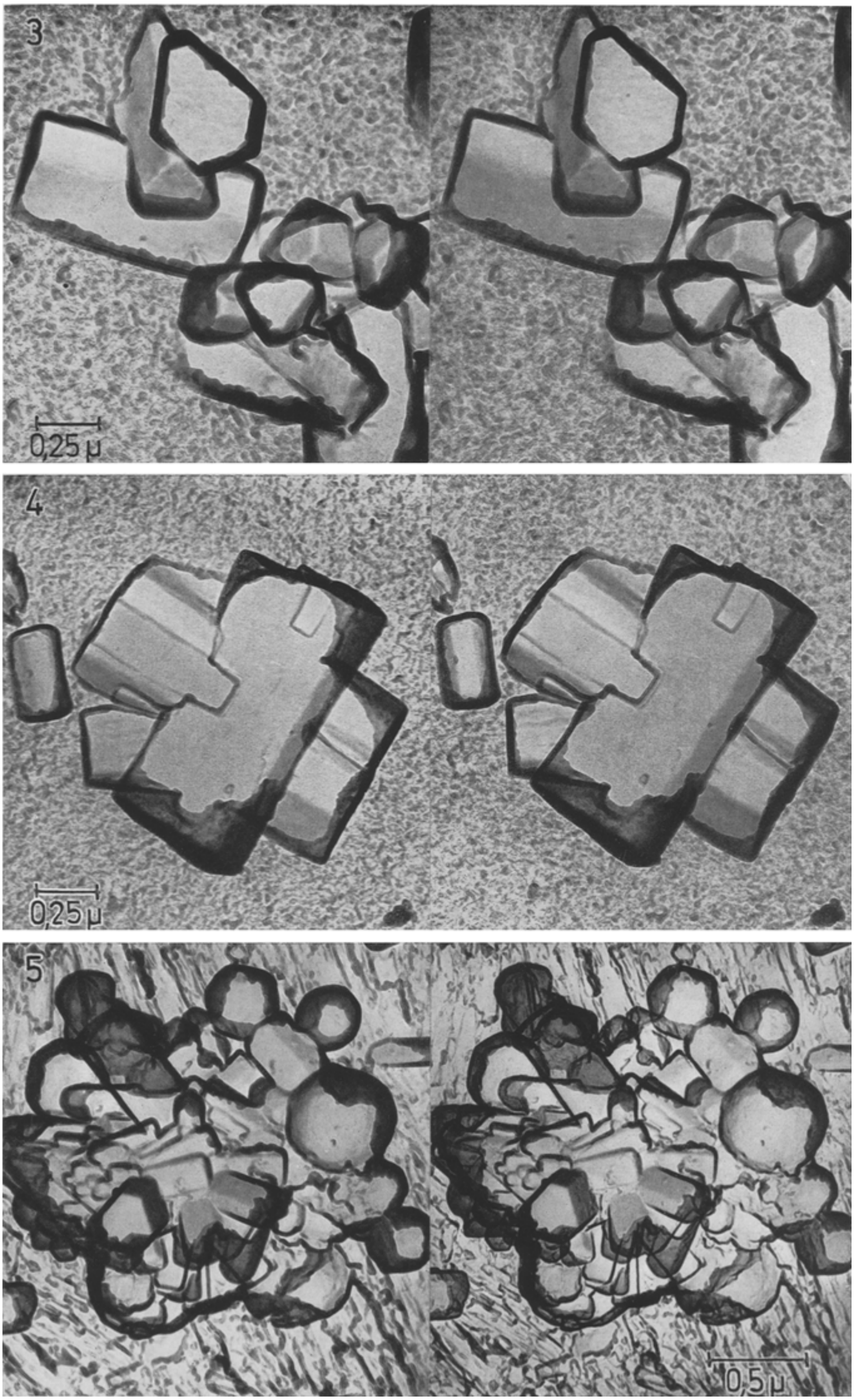
Fig. 2. Stereo-pair transmission electron micrograph of carbon replica of fractured carious enamel surface. Part of Fig. 1 to show three-dimensional form of the caries crystals

when exposed for replication, contribute to a characteristic, intricate and repetitive three-dimensional step-like pattern (Fig. 2). Preliminary measurements of the face angles of the large faces, obtained both directly from the images and indirectly via the *Stereosketch* (see Boyde, 1967) suggest a variation inconsistent with a single crystal moiety. This aspect must be studied further however, for we are unable as yet to distinguish with certainty between faces or facets of these crystals which are truly growth faces and those which are a result of fracturing during our preparation of the material.

The second type of caries crystal in the enamel is prismatic in form and hexagonal in cross-section (the 6 sides of the flat end-face are not usually equi-dimensional but the face angles between them are consistently 120°) (Figs. 3, 4). These crystals do not form extensive clusters as do the flattened "rhombohedral" type described above, but occur singly or in small groups (Fig. 5). The diameter of these crystals varies from ca. 1.5 μ down to a dimension approaching that of the typical hydroxyapatite crystallite of "sound" enamel (Fig. 6). Although the end-faces of these hexagonal crystals are characteristically flat, there are isolated examples of pointed terminations: the angle formed at the point was measured as 82°, the measurement being made directly from photographs in which the body of the crystal could be determined to lie in the horizontal plane.

Small, less regular crystals which cannot be easily identified with either of the two crystal types described above comprise a third group and are found constituting *en masse* what appear to be bacterial forms (Figs. 7, 8). In their external

Fig. 1. Transmission electron micrograph of carbon replica of fractured carious enamel surface showing a large cluster of "rhombohedral" caries crystals and, at top left, the closely packed, elongated crystallites typical of "sound" enamel. *c* caries crystal; *b* bodies strongly resembling bacteria; *id* intertubular dentine; *pd* peritubular dentine; *t* dentinal tubule; *en* "sound" enamel



Figs. 3—5. Relatively high magnification stereo-pair transmission electron micrographs of carbon replica of carious enamel surfaces. Note the small clusters and the form of the hexagonally-based caries crystals. Fig. 5 also shows (at far right) smooth-surfaced bodies resembling coccal bacteria

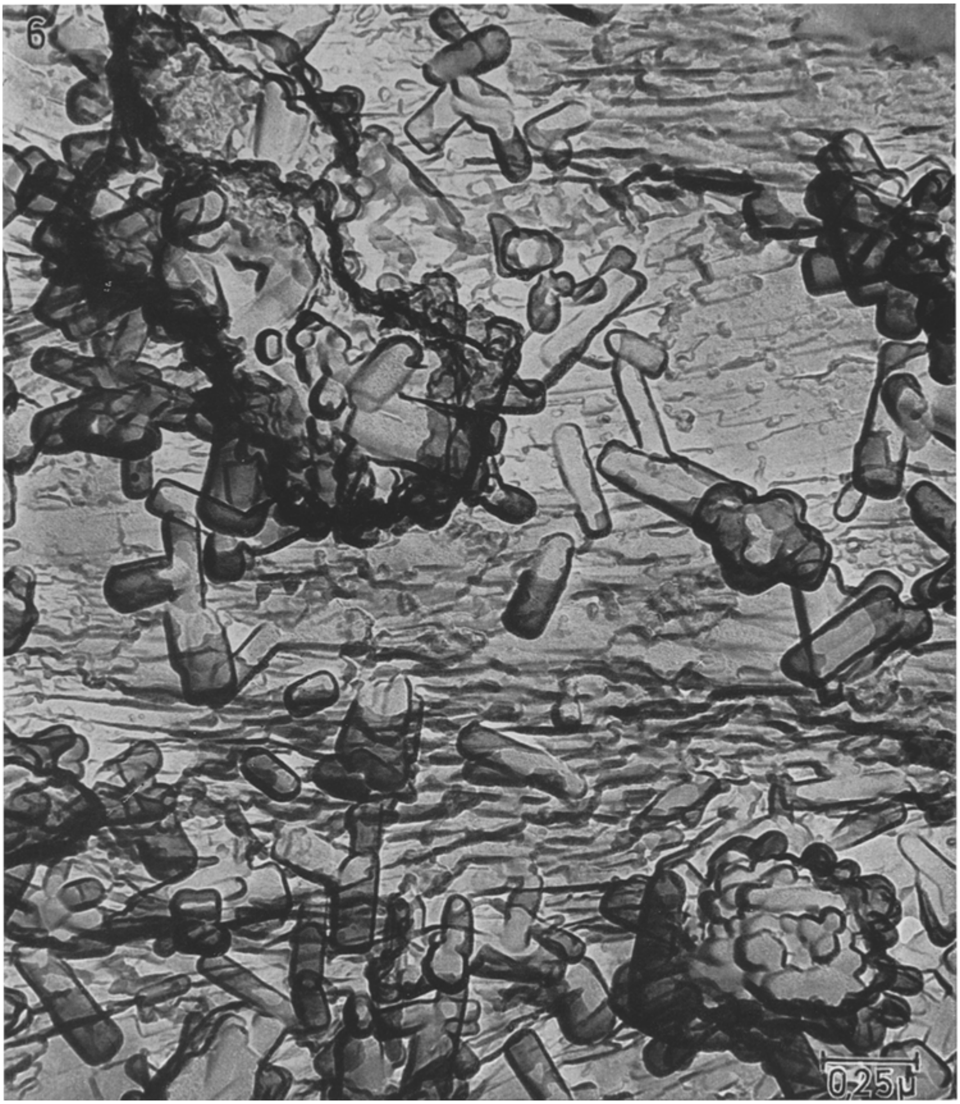
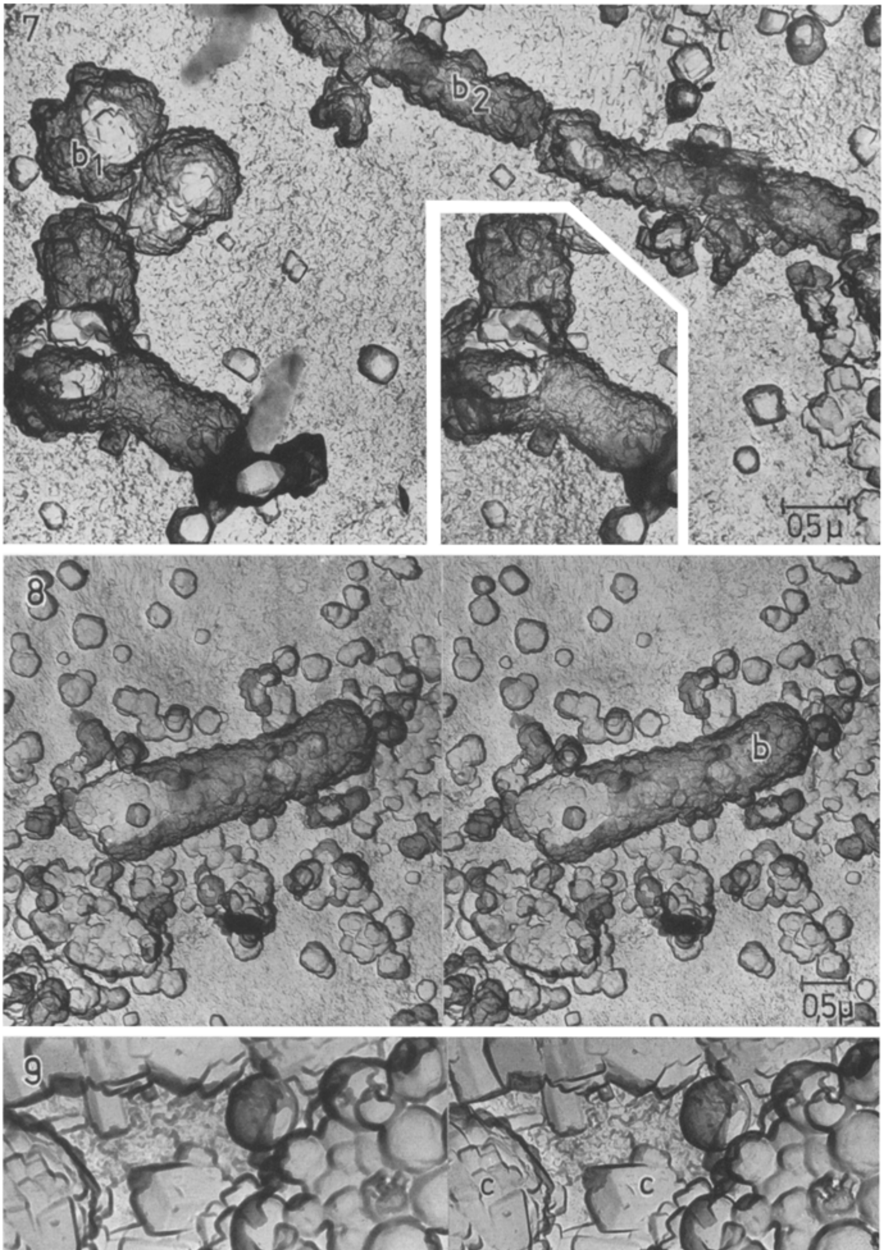
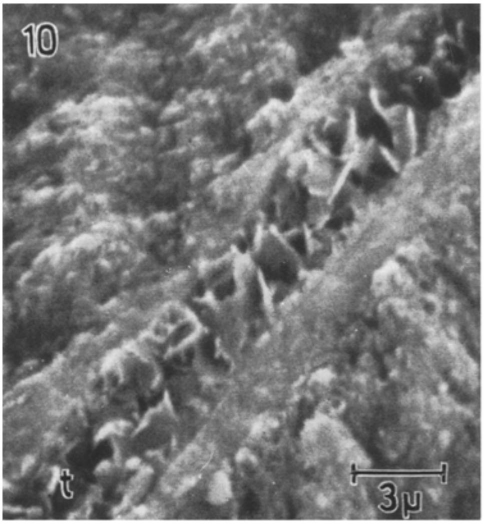


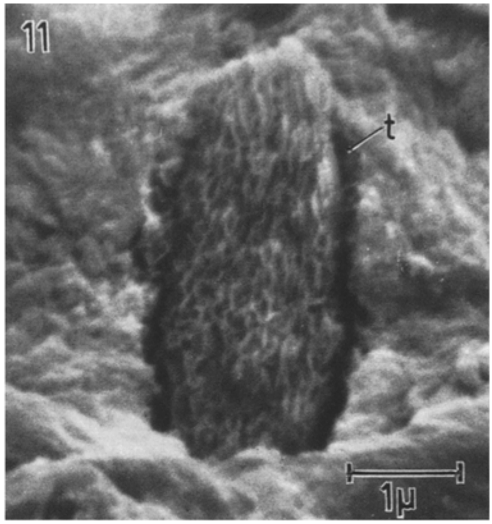
Fig. 6. Transmission electron micrograph of carbon replica of carious enamel surface. Small rod-like crystals of the hexagonal system which (at top left and bottom right) are aggregated into masses similar in size to the bacterial forms of Figs. 7 and 8

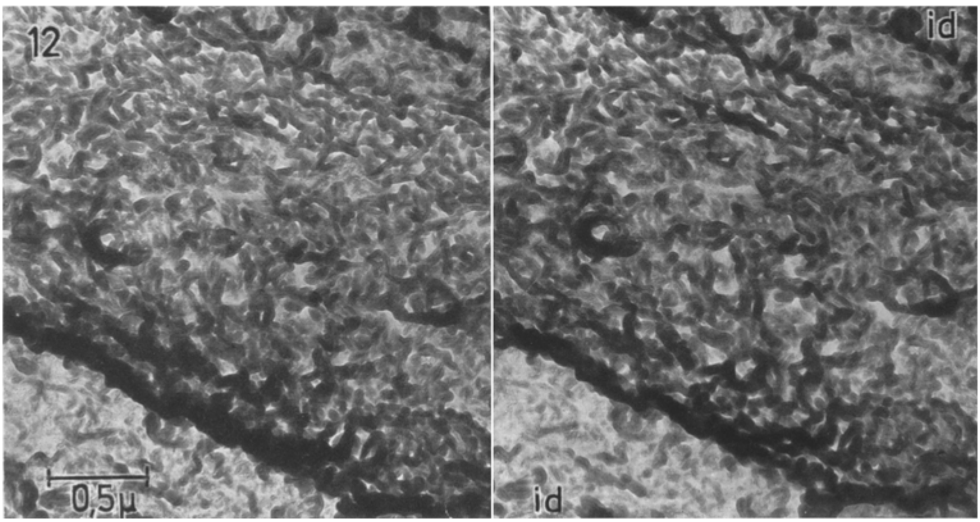
morphology, these bodies resemble coccal, and chain-forming, bacillus-like bacteria. We cannot determine, from the present results alone, whether these bacteria-like bodies are mineralized solid or whether the crystal faces represent an external coat only. Interpretation of these structures in these terms is complicated by the fact that over the same enamel surfaces, well-circumscribed bundles of crystals may be found in close proximity to smooth, rounded bodies with no visible crystalline component at all (Fig. 9 and cf. Fig. 6).



Figs. 7—9. Transmission electron micrographs of carbon replica of carious enamel surfaces







Figs. 10 and 11. Scanning electron micrographs of deliberately fractured surfaces of dentine adjacent to the caries cavity

Fig. 10. Thin, plate-like crystals in the tubule lumen

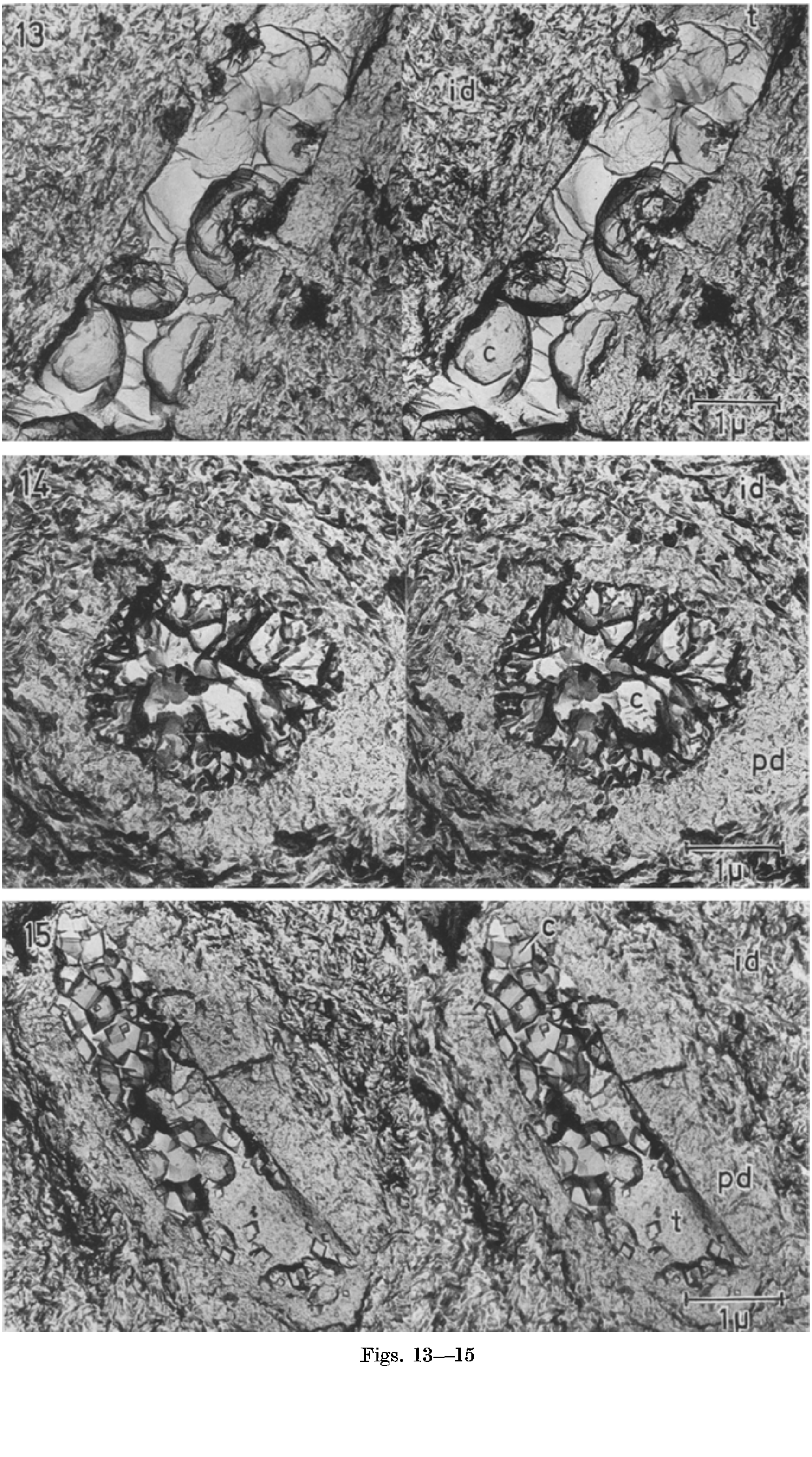
Fig. 11. Small, rod-like crystals forming a delicate meshwork and occluding the tubule lumen

Fig. 12. Stereo-pair transmission electron micrograph of carbon replica of deliberately fractured surfaces of dentine adjacent to the caries cavity showing small irregular rod-like crystals projecting into the lumen of the tubule

Fig. 7. Bodies resembling coccal (b_1) and bacillus-like (b_2) bacterial forms and with a surface pattern made up of small, individual, crystals. *Inset:* forms stereo-pair with adjacent part of figure

Fig. 8. Stereo-pair of bacillus-like bacterial form. Note the similarity of its surface pattern to the faces of the scattered crystals surrounding

Fig. 9. Stereo-pair showing smooth surfaced coccal-like forms and caries crystals, the latter occurring both singly and in well-circumscribed bundles



Figs. 13—15

Dentine. Four different types of inclusion, each with a distinctive crystalline form, are found in tubules of the dentine exposed by deliberate fracture of the tooth in the region of the carious cavity. The scanning electron microscope is of particular value in searching for these dentinal "caries crystals" for the reason that whole fractured tooth surfaces may be examined and the sampling rate thus obtainable is far superior to that of the replica technique.

The inclusions found in the dentinal tubules take the form of:

(i) thin, plate-like crystals which form septae across the tubule lumen and are best illustrated by the scanning electron microscope image (Fig. 10);

(ii) a delicate meshwork or honeycomb of crystals which may fill the tubule lumen completely (Fig. 11, Fig. 12 and c.f. Fig. 3 of FRANK, WOLFF and GUTMANN, 1964);

(iii) massive crystals (up to 2μ in diameter) which are slightly rounded and without definite face angles (Figs. 13, 14);

(iv) a rhombohedral form of caries crystals which may be scattered along the walls of a dentinal tubule (Figs. 15, 16) or which may occlude the lumen completely (Figs. 17, 18) and which has been described previously by a number of workers (for example, LENZ, 1955; HÖHLING, 1961a; TAKUMA and KURAHASHI, 1962; and HERTING, 1966).

Crystals are not confined to tubule lumens but are occasionally found in intertubular dentine regions (Fig. 19) where they are typically less regular in form.

No attempt has been made in the present study to relate the various types of tubule inclusion encountered to a particular stage of progression of the carious lesion. However, it was readily apparent that any one of the above deposits rarely occurs alone, so that in the length of say 10μ of an affected tubule, there is commonly a combination of a number of them. Further, over a group of adjacent tubules, the presence or nature of the inclusion material bears little or no relationship to its neighbours (Fig. 20).

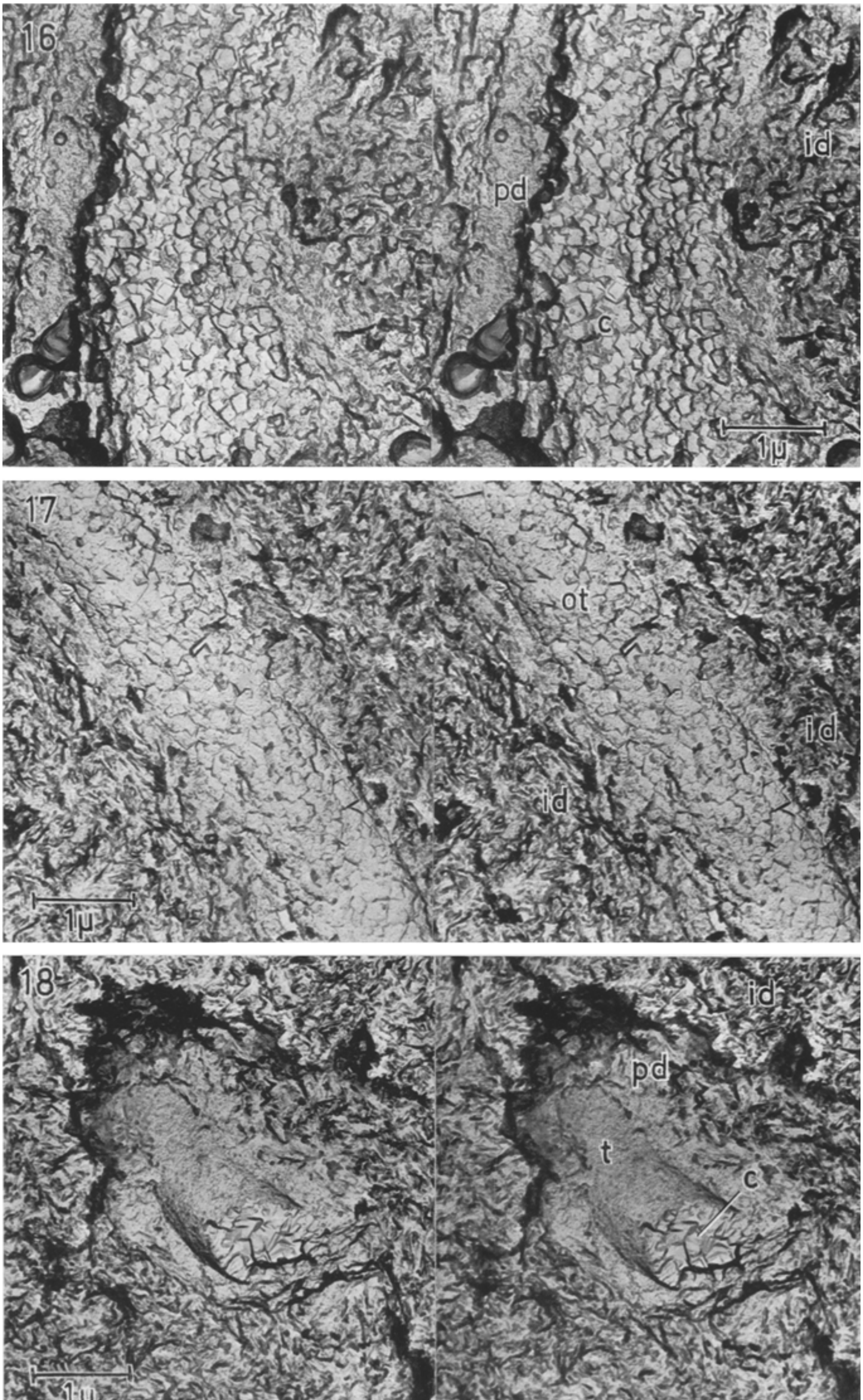
The intratubular accumulation of what is commonly termed "peritubular dentine" is known to occur independently of caries and for that reason is not included in the list above, although it was a common finding in the teeth examined in this study. Where "peritubular" (better intratubular) dentine does come to occlude the tubule lumen, occlusion material often projects as a hom ogeneous rod from the prepared fractured surface (Figs. 21, 22). In such instances, the occlusion material presents a relatively smooth surface and there is characteristically a plane of separation or cleft between it and the surrounding dentine, which may be either intertubular or peritubular dentine.

Figs. 13—15. Stereo-pair transmission electron micrographs of carbon replica of deliberately fractured surfaces of dentine adjacent to the caries cavity

Fig. 13. Massive, slightly rounded crystals in tubule lumen

Fig. 14. Crystals similar in form to those in Fig. 13 and completely occluding the tubule lumen

Fig. 15. Rhombohedral crystals in tubule lumen



Figs. 16—18. Stereo-pair transmission electron micrographs of carbon replica of deliberately fractured surfaces of dentine adjacent to the caries cavity

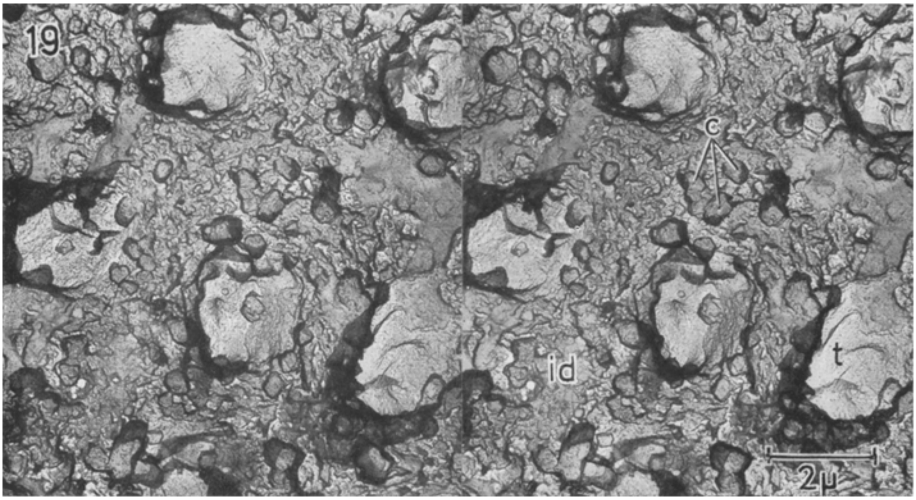


Fig. 19. Stereo-pair transmission electron micrograph of carbon replica of deliberately fractured surface of dentine adjacent to the caries cavity. Transverse fracture showing irregularly shaped crystals confined mainly to areas of intertubular dentine

Discussion

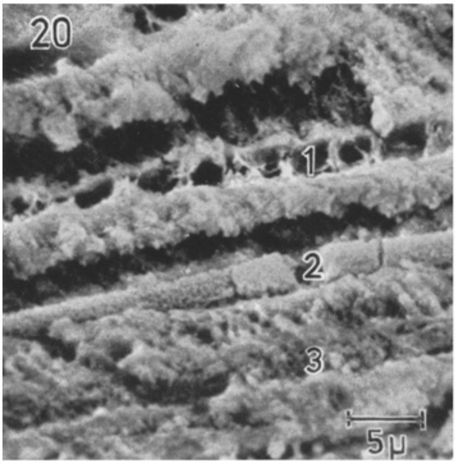
A major advantage to studying hard tissue morphology by a combination of scanning electron microscopy and a replica technique for the transmission electron microscope is that the scanning microscope provides a previously unobtainable sampling rate which can then be selectively reinforced in terms of improved resolution by the replica technique. This combination of methods is especially suited to visualizing the three dimensional morphology of delicate mineralized structures and involves minimal disturbance to their original configuration. Thus these methods may be used to provide clear three-dimensional images of "caries crystals" from which we may appreciate the morphological variations and unexpected profusion of these structures.

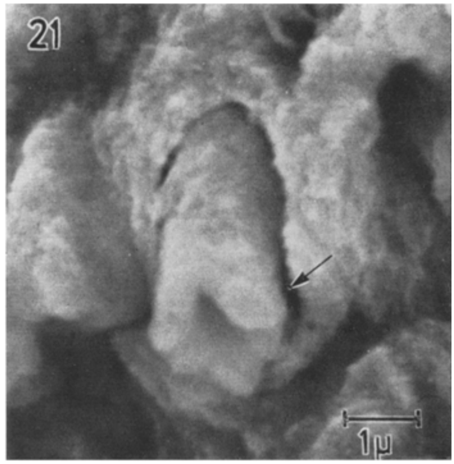
The present results point to the existence in carious enamel of a much larger proportion of "reprecipitated mineral" in "flattened rhombohedral" form (Fig. 1) than has previously been indicated (see for example, Helmcke, 1955, 1960, 1962; Torell, 1957; Höhling, 1961 b). Further, it is evident that a second morphological type of enamel caries crystal exists, which has a hexagonal cross-section and does not have the same tendency to form large clusters (Fig. 3). A third crystal type is associated with what are interpreted as bacterial forms (Figs. 7, 8) and this finding and its attendant implications are discussed more fully below. In carious dentine there are at least *three distinct morphological categories of crystal* (Figs. 10, 11, 13) in addition to the rhombohedral crystals generally described

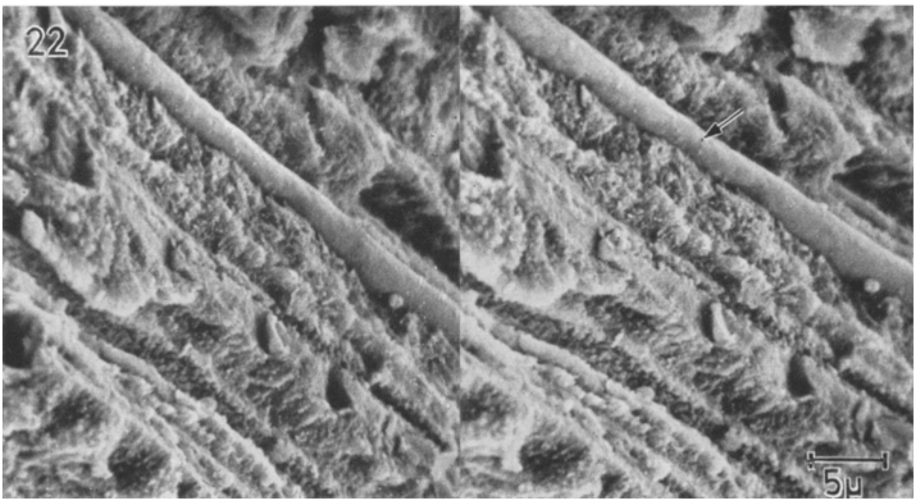
Fig. 16. Note the accumulation of rhombohedral caries crystals in the exposed lumen of the tubule

Fig. 17. This shows the rounded surface of an occluded tubule (*ot*). The outline of the rhombohedral crystals constituting this occlusion are clearly seen

Fig. 18. An occluded tubule fractured transversely and showing its constituent rhombohedral crystals. Note also the different natures of the surrounding intertubular and "peritubular" dentine







Figs. 20—22. Scanning electron micrographs of deliberately fractured surfaces of dentine adjacent to the caries cavity

Fig. 20. A variety of inclusion in neighbouring tubules (cf. 1, 2 and 3)

Fig. 21. Tubule partially occluded with "peritubular" dentine-like material which has fractured out of the tubule to present a typical smooth surface and with cleft (at arrow) separating it from the surrounding dentine

Fig. 22. Stereo-pair showing a smooth-surfaced occlusion (at arrow) which has remained in its tubule bed after fracture of the specimen

in the lumens of dentinal tubules (Lenz, 1955; Höhling, 1961a; Takuma and Kurahashi, 1962; Herting, 1966) and identified as β-tricalcium phosphate by Vahl, Höhling, and Frank (1964).

In the present study, the surface layer of material remaining after 1:2-diamino-ethane extraction was harvested from six carious teeth and examined by X-ray diffraction. The most abundant crystalline species found as a contaminant was identified as β-tricalcium phosphate (Whitlockite) and we presume this to correspond to the rhombohedral crystallites. More precise measurements of this X-ray diffraction pattern reveals a significant Mg content to this Whitlockite (ELLIOTT — in preparation).

It is clear moreover, that the occurrence of these rhombohedral crystals (Fig. 15) in the dentine tubules is not limited either to carious or to human teeth, as is shown by their demonstration both in enamel cracks of sound human teeth (KATTERBACH et al., 1965) and in "clinically sound" dolphin teeth (LESTER and BOYDE, 1968). This would suggest that the environmental conditions favouring their formation are more widespread even than caries — or at least that clinical manifestation which is designated as caries.

Crystal habit is of course notoriously variable even for a single crystal species, since it can be varied according to the presence of certain contaminants in solution or to certain physico-chemical conditions which restrict crystal growth. A great many studies have been made of the conditions under which various calcium phosphates crystallize, but the identity of the calcium phosphate is most often determined by X-ray diffraction sudies without much attention being given at an electron microscopical level to the details of crystal habit (NEWESELY, 1965, is a notable exception to this). The most plausible method at present for identifying these *in vivo* crystal types would seem to be by selected area electron diffraction of either very thin sections (for example, VAHL, HÖHLING and FRANK, 1964) or of "pseudo" — or "extraction" — replicas. Unfortunately, the resolution of selected area electron diffraction (at say 5 μ) is not good enough to analyse crystals of only one particular morphology. Furthermore, the sampling of caries crystals in both cases (that is, in sections and in replicas) would not approach 100%, as the larger crystals could not be sectioned and the crystals of varying sizes and shapes would have varying tendencies to be stripped with the pseudo-replica. We hope therefore, that the present results might serve to encourage the further study of crystals under a wider range of conditions *in vitro* and in which both the crystal species and the three-dimensional crystal morphology might be assessed.

The intimate association of caries crystals with the caries process leads one to consider the variety of pH conditions and ionic concentrations which must exist in and about the carious lesion. One would expect that at a site of recrystallization, the pH or the ionic concentration product or both would differ from that at a site of active dissolution of the original mineral component; and yet caries crystals form in advance of the actual clinical lesion as well as at the surface of the cavity itself. It is a fact, however, that because of the nature of the caries process and the consequent loss of tissue, small but unnatural clefts or planes of separation occur in the normally close-packed enamel and dentine and these may constitute preferred fracture planes. Thus, and especially in the interpretation of replica from carious enamel pretreated with 1:2-diamino-ethane, there is always the important reservation that one can not be certain whether the surface represented is of tissue fractured "de nouveau" or whether the cleavage plane has involved a previously existing cleft brought about by the caries process. For this reason, we do not attempt in the present study to interpret the material with respect to distinguishing zones which may be strictly related to the advance and, therefore, to the mechanism of the caries process.

TAKUMA and KURAHASHI (1962) interpreted occluded dentinal tubules as a calcification of the odontoblast process, presumably because of the presence of a peripheral, more electron-dense crystalline zone which was quite distinct from the tissue immediately surrounding, which they interpreted as "peritubular" dentine. We would assume the dense peripheral zone reported by these workers to correspond to the level of the plane of fracture which is characteristic of occluded dentinal tubules in our preparations (Figs. 21, 22) — occluded dentinal tubules fracture so that the inclusion material presents a smooth surface which is thus separated from the surrounding dentine, which may be either intertubular or "peritubular". The question of whether the odontoblast provides an intracellular matrix for mineralization within its tubule whilst undergoing a process of degeneration (see BERNICK, WARREN and BAKER, 1954; and FRANK et al., 1964) or is forced to retract (providing as it does so an extracellular matrix for mineralization ?), seems to us a basic and important question which might well be decided by the examination of freeze-etched material.

Attention has been recently refocussed on the question of "bacterial calcification" by ENNEVER and CREAMER (1967), who use the term to designate the accumulation within a microbe of crystalline material which they and others have identified as a calcium phosphate (see ENNEVER, 1960; TAKAZOE, KURAHASHI and TAKUMA, 1963). Bacterial califìcation is known to occur naturally in the bacterial plaque associated with teeth, both sound and carious, and material of this nature has been studied previously by conventional sectioning techniques for electron microscopy (for example, GONZALES and SOGNNAES, 1960; ZANDER, HAZEN and SCOTT, 1960; KATTERBACH et al., 1965; SCHROEDER, 1965; FRANK and BRENDEL, 1966). However, as far as can be determined, no information is available on the surface morphology of the affected microbes.

There is general agreement in the description of the crystalline components involved in bacterial calcification as "fine and needle-like", although the dimensions reported vary (cf. ZANDER, HAZEN and SCOTT, 1960; RIZZO, SCOTT and BLADEN, 1963; TAKAZOE, KURAHASHI and TAKUMA, 1963; THEILADE et al., 1964; SCHROEDER, 1965). Our results show that certain forms strongly reminiscent of bacteria, and associated with carious enamel surfaces, display crystal faces at their surface of diameter ca. 1000° A (Figs. 7, 8). The question arises: are these bacteria, and if so, are they "calcified" solid with the crystals extending through their substance, or, are the crystals plate-like (cf. SCHROEDER, 1965) and confined to the surface of the microbe (cf. RIZZO et al., 1963; KATTERBACH et al., 1965) ? The situation is complicated by the finding on these same enamel surfaces both of well-circumscribed bundles of prismatic crystals (strongly resembling the hexagonal caries crystal crystals described in this paper) (Figs. 6, 9) and of smooth-surfaced ovoid bodies resembling coccal bacteria (Fig. 5).

We cannot hope to answer these questions at present, but are pursuing the problem with special reference to the bacteria associated with caries plaque and to the precise effect on these bacteria of 1:2-diamino-ethane extraction.

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