CRYSTAL GROWTH IN RAT ENAMEL

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ABSTRACT

Observations have been made, using electron microscopy and x-ray diffraction, on the changes in crystal size and shape which occur in developing rodent enamel during mineralization. Small enamel pieces isolated from ground sections of rat molars and incisors were either embedded in methacrylate and sectioned with a diamond knife for electron microscopy, or they were mounted intact on glass fibers in a Debye-Sherrer type powder camera for x-ray diffraction. By either approach it was found that the apatite crystals were very long in the c axis direction from the beginning of enamel mineralization. Morphologically, the early crystals took the shape of extremely thin, long plates arranged in such a manner that there seemed to be little room for any further length-wise growth. It was demonstrated clearly, on the other hand, that the crystals increased in both thickness and width with advancing mineralization. As a result, the thin crystal plates gradually developed into hexagonal rods, which in the most mature enamel examined measured 500 to 600 A in width and 250 to 300 A in thickness.

Enamel was one of the first tissues examined with the electron microscope. While the initial studies dealt with the morphology of mature enamel (5, 9, 20), later investigators were more concerned with its development. Interest was centered primarily on differentiation of the ameloblasts and on ensuing cytoplasmic changes associated with matrix formation and crystal nucleation (2, 3, 12, 14–16, 21, 22). Only recently with improvements in both techniques and instrumentation has it become possible to visualize the sequence of events which transform the organic matrix, a secretory product of the ameloblasts, into a highly mineralized tissue. It now appears that maturation of the enamel is due largely to crystal growth rather than to an increase in the total number of crystals (17). Agreement has not been reached, however, on the manner in which growth occurs (7, 17), on the shape of the most mature crystals (7, 11, 17), and on the relationship between the organic matrix and the apatite crystals (4, 7, 13, 18, 19).

The data to be presented in this paper on enamel crystal growth are from observations which were made in the course of a study of rat enamel mineralization by microradiography, x-ray diffraction, electron microscopy, and electron diffraction. Only observations on changes in crystal size and shape will be dealt with here. The general mineralization pattern and the interrelationship between the organic and inorganic fractions of enamel will be the subject of other reports.

MATERIALS AND METHODS

The material consisted of developing lower first molars of 8-day-old and upper incisors of 75-day-old albino rats (Sprague-Dawley strain) kept on a normal diet. The teeth were dissected out quickly, fixed in 10 per cent neutral formalin for about 24 hours, and embedded in Ward's Bioplastic. Planoparallel ground sections, varying in thickness between 20 and 100 microns, were prepared by manual grinding. The majority of the molars were sectioned in a buccolingual direction through the two middle cusps of the teeth. Most incisors were prepared to give longitudinal sections containing the middle of the teeth. Some cross-sections of both molars and incisors were also made.

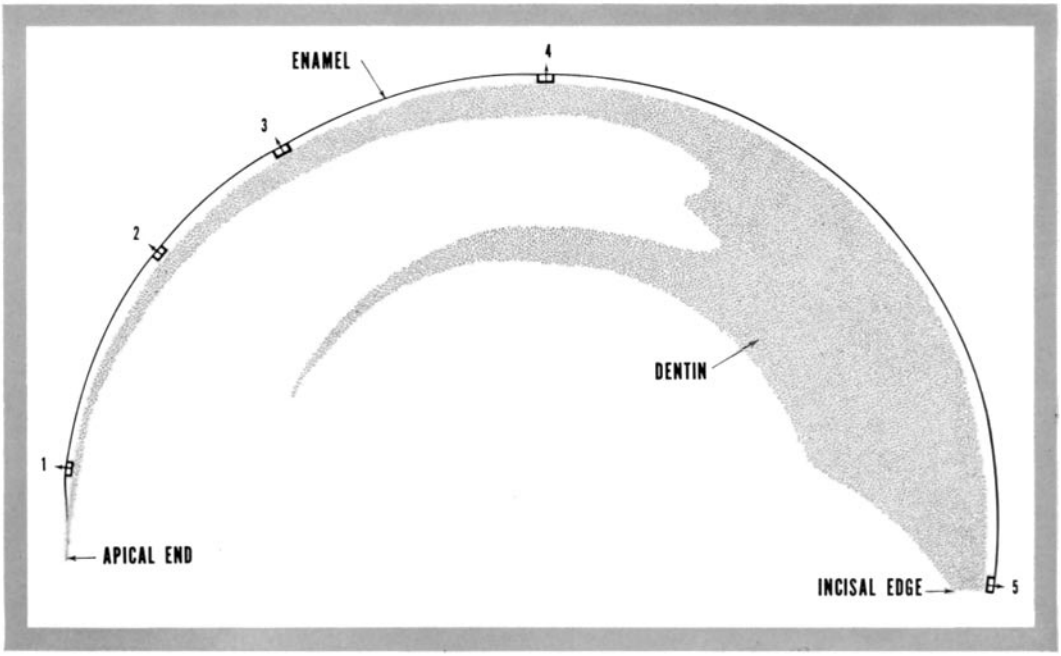


FIGURE 1

Schematic representation of an upper incisor from a 75-day-old rat. The numbered areas in the enamel indicate the location of the five samples used for x-ray diffraction. The resulting patterns are seen in Fig. 18.

Microradiographs were taken of all the ground sections, following which small pieces, usually comprising the entire width of the enamel plus a little dentin, were dissected from the ground sections. In the case of the incisors, which are continuously growing teeth, the samples selected covered the area of development from the apex halfway to the incisal edge (Fig. 1). The isolated enamel pieces were placed on the bottom of gelatin capsules, which were subsequently filled with prepolymerized butyl–methyl methacrylate (8:2) and polymerized overnight under nitrogen at 45°C. Sectioning was done with diamond knives on an LKB ultrotome. The sections were picked up on carbon-covered specimen grids and examined in the Siemens Elmiskop I. In most instances the microscope was operated at 100 kv, and selected area electron diffraction was carried out at 100 kv exclusively. The cold stage adjusted to maintain a temperature of −90°C was used frequently.

FIGURE 2

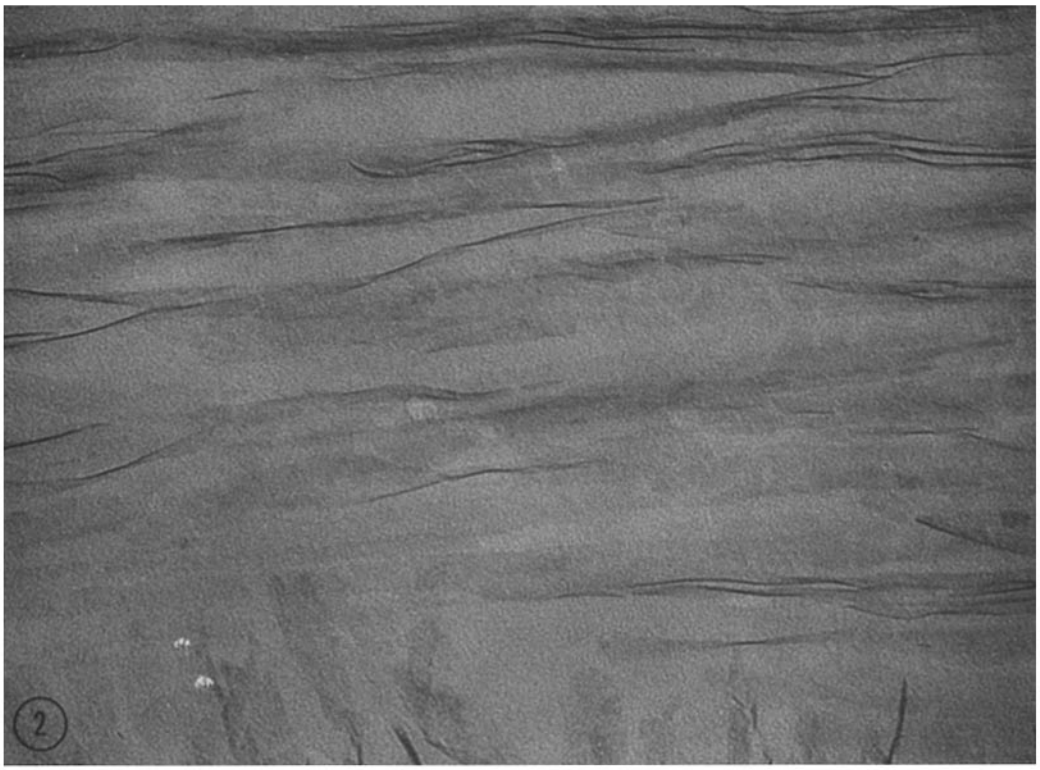
The earliest stage of crystal formation observed. Note the extreme thinness of the plate-like crystals as evident from the narrowness of the dense profiles of crystals seen on edge and the much lower density of those viewed from the broadside. The cutting direction was perpendicular to the long axis of the crystals. × 200,000.

FIGURE 3

Distortion in the crystal ribbons produced by cutting in a direction parallel to their long axis. × 100,000.

FIGURE 4

Selected area diffraction pattern obtained from a region similar to that shown in Fig. 2.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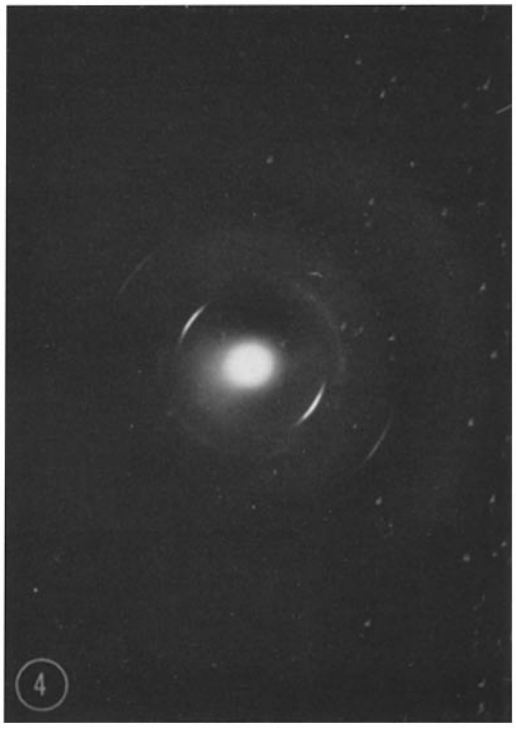
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For x-ray diffraction five enamel pieces, approximately identical in size and shape, were dissected from a longitudinal ground section of an incisor (Fig. 1). Care was taken to remove all the dentin from the enamel. Each specimen was mounted with Canada balsam on the end of a tapered glass fiber so that the long axis of the enamel section was parallel to the fiber. Diffraction photographs were taken with a Debye-Sherrer type powder camera (114.6 mm diameter) using Ni-filtered Cu $K\alpha$ radiation (λ = 1.54 A). Exposure time varied from 9 to 29 hours depending on the degree of mineralization of each sample.

RESULTS

Electron Microscopy

All areas of the samples examined contained inorganic crystals arranged in groups corresponding to the basic enamel rod pattern. Within each group the crystals were oriented with their long axis approximately parallel to the rod direction. A definite difference in size and shape existed between the crystals found in newly secreted enamel matrix and those of mature enamel. Between these two points, a range of intermediary forms could be followed, the striking feature being that within a given area or layer all the crystals were of nearly the same size and shape. Identical observations were made in sections of molar and incisor enamel.

The youngest crystals, which were found in the outer surface layer of enamel that had not reached its full thickness, took the shape of long plates. They appeared in sections as parallel ribbons of very low density, when viewed from the broadside, or as more dense, narrow profiles, when seen on edge (Fig. 2). The crystals were from 200 to 300 A in width and had a thickness of about 10 A. They seemed to form long, straight rows with such a close end to end relationship of the individual crystals that it was difficult to assess their length (Fig. 2). In some areas, however, the rows were folded and consisted of smaller segments, 1000 to 3000 A long (Fig. 3). There was a strong possibility that this was the result of sectioning since the latter was generally the case when the crystals were oriented with their long axis parallel to the cutting direction.

Selected area diffraction patterns obtained from such young enamel indicated that the crystalline material was hydroxyapatite although they contained too few lines to permit positive identification. The strongest lines present corresponded to the 002 and 004 reflections. A high degree of preferred orientation in the c axis direction was evident in the arcing of these rings (Fig. 4). When related to the areas from which patterns were made it became clear that the c axis direction was identical with the long axis of the crystal rows.

In the early phases of crystal growth an increase in thicknes occurred, which was evidenced directly by a gradual change in dimension of the dense profiles and indirectly by an enhanced density of the ribbons (Figs. 5 to 7). At these stages the arrangement of the crystals in long parallel ribbons was more readily seen, and it was apparent that from the onset there was essentially no space available for additional lengthwise growth. In contrast, ample space was found between the crystal rows.

Selected area electron diffraction patterns from regions similar to that seen in Fig. 7 contained now sufficient reflections to permit positive identification of the mineral as hydroxyapatite. The patterns demonstrated the same high degree of orientation in the c axis direction (Fig. 8).

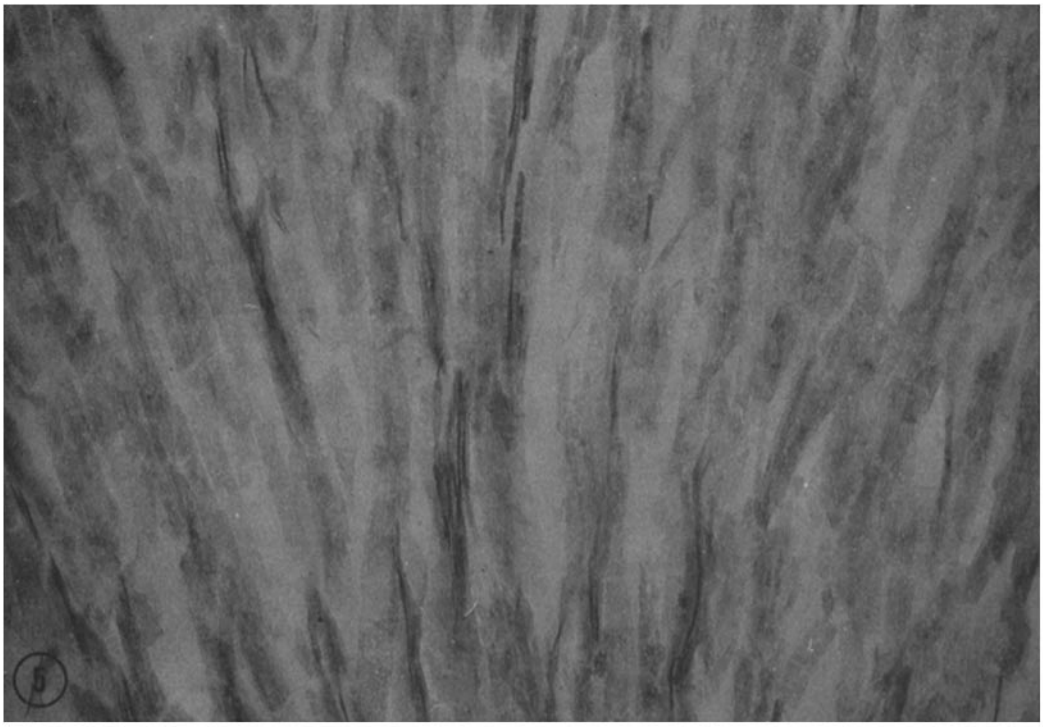
When the crystals became thick enough to be seen clearly, it became evident that the sections contained crystals cut both lengthwise and crosswise as was to be expected from the known histology of rodent enamel (10, 21). Selected area diffraction patterns of cross-cut crystals did not contain the 002 and 004 reflections, verifying that the c axis was parallel to the electron beam (Fig.

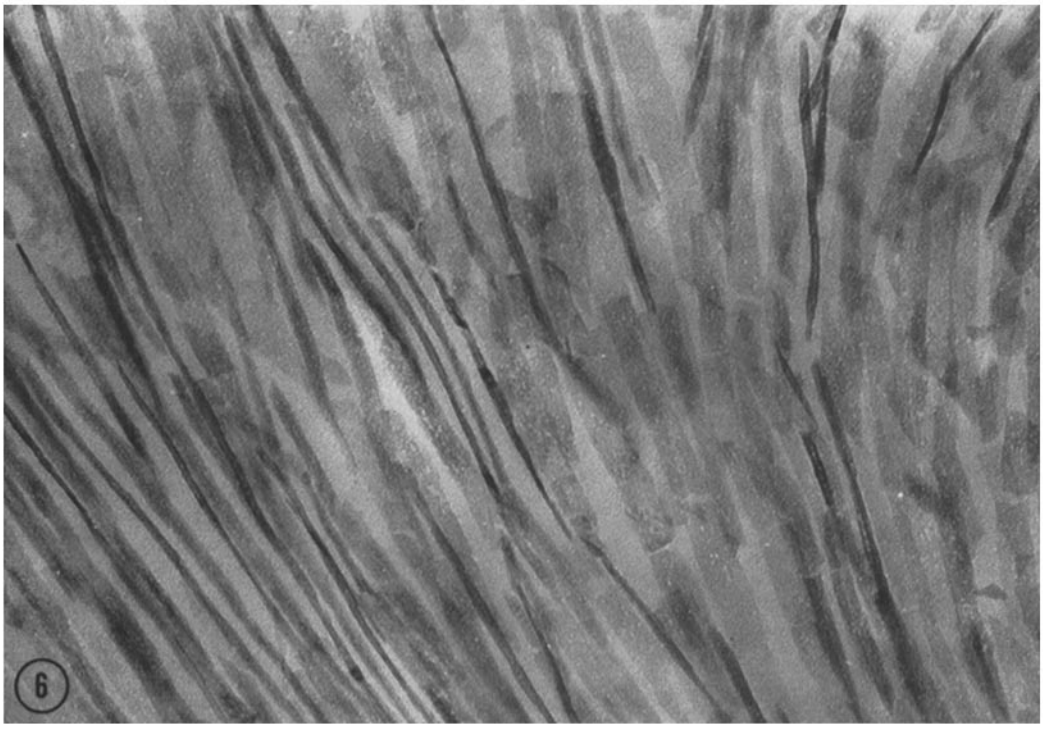
FIGURE 5

Early stage of crystal growth. The crystal ribbons are seen more clearly due to an increase in their thickness to 30 A as measured from the dense profiles. × 150,000.

FIGURE 6

A somewhat later stage in which the thickness of the crystals has increased to approximately 80 A. × 150,000.





9). Both the electron diffraction patterns and the microscopic image showed that the crystals, when viewed in cross-section, were randomly oriented about this axis (Figs. 9 and 10).

Once identification of cross-cut crystals was possible, further increases in size could be followed more accurately. At a growth level corresponding to that observed in Fig. 7, the cross-sectioned crystals were roughly oblong with dimensions on the order of 200 to 300 A by 80 to 100 A (Fig. 11). With continued growth the shape of the cross-sectioned enamel crystals became clearly hexagonal (Fig. 12). At first the widest diameter of the hexagons measured from 200 to 300 A and their shortest diameter from 100 to 120 A. With further mineralization the crystals appeared to grow in width as well as in thickness since the hexagons were found to increase in size in all directions (Fig. 13 and 14). As can be seen from the micrographs, the thickness of the individual crystals at the various levels of development was much more uniform than their width. Often a thin dense line could be observed bisecting the hexagons along their widest diameter.

In regions with longitudinally cut crystals, breaks occurred along the lengths of the crystals with increasing frequency the thicker they became. This was most prominent where the direction of their long axes was the same as the cutting direction (Fig. 15). The fracture lines were quite evenly spaced and were about 1500 A apart. The widths of the broadest and narrowest ribbons which were observed in such areas corresponded at any time to the longest and shortest diameters of the hexagons in neighboring areas with cross-cut crystals (Fig. 16). A dense line similar to the one seen cutting across the hexagons could occasionally be followed along the middle of the narrowest ribbons, but was never seen in the broader ones (Fig. 17).

In the most advanced stages of development studied the crystals were 250 to 300 A thick and from 500 to 600 A wide (Fig. 14). Very little difference in these dimensions was apparent throughout the entire thickness of the enamel.

X-Ray Diffraction

The x-ray diffraction lines obtained from all five samples were those of hydroxyapatite (Fig. 18, 1 to 5). The patterns, however, showed differences in breadth of the various lines. In general, the diffraction lines sharpened, indicating crystal growth and/or improvement in crystal perfection as mineralization progressed. The only exception was the 002 line, which was relatively sharp even in the first sample. The most pronounced change was found between the patterns of the first and second sample, while no difference could be observed between the patterns of sample 4 and 5.

The lines from the 211, 112, 300, 202, 301, and 310 sets of diffraction planes showed the progressive sharpening most clearly (Fig. 18). Thus in pattern 1 the 211 and 112 lines could not be resolved by visual estimation. In pattern 2 these lines were separate, and both were sharper than the 300. In patterns 4 and 5 all three lines seemed to be of equal breadth. Similarly, the 310-212 doublet was unresolved in pattern 1, barely discernible in pattern 2, and easily resolved in patterns 4 and 5.

DISCUSSION

Previous studies have shown that in both human and rodent enamel formation crystal nucleation takes place in the immediate vicinity of the ameloblasts (2, 14, 17, 21) and that the crystals very rapidly obtain the shape of long plates, less than 400 A wide and from 15 to 30 A thick (17, 21). Although the manner in which the present

FIGURE 7

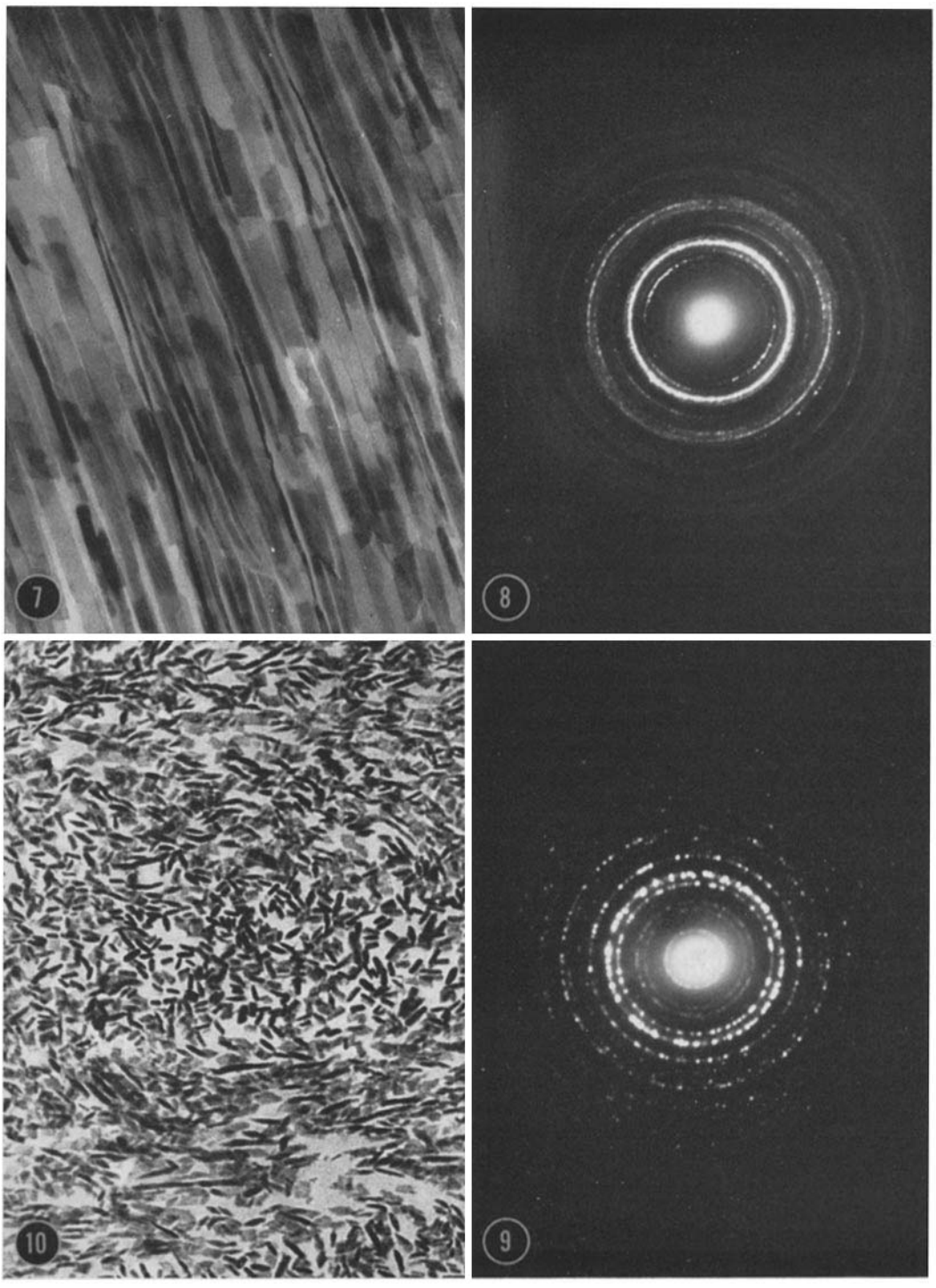
Parallel crystal rows oriented with their long axis in the plane of the section. Their has been a further increase in thickness to about 100 A. × 75,000.

FIGURES 8 AND 9

Selected area diffraction patterns matching the micrographs to their left. Preferred orientation of the crystals in the c axis evident from Fig. 8.

FIGURE 10

An area with cross-sectioned crystals. Note random orientation. × 75,000.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material was collected precluded direct localization with respect to the related cells, the crystals found in the peripheral developing enamel of both molars and incisors were similar in shape and size to these plates and undoubtedly represent the first stage in crystal growth.

The low density of these crystals when viewed from the broadside, their extreme thinness when seen in profile together with artefacts introduced in cutting, made it difficult to determine their dimensions accurately. It appeared, however, that the crystals formed continuous ribbons over large areas leaving little if any room for further lengthwise extension. This supports previous observations that the crystals have already acquired their adult length at this early stage.

The thickness of the young crystals, on the other hand, was about 10 A, which would account for their low density when viewed from the broadside. It would also explain the absence in the electron diffraction patterns of reflections from planes other than those perpendicular to the long axis of the c axis of the crystals. Actually, since the a axis length of hydroxyapatite as determined by x-ray diffraction is 9.44 A (1), the plates are only slightly more than one unit cell thick.

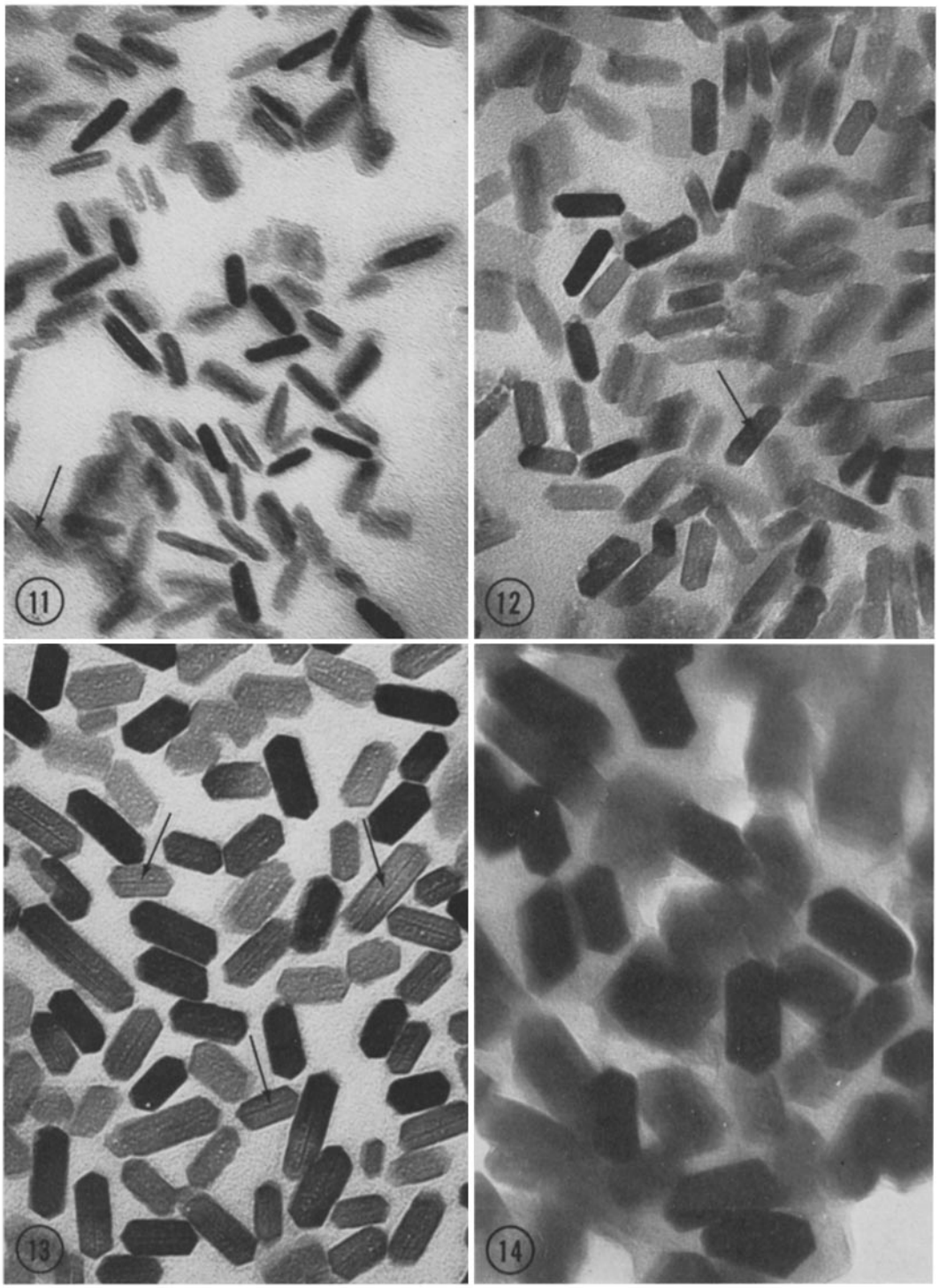
Crystal growth resulted in the formation of hexagonal rods through a gradual increase in thickness and width of the thin plates. Since the difference between width and thickness found in the youngest crystals appeared to be retained in the older ones, a uniform rate of growth on all surfaces is indicated. No evidence was found of fusion between individual crystals as reported by Frank *et al.* (7) and Rönnholm (17, 18). While such a process might occur in the rapid formation of the long, thin platelike crystals it is more difficult to imagine a side to side union between the latter. The random orientation of the crystals about their c axis together with the high degree of crystal perfection observed in cross-cut crystals (13) are findings which make it difficult to accept the fusion theory. As a matter of fact, Nylen and Omnell (13) and Scott and Nylen (19) have demonstrated the existence of an organic layer in intimate contact with the crystal surface, and it is conceivable that this layer may serve to prevent fusion between the crystals. Nor did growth appear to result from the addition of one or more unit cells at a time as suggested by Rönnholm (17). The latter concept was based on the observation of stepwise increases in crystal thickness at various distances from the related cells. There are many indications, however, that enamel matrix formation itself is an incremental process. It would not be unexpected, therefore, if this was reflected in the sizes of the crystals from the different enamel segments. Furthermore, it appears much more likely, based on physicochemical principles, that crystal growth results from the addition of individual atoms and not whole unit cells.

The crystal growth pattern deduced from the x-ray diffraction studies corresponded to that observed directly in the electron microscope. Since the sharpness of a diffraction line as measured by its breadth is inversely proportional to the mean thickness of the crystals perpendicular to the reflection planes involved, assuming that the strain factor is absent, variations in line broadening can help determine the direction in which a crystal grows. Thus the narrowness of the 002 line in the patterns obtained from the rat incisor shows that even in the apical end of the tooth the crystals are many atomic layers long in the c axis direction. The width of the line approaches, however, the limiting value for the camera system used. Consequently, it cannot be said that no further lengthwise growth has occurred from the apical to the incisal end of the tooth even if the width of the 002 line remained constant in all five patterns. The decreasing broadening of such lines as the 300, 310, and 301, on the other hand, clearly indicates that considerable increases in dimensions perpendicular to the c axis have taken place.

The shape of the mature crystals was definitively shown to be that of long hexagonal rods, as previously indicated by Frank *et al.* (7) and by Höhling (11). However, in contrast to the observations of the former, the crystals did not become

FIGURES 11 TO 14

Progressive stages in crystal growth illustrated through changes in the cross-sectional dimensions of the crystals. At first, the cross-cut crystals are oblong (Fig. 11), then they become hexagonal (Fig. 12). Finally the hexagons grow in all directions (Figs. 13 and 14). Note dense bands bisecting hexagons (arrows). × 300,000.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equilateral in rat enamel. This conclusion is based not only on the cross-sectional appearance of the crystals, but also on the close correspondence between their dimensions in both lengthwise and cross-cut aspects. Since the width of the broadest ribbons always was identical with the longest diameter of neighboring hexagons it is quite evident that the latter represent true profiles of crystals sectioned perpendicular to their long axis. This was also borne out by the absence of the 002 and 004 reflections in the selected area electron diffraction patterns of such cross-cut crystals.

On this basis it was determined that the most mature crystals observed electron microscopically had a width of 500 to 600 A and a thickness in the range of 250 to 300 A, dimensions which agree with those previously recorded for fully mineralized rodent enamel (2, 6). Since in the incisor no difference was found between the x-ray diffraction patterns obtained from the incisal enamel (Fig. 1, stage 5) and from the most mature enamel which was studied morphologically (Fig. 1, stage 4), it is suggested that little if any further crystal growth took place past the latter stage. If this is correct, one has to accept the existence of much larger intercrystalline spaces in enamel than previously anticipated. As pointed out by Scott and Nylen (19) it is difficult to visualize how the organic matrix, which constitutes a very small percentage by weight (1 to 3 per cent), could fill the proportionate volume depicted here. Fibrous proteins especially are highly condensed. It is known, on the other hand, that hydrated gels can occupy tremendous volumes per unit weight, and it is conceivable that at least part of the organic matrix may take this form.

The length of the crystals could not be established with certainty. While they appeared very long in intermediate stages of growth, the more mature crystals broke into quite regular pieces when sectioned. It is possible that the effect of the cutting is to separate individual crystals, otherwise packed in an extremely close end to end relationship. If this should be the case, the average length of the crystals would be 1500 A, a value identical with the long dimension given by Rönnholm (17) and close to the one determined by Glas and Omnell (8).

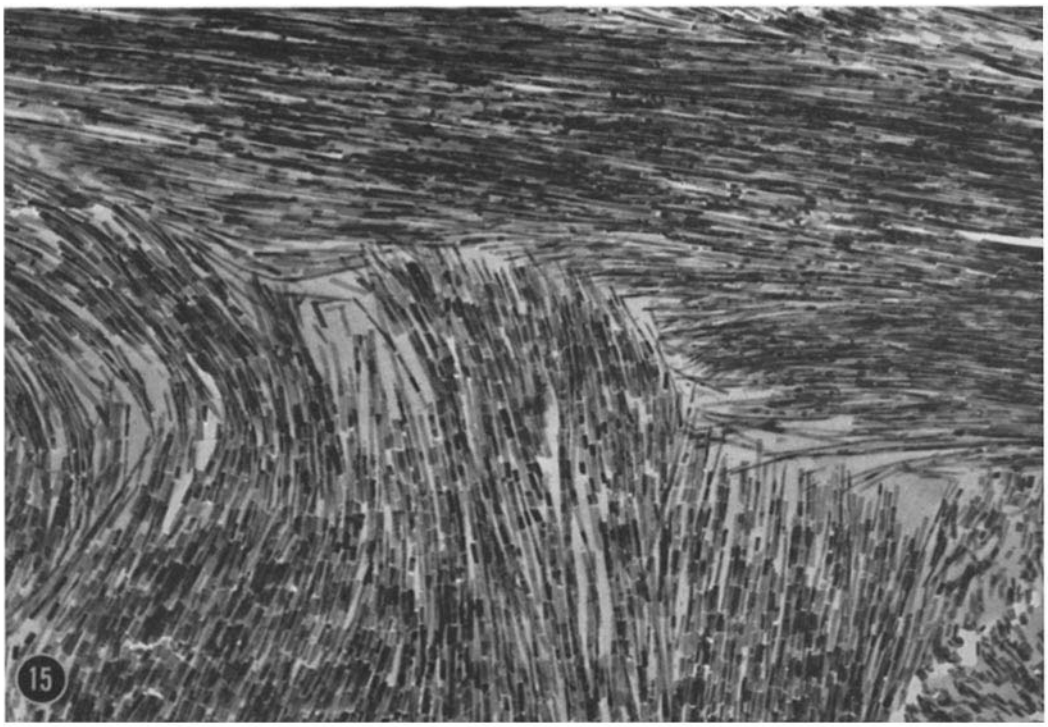
Depending on their orientation with respect to the electron beam a thin, dense line was occasionally seen bisecting the crystals. A similar line, 25 A thick, was observed by Rönnholm 17 and interpreted as remnants of a calcified organic matrix or as due to difference in crystal composition of the first crystallites formed. Preliminary studies with through focus series indicate, however, that the line disappears at exact focus. This throws considerable doubt as to whether or not it represents a real structure of the dimensions given, and suggests that it may be an interference pattern due to a phase discontinuity in the crystal. One possible explanation is that the enamel crystals are actually twin crystals and that the twinning plane is the source of the phase discontinuity. Further support for the

FIGURE 15

Fairly mature crystals oriented with their long axis in the plane of the section. The direction of cutting was parallel to the broken crystal rows in the lower half and perpendicular to the more intact rows in the upper half of the micrograph. × 35,000.

FIGURE 16

Rather mature crystals sectioned parallel to their long axis. The variation in thickness of the ribbons reflects the random orientation seen in groups of cross-sectioned crystals. Their width (W) and thickness (T), based on the dimensions of the broadest and narrowest ribbons, are 400 A and 200 A, respectively, values which correspond to the widest and shortest diameter of neighboring cross-cut crystals. × 150,000.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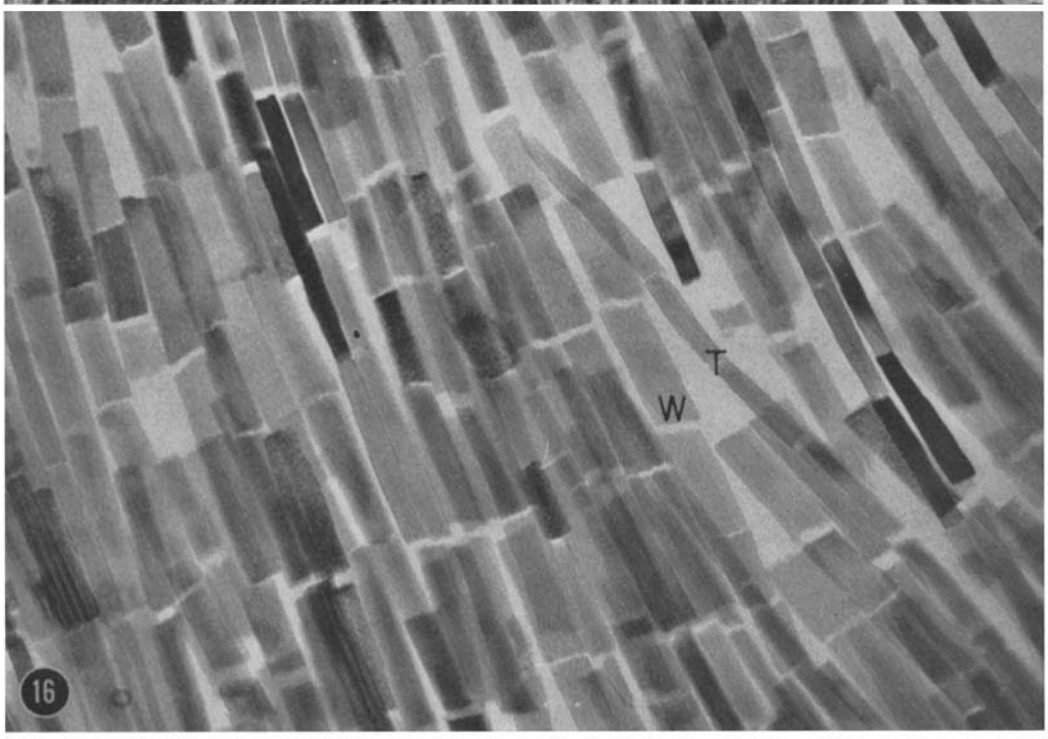
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interference pattern effect is given by the fact that the line is seen only in crystals, which have been cut in such a way that the broadest surfaces of the hexagonal rod lies in a plane perpendicular to that of the section.

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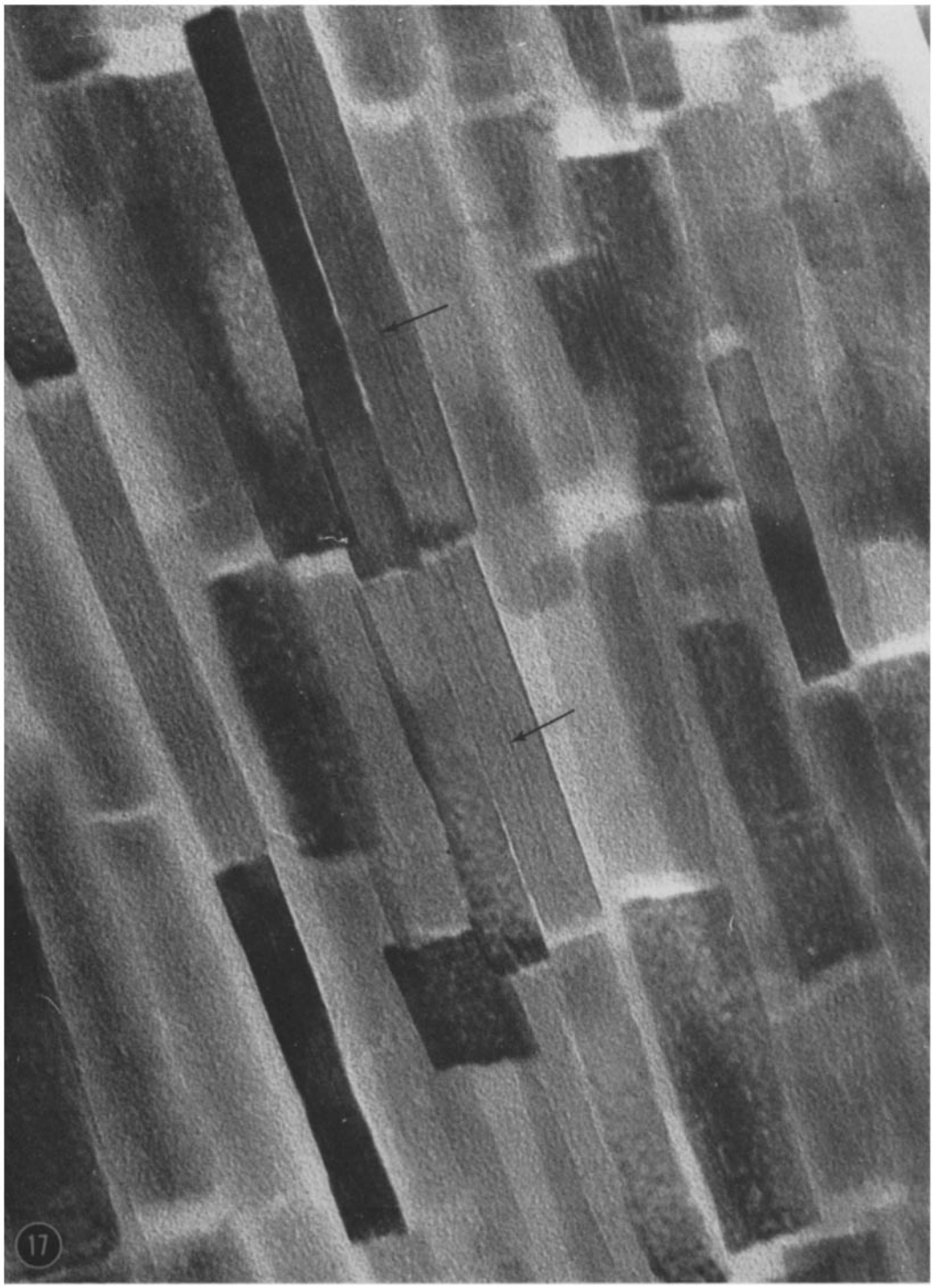
FIGURE 17

Higher power of region similar to that shown in Fig. 16. The arrows indicate the dense line which is occasionally seen bisecting the narrowest ribbons, but never the wider ones. Its presence depends apparently on a suitable orientation of the crystals to the electron beam. × 480,000.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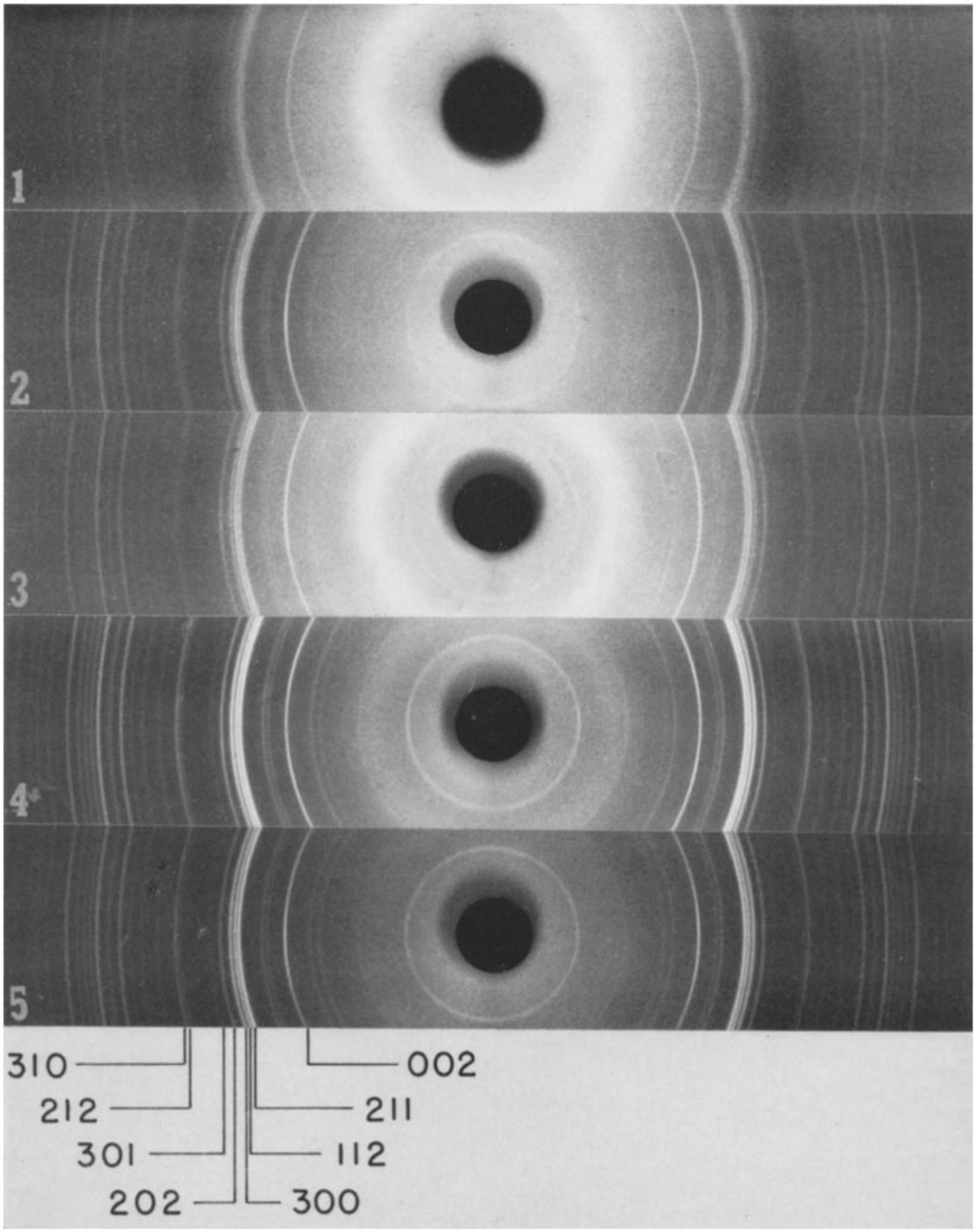

FIGURE 18

X-Ray diffraction patterns from five samples representing in order (1 to 5) advancing stages of incisor enamel development. The localization of the samples is recorded in Fig. 1. Note the constant width of the 002 line in all 5 patterns and the progressive sharpening of the other identified lines.

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