

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 inter-tubular 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).

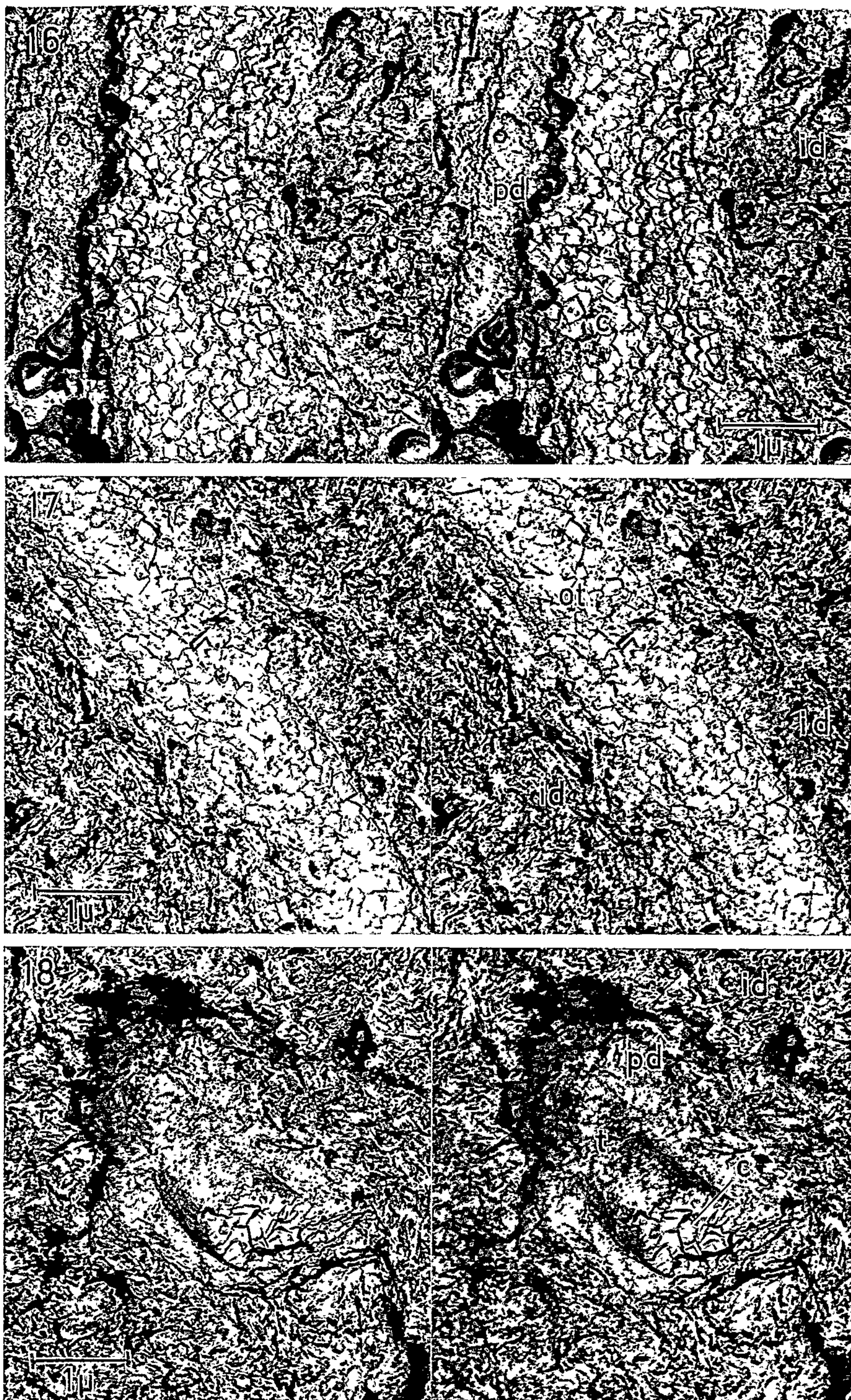
The intratubular accumulation of what is commonly termed "peritubular dentine" is known to occur independently of caries and for that reason is not included in the list above, although it was a common finding in the teeth examined in this study. Where "peritubular" (better intratubular) dentine does come to occlude the tubule lumen, occlusion material often projects as a homogeneous 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

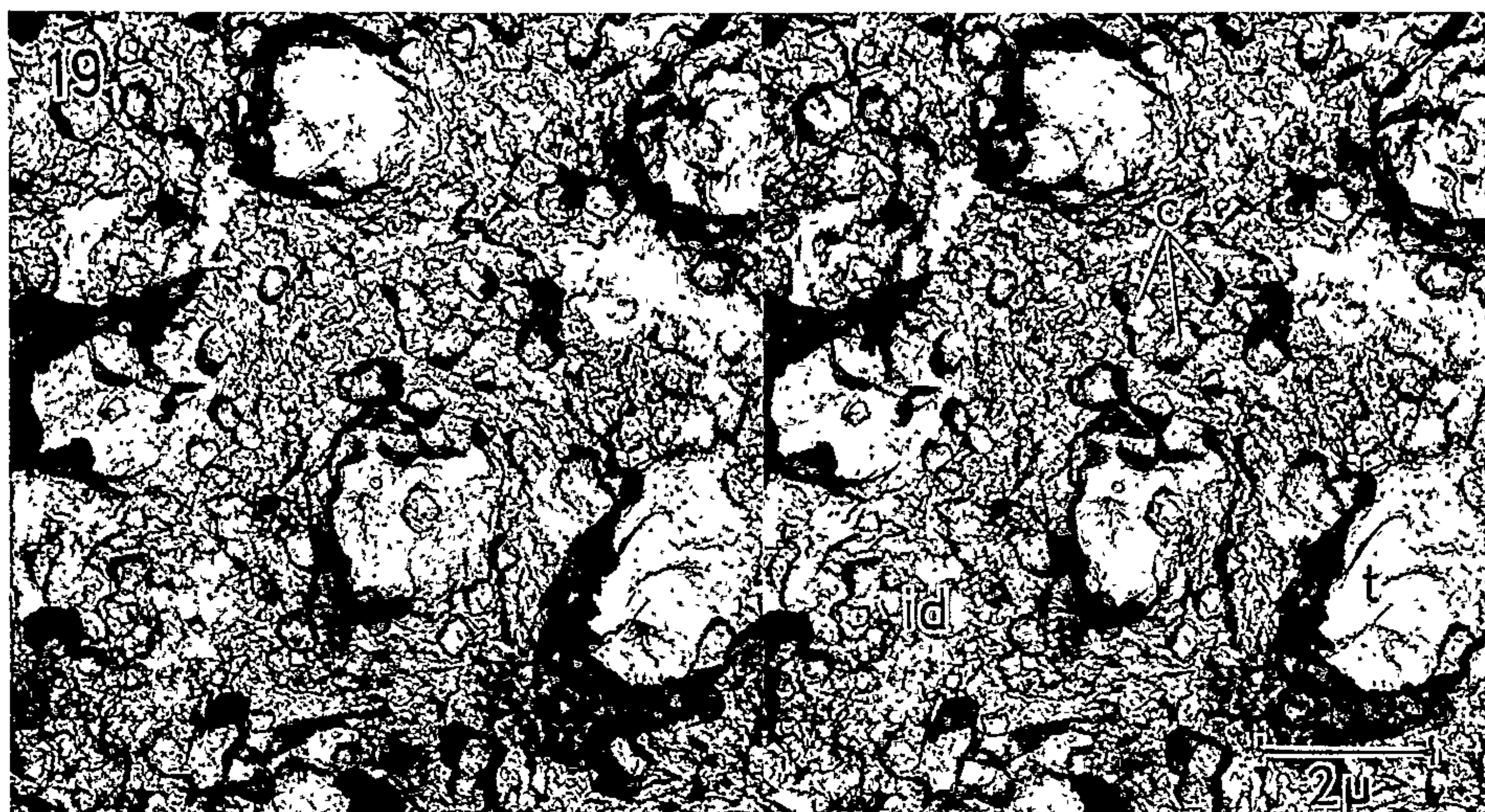


Fig. 19. Stereo-pair transmission electron micrograph of carbon replica of deliberately fractured surface of dentine adjacent to the caries cavity. Transverse fracture showing irregularly shaped crystals confined mainly to areas of intertubular dentine

Discussion

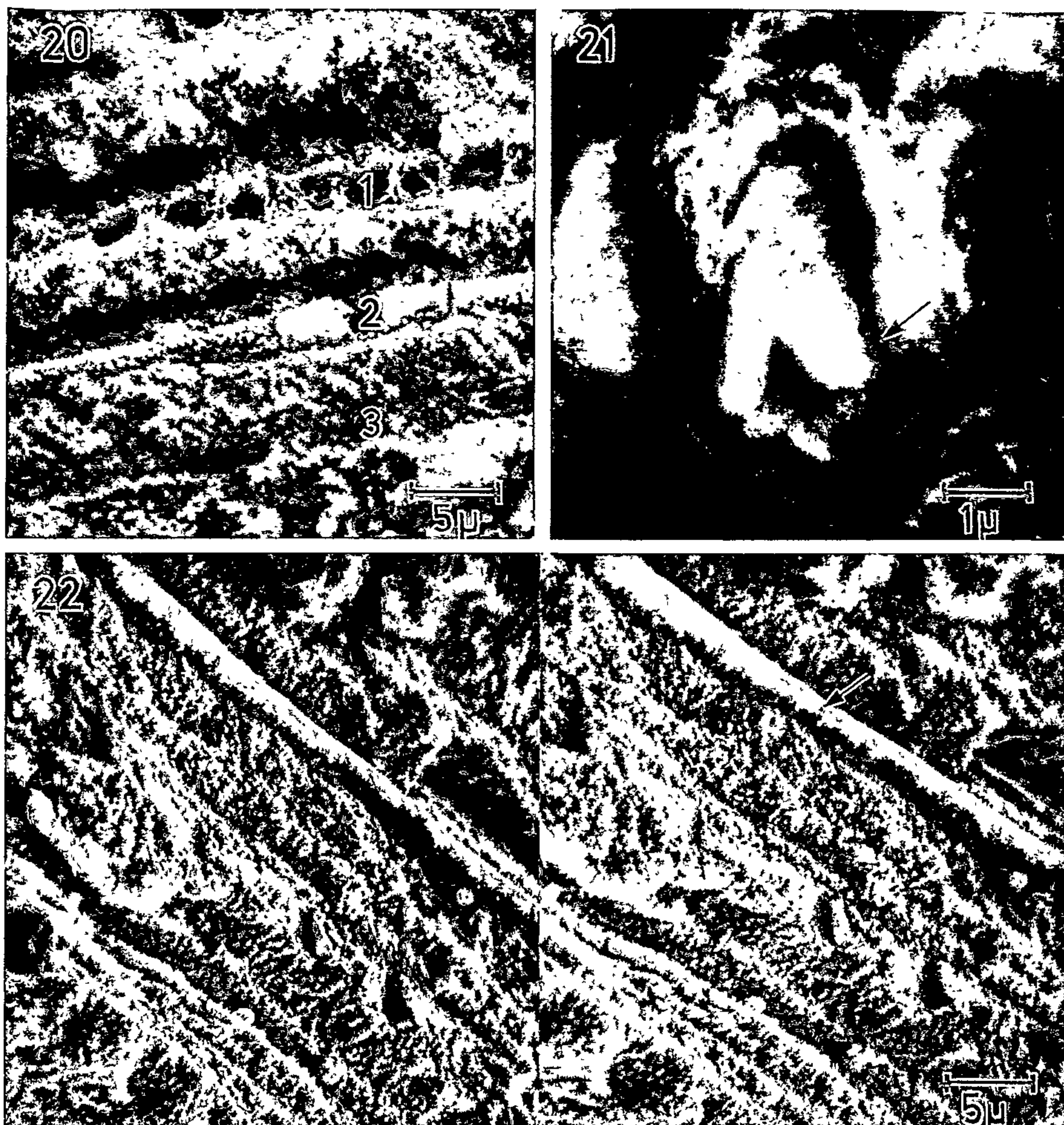
A major advantage to studying hard tissue morphology by a combination of scanning electron microscopy and a replica technique for the transmission electron microscope is that the scanning microscope provides a previously unobtainable sampling rate which can then be selectively reinforced in terms of 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-diaminoethane 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 corres-

pond 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 studies without much attention being given at an electron microscopical level to the details of crystal habit (NEWSELY, 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 5μ) 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 calcification 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 SOGNAES, 1960; ZANDER, HAZEN and SCOTT, 1960; KATTERBACH et al., 1965; SCHROEDER, 1965; FRANK and BRENDDEL, 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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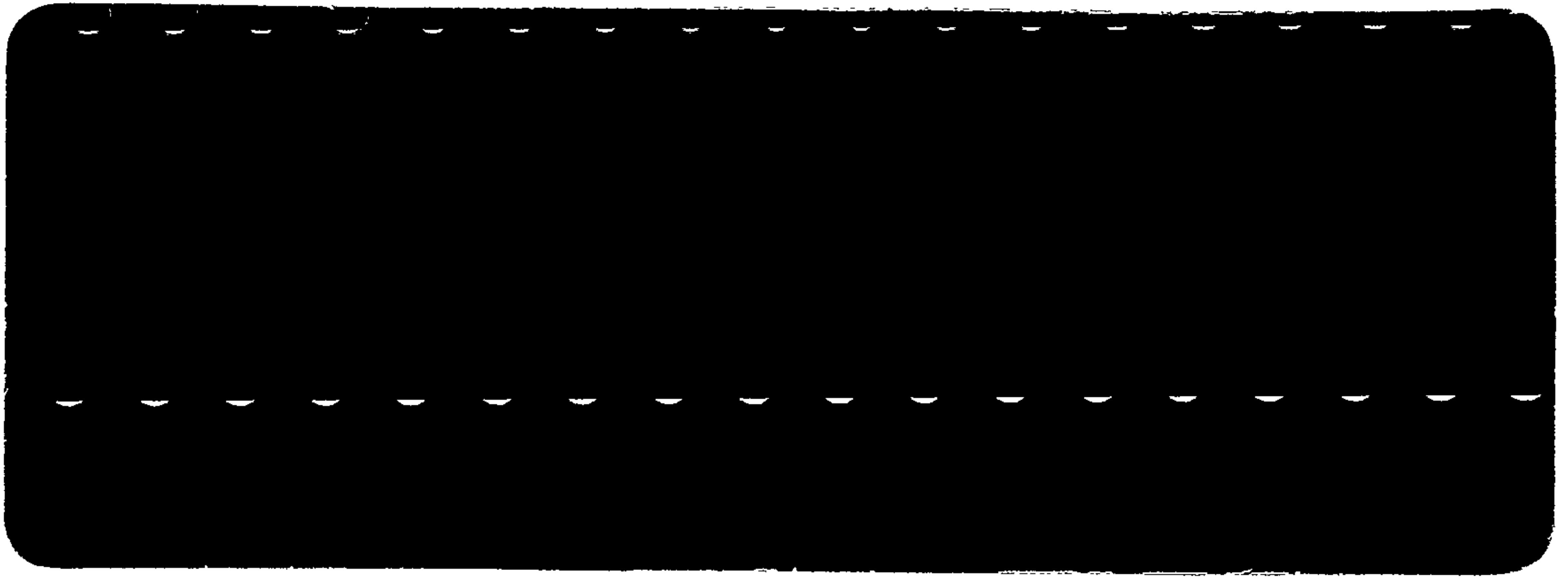
Scanning Electron Microscopy of Some
Components of Caries-Associated Plaque:

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Untreated dried plaque associated with caries does not have morphologically recognizable detail when examined by surface electron microscopy. It was found that prior treatment of whole human carious teeth with 1:2 diamino-ethane at 112° C. removed both the intermicrobial material and the contents of some bacterial cells and, possibly, some of the bacteria in their entirety. This procedure leaves a skeletal framework of bacterial forms that in the present study were examined directly in the scanning electron microscope after the application of a conducting coat that consisted of layers (ca 200 Angstroms) of gold. Stereo-pair images were recorded at a tilt angle of ten degrees. In this bacterial "matrix", coccal, bacillary, and filamentous forms of various sizes could be identified. Some of the bacillary types formed chains and some of the filamentous forms were hollow, as frequent patent endings demonstrated. Whereas these two forms were smooth-surfaced, the coccuslike bacteria had a surface pattern seemingly made up of large plate-like segments. The interweaving

of the filamentous types was a notable feature. It is suggested that, in combination with more selective (enzymatic?) digestive procedures, this method holds promise for the identification of the bacterial species and assessment of the percentage volume occupied by them in the plaque.

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A METHOD OF PREPARING BACTERIAL PLAQUE LINING CARIOUS CAVITIES FOR EXAMINATION BY SCANNING ELECTRON MICROSCOPY

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Summary—Scanning electron microscopy can be used to study the morphology of dental plaque *in situ* on a tooth, if the specimen is first treated with ethylene diamine. It confirms the mixed nature of the microbiological flora. The suggestion is made that selective digestion may give more precise information on the types and numbers of organisms present.

INTRODUCTION

THE METHOD reported here, for preparing some components of caries plaque for examination by scanning electron microscopy whilst they remain *in situ* on the tooth and cavity surface, was found whilst preparing specimens for studies reported elsewhere (LESTER and BOYDE, 1968). Although the precise mode of action of the chemical agent on the components of the plaque is unknown, the empirical results so far promise to be of sufficient value in a number of lines of enquiry to warrant a description of the procedure.

MATERIALS AND METHODS

Deciduous and permanent human teeth with extensive carious lesions were fixed upon extraction in neutral formol-saline and stored in 70 per cent ethanol. The carious cavity in each of these teeth involved extensive loss of enamel and dentine and the pulp chamber was "exposed" in most instances. The whole teeth were placed in glass extraction thimbles in a Soxhlet condenser and subjected to 1,2-ethane diamine ($\text{NH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{NH}_2$, commonly known as ethylene diamine) at a temperature of 112°C , the organic solvent being distilled through this apparatus continually for a period of at least 14 days. At the end of this time, three changes of absolute ethanol were distilled through in place of the 1,2-ethane diamine in order to wash the specimens, which were then allowed to dry in air and stored in a desiccator until required.

For examination in the scanning electron microscope (Cambridge Instrument Co. Stereoscan, operated at 10 kV) specimens were given an electrically-conducting coat of $\sim 200 \text{ \AA}$ of carbon and then $\sim 300 \text{ \AA}$ gold, which were evaporated *in vacuo* on to the specimens whilst they were rotating and facing at an angle of approximately 45° to the evaporation sources. Stereo-pair images were recorded with a tilt angle of 10° .

OBSERVATIONS

Untreated, dried, plaque surfaces prepared in the way described above, but with the omission of the 1,2-ethane diamine extraction process, presented a totally unrecognisable appearance when examined by scanning electron microscopy. In the 1,2-ethane diamine treated material, however, a thick layer consisting of a variety of delicate interlocking forms was evident, even at low magnification examination, over the enamel and dentine walls of the caries cavity and, in some instances, over the external surface of the tooth about the margins of the cavity (Figs. 1-3). These delicate structures we interpret as bacteria of the caries plaque, on the basis of the scanning electron microscope studies of cultured caries plaque organisms by BOYDE and WILLIAMS (1968). The occurrence of this plaque material is irregular and no attempt has been made in this preliminary study to analyse its distribution. The forms constituting the plaque after 1,2-ethane diamine extraction are readily divisible into the following apparently different morphological types:

(i) an elongated rod-like or bacillary form which is segmented (units $\sim 5 \mu$ in length) (Fig. 1);

(ii) a rod-like or bacillary form of lesser diameter than (i) ($\sim 0.5 \mu$ for fifty measured) and with short side-branches (Figs. 1 and 3);

(iii) an unsegmented tubular form which is hollow, as frequent patent endings demonstrate (Figs. 2 and 4);

(iv) an elongated ribbon-like form of lesser diameter than (iii) ($\sim 0.6 \mu$ for fifty measured) and which gives the appearance of being fragile and having collapsed in places (Fig. 5); and

(v) an ovoid or coccus-like form especially common over enamel and dentine cavity surfaces and which varies in size such that the figures for the apparently least and the apparently largest long diameter, for fifty organisms measured, range from 0.3μ to 1.8μ (Figs. 6-9).

The surfaces of the bacillary and filamentous organisms are smooth and structureless. The surfaces of the coccal forms, although relatively smooth overall and rounded, appear on higher magnification examination to be made up of the collective contributions of large, individual, plate-like segments (see for example, Fig. 7).

In addition to the above forms, a few isolated examples of what we interpret as yeast cells were noted.

DISCUSSION

It is generally accepted that the initial attack in the caries process stems from within the bacterial plaque at the enamel surface, and that the adherence of the plaque must, therefore, be a factor of importance in this process. This adherence is often explained in terms of the adhesive properties of the polysaccharide components (see GUGGENHEIM and SCHROEDER, 1967) of the plaque, and of the strengthening with filamentous organisms (see for example, SNYDER, BULLOCK, and PARKER, 1967). The present results lend additional weight to the latter explanation of the mechanical

properties of the plaque, for the complex interweaving of the filamentous bacterial forms (Figs. 1, 2 and 5) must act in stabilizing the plaque and, by providing support, help to maintain a favourable environment in which the viable constituents may continue to function.

It is clear, from examination of those structures remaining on the cavity surfaces so far examined, that the intermicrobial material (at least that in an unmineralized state) is effectively dissolved by 1,2-ethane diamine. Preliminary results of digestion experiments on dried droplets of suspensions of formalin-fixed, typed bacteria common to caries plaque indicate that certain bacteria are wholly digested by 1,2-ethane diamine, whilst in other species the bacterial cell wall is insoluble although the cell contents are removed. In following this line of investigation, it was subsequently found that gross samples of two commonly-occurring components of bacterial cell walls, namely chitin (in the form of beetle elytra) and cellulose (in the form of filter paper and newsprint) were not digested by 1,2-ethane diamine.

Calcified areas are known to be present in dental plaques (ENNEVER and CREAMER, 1967; GONZALES and SOGNAES, 1960; ZANDER, HAZEN and SCOTT, 1960; KATTERBACH *et al.*, 1965; SCHROEDER, 1965; FRANK and BRENDL, 1966) and it was expected that these would remain in the present material after 1,2-ethane diamine treatment. Further, in studies on "caries crystals" using a carbon replica technique for the transmission electron microscope, structures were found which strongly resembled bacteria and which displayed what appeared to be crystal faces at their surfaces (LESTER and BOYDE, 1968). Thus, the possibility that at least some of the bacteria remaining after 1,2-ethane diamine extraction are calcified cannot be entirely excluded.

The presence of relatively larger numbers of filamentous and bacillary forms in the bulk of the plaque material, with a predominance of coccal bacterial forms closer to the enamel surface, agrees well with the description of the predominant organisms by other workers (for example, SCOTT and ALBRIGHT, 1954; FRANK, 1955; AWAZAWA, 1964). However, the voluminous literature on the microbial content of dental plaques leaves a very clear impression that it would at present be unwise to attempt to identify naturally occurring organisms by comparison of their morphology with that of cultured organisms (the latter being known, in general, only by light microscopy—see BURNETT and SCHERP, 1957; GILMOUR, HOWELL and BIBBY, 1961; KEYES, 1962; THEILADE, THEILADE and SCOTT, 1962; SNYDER, BULLOCK and PARKER, 1967). Nevertheless, it may be predicted that the application of the techniques of surface electron microscopy to the problem of identifying microbes on morphological grounds (see GRAY, 1967; BARTLETT, 1967) will contribute to further understanding. Furthermore, the present qualitative results point to the possibility of obtaining quantitative data on dental plaque, such as the proportion of its volume occupied by bacteria belonging to various genera. This approach may be furthered by developing methods of digesting out various plaque components more selectively. In this way, it might be possible to remove one bacterial species at a time with suitably specific enzymes.

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Résumé—La microscopie électronique par "balayage" peut être utilisée pour étudier la morphologie de la plaque dentaire *in situ*, lorsque le spécimen est traité préalablement avec de l'éthylène diamine. Une telle étude confirme le caractère mixte de la flore bactérienne. Il semble que la digestion sélective donne des informations précises, concernant les types et le nombre des micro-organismes présents.

Zusammenfassung—Mit Hilfe der Raster-Elektronenmikroskopie ist es möglich, die Morphologie der Zahnplaques auf einem Zahn *in situ* zu untersuchen, wenn das Objekt zuerst mit Äthylendiamin behandelt wird, so daß die Mischflora erhalten bleibt. Es wird erwogen, daß der selektive Abbau genauere Informationen über Arten und Zahlen der vorhandenen Organismen ergeben dürfte.

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PLATES 1-2 OVERLEAF

PLATE 1

FIGS. 1-4. Scanning electron-micrographs of some bacterial components of plaque on the enamel walls of carious cavities after 1,2-ethane diamine extraction.

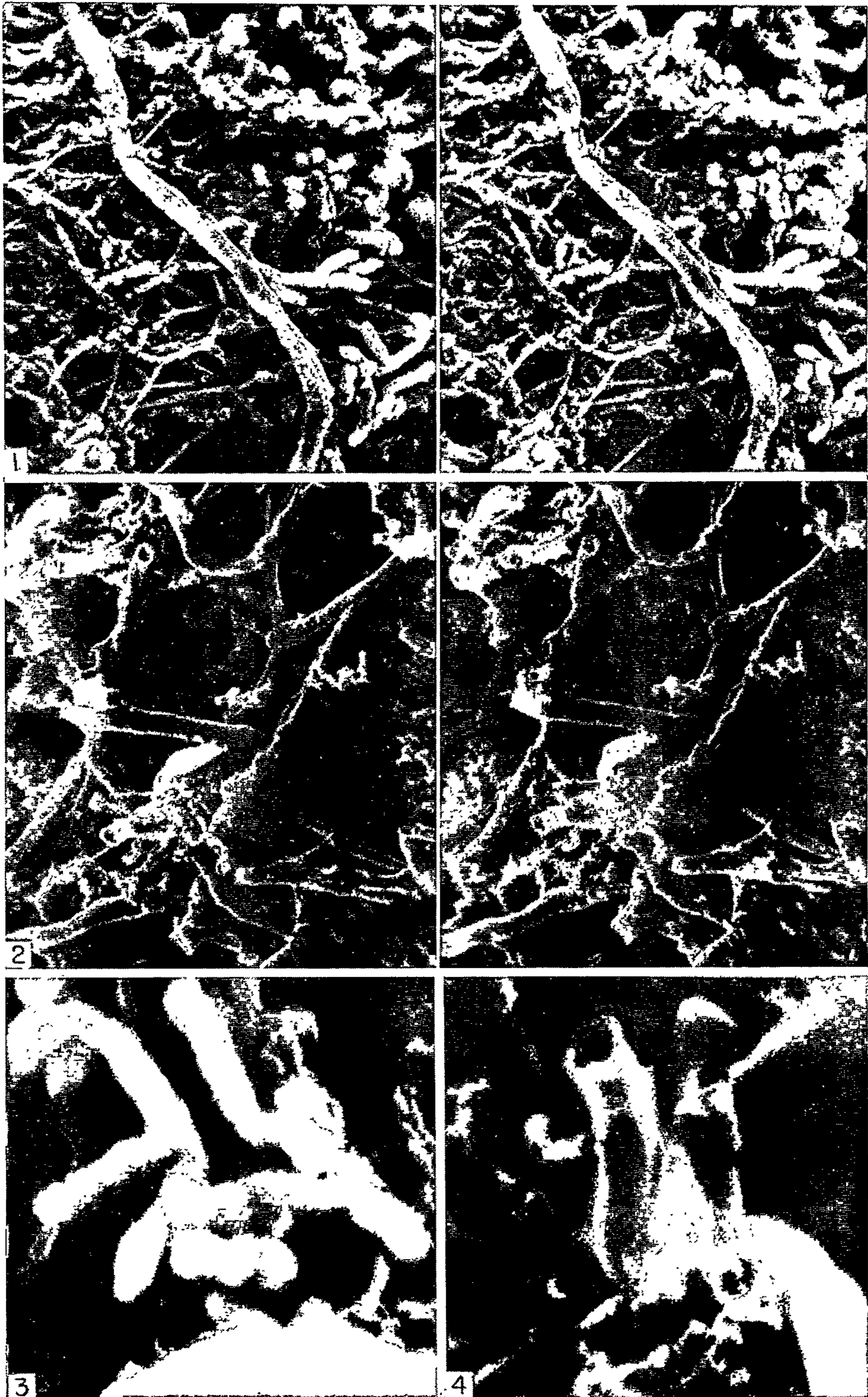
FIG. 1. Stereo-pair showing the depth and delicate nature of the plaque. Various rod-shaped or bacillary bacterial forms arranged in chains. $\times 2900$.

FIG. 2. Stereo-pair showing hollow smooth-surfaced filamentous microbes. $\times 3700$.

FIG. 3. Enlargement of part of Fig. 1 to show side-branching and rounded ends of rod-shaped organisms. $\times 12,300$.

FIG. 4. Enlargement of part of Fig. 2 to show hollow nature of filamentous organisms. $\times 9300$.

SCANNING ELECTRON MICROSCOPY OF PLAQUE BACTERIA



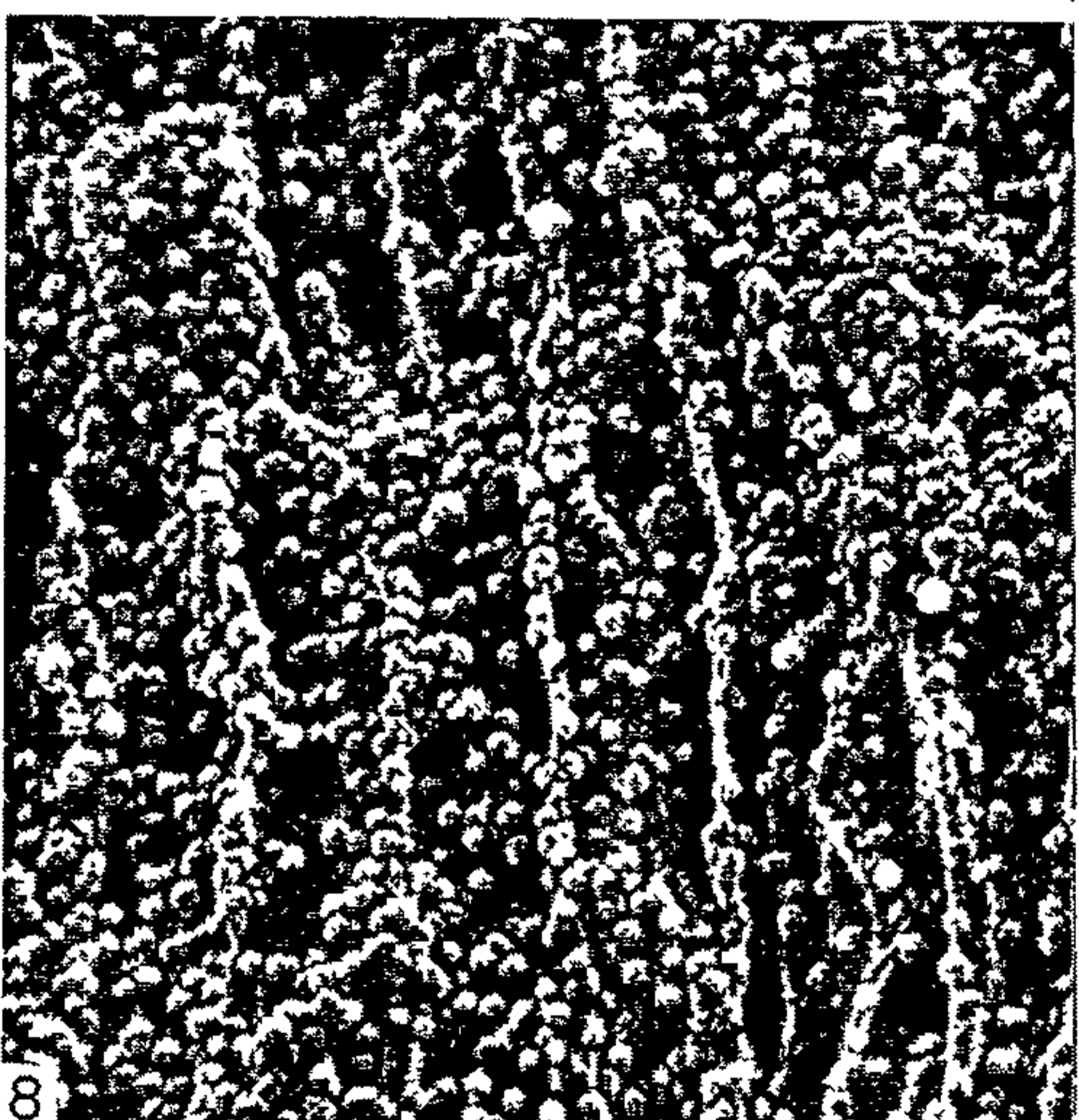


PLATE 2

FIGS. 5-9. Scanning electron-micrographs of some bacterial components of caries plaque after 1,2-ethane diamine extraction.

FIG. 5. Filamentous organisms over the enamel wall of a carious cavity, showing the extent of their interweaving. $\times 3000$.

FIG. 6. Coccal organisms over the enamel wall of a carious cavity, showing an embossed surface pattern. $\times 10,000$.

FIG. 7. Coccal form showing detail of surface pattern. $\times 11,400$.

FIG. 8. Low magnification of carious enamel surface showing the prominent prism sheath regions and the profusion of coccal bacterial forms. $\times 1140$.

FIG. 9. Higher magnification stereo-pair of Fig. 8. $\times 3300$.

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The Structure and Development of Marsupial Enamel Tubules

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Summary. The development and structure of marsupial enamel tubules has been studied in a number of species by a variety of microscopical techniques. The results were as follows.

1. The undoubted continuity of dentinal and enamel tubules could be traced in all species examined.

2. The tubules leave more residue than the surrounding enamel when decalcified.

3. The tubules are permeable to dyes in extracted teeth.

4. The dyes methyl blue and trypan blue did not reach the enamel tubules from the pulp or blood-stream in *in situ* adult teeth of *Metachirus nudicaudatus*.

5. The tubular nature of the tubules is well demonstrated in scanning electron micrographs and replicas of fractured enamel and also in replicas of argon-ion beam eroded *Macropus* molar enamel surface.

6. The tubules are situated within the enamel prisms.

7. The tubules may be recognized in electron micrographs of developing enamel as regions in which crystallites do not develop.

8. The study of enamel tubule development revealed no special features of the ameloblasts or of the nature of the first secreted enamel.

Introduction

JOHN TOMES (1849) discovered that in the marsupials with the sole exception of the wombat "the greater number, if not all, of the dentinal tubes are continued into, and constitute a considerable portion of the enamel". The variety of views expressed since that time concerning the position and origin of the tubules in marsupial enamel are summarised in Table 1. It will be noted that whereas the majority of previous workers have been convinced of the continuity of the enamel and dentinal tubules, opinion has been equally divided on the question of whether they originate from the dentine or are of purely enamel origin. Very little developmental

Explanation to Table 1.

A) "Indeed in all teeth the enamel fibre is at an early stage of formation partially tubular". (TOMES, 1849).

B) v. EBNER invoked a resorption of the first formed dentine to account for the continuity with the dentinal tubules. He also described the positive birefringence of marsupial enamel for the first time.

C) Noted that the "honeycomb" of developing enamel is of greater thickness in marsupials.

D) Canals not bound to individual prisms.

E) "Tubules" are ground section artefacts.

F) "Kittsubstanz" of many earlier authors is equivalent to prism sheath substance of modern terminology, rather than to interprismatic regions.

Table 1. Summary of previous views on the position and origin of the tubules in marsupial enamel

	e = enamel origin d = dentinal origin	- = no continuity with dent. tubules + = continuous with dent. tubules	p = in prisms i = in interprismatic regions s = in prism sheath substances	Name adopted (Canal = Kanälchen, canal, or canaliculus)	+ = developing tissue studied	Other remarks
A) J. TOMES (1849, 1856)	e	+	p	tube and fibril		note A
B) V. EBNER (1890)	e	+	s	canal		note B 3—5 round each prism
C) C. S. TOMES (1897, 1904)	e	+	p	fibre and fibril	+	note C 1 in every 4 prisms
RÖSE (1893, 1897)	d	+	s	canal		
PAUL (1896)			s	tubule		
WILLIAMS (1897, 1923 and cit. by CARTER, 1920)	d	+	p and s	fibril canal		
D) WALKHOFF (1898)				canal		denied existence of "Kittsubstanz"
MUMMERY (1914, 1915, 1919)	d	+	s	tube and fibril	+	penetration of dentinal fibril
ADLOFF (1914)			s	canal		largely substitute for prism sheath substance
CARTER (1917, 1920, 1922)			p and s	tube and fibril	+	direction not dependent on prisms
WEIDENREICH (1926)	d	+	pis	canal		part of same system as tufts and lamellae
MUNCH (1929)			—	canal		parallel prism direction
SPRAWSON (1930)		+	i	tube		stain penetration d.ts. → e.ts.
MARCUS (1931)	d	+	p	axial fibril		
HÄUSELE (1932)			p			
SKUES (1932)	e		p	fibril	+	
MCCREA and RO- BINSON (1935/36)		+	—			stain penetration d.ts. → e.ts.
E) MOSS and APPLE- BAUM (1963)	e	—	p	fibre	+	≡ to tufts, (note E) uncalcified rods
BOYDE and LESTER (1967)	e	+	p	tubule	+	deficient crystallite formation locally

Explanation see p. 558

material has been studied and there have been no reports of the investigation of this problem with modern techniques for elucidating ultrastructure. Further, although the permeability of the enamel tubules has been demonstrated *in vitro* (SPRAWSON, 1930; McCREA and ROBINSON, 1935/36), no previous attempts have been made to demonstrate a physiological continuity of the dentinal and enamel tubules *in vivo*.

Materials and Methods

The marsupial species studied and the method of their study are listed in Table 2.

Table 2. *Materials and methods*

Refer- ence numbers*	Species studied	Type of Examination				
		Electron micro- scopy of tubule de- velopment	Electron micro- scopy of adult structure	Light micro- scopy of tubule de- velopment	Light micro- scopy of adult structure	Dye diffusion experi- ments
B 61	<i>Metachirus nudicaudatus</i>	+	+	+	+	+
B 64	<i>Didelphys marsupialis</i>			+	+	
B 119	<i>Vombatus ursinus</i>		+		+	
B 151	<i>Trichosurus vulpecula</i>	+	+	+	+	
B 154	<i>Acrobates pygmaeus</i>			+		
B 169	<i>Antechinus swainsoni</i>			+		
B 177	<i>Pseudocheirus convolutor</i>			+		
B 215	<i>Protemnodon rufrogrisea</i>			+		
	<i>Macropodidae</i> (various species)		+	+	+	+

* (See "The Mammals" — Desmond Morris).

Electron Microscopy of Tubule Development

Tooth germs were fixed in Palade's and Dalton's fixatives and embedded in methacrylate. Approximately 500 Å thick sections were cut on a Porter-Blum microtome and examined at 60 kV in a Siemens Elmiskop I. $\frac{1}{2}$ μ thick sections were stained with crystal violet and basic fuchsin for light microscope controls.

Electron Microscopy of Adult Structure

Single-stage carbon replicas of fractured whole adult teeth were prepared by a method described by BOYDE (1967). Stereo-pair micrographs (tilt angle usually 8° 40') were taken at 40 kV.

Fractured whole teeth and teeth extracted for prolonged periods with hot 1:2 diaminoethane were examined in a scanning electron microscope (Cambridge Instrument Company "Stereoscan"). A conducting coat of ca. 200 Å carbon and 300 Å gold was applied by vacuum evaporation with the specimens rotating so as to ensure good coverage of the surface to be examined. Stereo-pair electron micrographs (tilt angle usually 10°) were taken at 10 kV.

One transverse ground section of a *Macropus* molar was bombarded with 5 keV argon ions in the specimen chamber of a scanning electron microscope (BOYDE and STEWART, 1962).

Light Microscopy of Tubule Development

Ground sections were embedded in Canada balsam and examined by ordinary transmitted light.

Dye Diffusion Experiments

The pulp chambers of extracted *Macropus* and *Metachirus* molars were filled with solutions of crystal violet and basic fuchsin and allowed to stand for 1 or 2 days until dry. The external surfaces of these teeth had been covered with wax to prevent centripetal access of spilt solution.

One 1 year old 3.5 kg opossum (*Metachirus nudicaudatus*) was given a series of four intraperitoneal injections of 1% aqueous trypan blue over a period of one week (total 40 mls 1% solution injected).

Cavities which exposed the pulp were prepared in the buccal surfaces of some of the cheek teeth of a second 1 year old opossum and an adult female opossum of unknown age. Dry methyl blue was "teased" into these cavities with a probe; moistened; and the cavity sealed with quick setting zinc oxide and eugenol cement.

For the first experiment, the teeth were extracted after an interval of one month following the final injection. The teeth were extracted after an interval of one week in the case of the intrapulpal cavities. Ground sections were prepared in both instances.

Results

Electron Microscopy

Marsupial enamel was found to bear a close resemblance to the ungulate pattern, with longitudinal rows of prisms separated by longitudinal inter-row sheets ("Pattern 2", BOYDE, 1964). It is characteristic of this pattern that the crystallites within prism domains diverge less from the prism direction than those in "Pattern 3" prisms. The orientation of the prism crystallites, following the overall cuspal or incisal inclination of the prisms, thus contrasts with that of the inter-row sheet crystallites which incline slightly cervically. The main difference between marsupial and ungulate enamels observed in this study was the more common occurrence of a joining of prism-sheaths between members of the same row of prisms in ungulate enamel, related to a greater prominence of the inter-row ridges at the developing surface.

The main interest centred on the identification of the so-called enamel tubules. Some trouble was experienced in establishing reliable criteria for their identification in ultra-thin sections, since they were only seen as defects in the generally even distribution of crystallites in the developing enamel and were difficult to distinguish from artefact tears in the sections. The tubules were eventually positively identified by tracing the same pattern of defects in serial sections. Fig. 6 shows a small part of a pattern of tubules that was identified in all of 35 serial sections.

The enamel tubules were found to develop and to be propagated at the mineralising front of the enamel as areas in which there was a failure to form crystallites (Figs. 1, 3). Their site of development in the cervical slopes of the depressions in the mineralising front, rather than in relation to the interrow ridges, determines their intraprismatic localisation. These discontinuities in the otherwise intact mineralising front were not matched by any apparent abnormality in the surface of the Tomes' processes of the ameloblasts or in the extracellular granular material (the enamel matrix gel). The tubules were found to be located anywhere within prism domains, even in positions confluent with the boundary between a prism and an adjacent inter-row sheet, but not at the prism sheath separating two prisms in the same row (Figs. 7, 8, 9, 19, 20, 21).

The only morphologically identifiable contents of the tubules were the occasional isolated crystallites which passed through or across them (Figs. 3, 4, 5). The



Figs. 1—9. Electron micrographs of ultra-thin sections of developing *Metachirus nudicaudatus* enamel

Fig. 1. Longitudinal section of early developing enamel. Note one "tubule" crossing from dentine to enamel. The inset figure shows the orientation of the section, and also a "tubule" at the mineralising front. *e* enamel, *d* dentine, *edj* enamel-dentine junction, *et* enamel tube, *dt* dentinal tube, *x* enamel crystallites, *a* ameloblasts, *Tp* Tomes' process, *em* enamel "matrix", *ptd* peritubular dentine, *lb* lateral branch of dentinal tubule, *tb* terminal bar apparatus, *irs* inter-row sheet, *p* enamel prism, *c* crack, *f* fold in replica film, *sz* surface zone

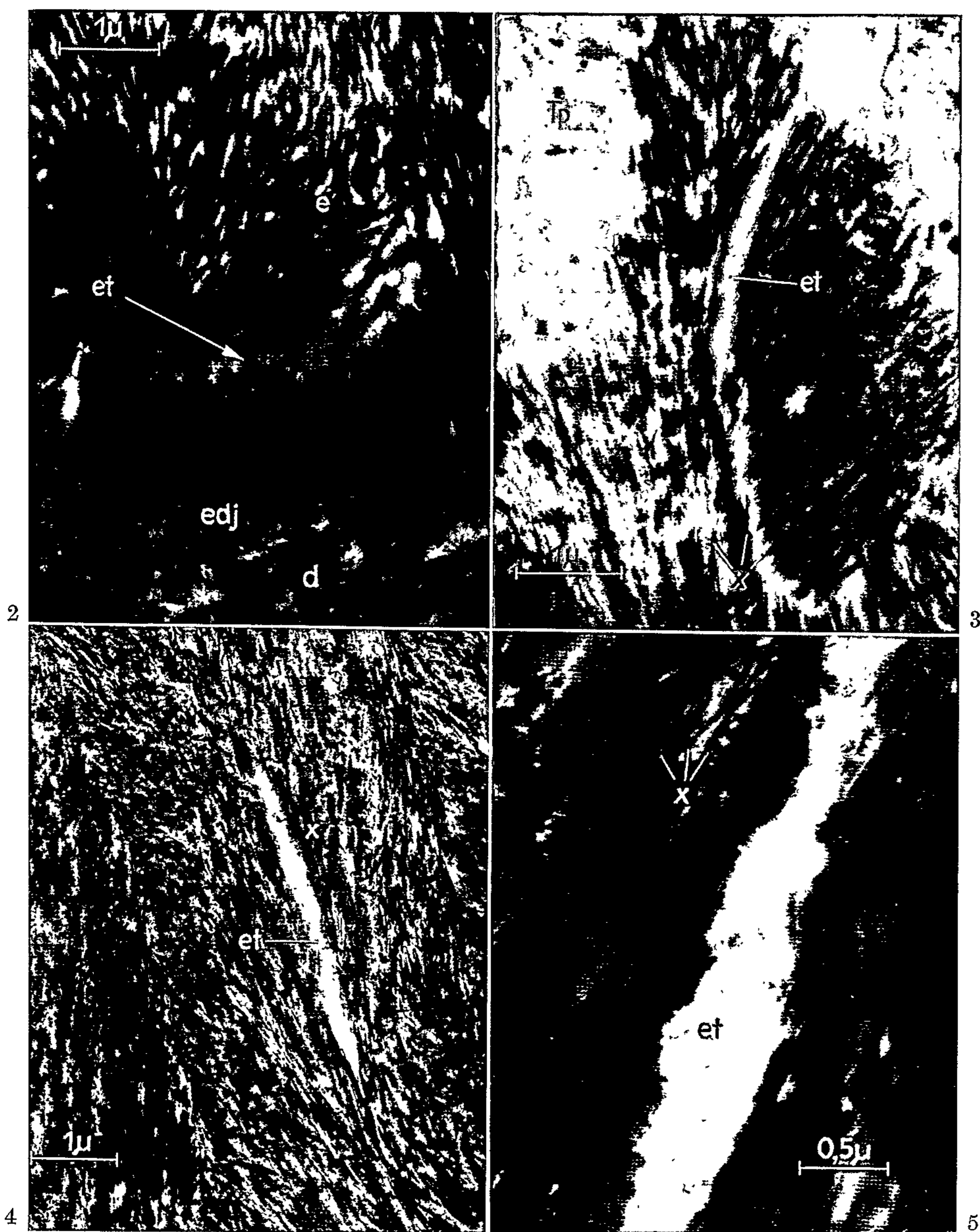


Fig. 2. A bend and bulge in an enamel tubule close to the enamel-dentine junction, showing the projection of crystallites into the "tubule lumen"

Fig. 3. A tubule continuous from the mineralising front for some distance into the enamel
 Figs. 4 and 5. Tubules in more mature enamel

material within the tubules appeared entirely similar to the rest of the organic matrix throughout the enamel.

The undoubted continuity of dentinal and enamel tubules could be traced in all the marsupial species we have examined (Figs. 1, 10, 11, 12, 16, 17, 18 and 27), except in *Vombatus ursinus* [wombat, the one marsupial reported by TOMES (1849)

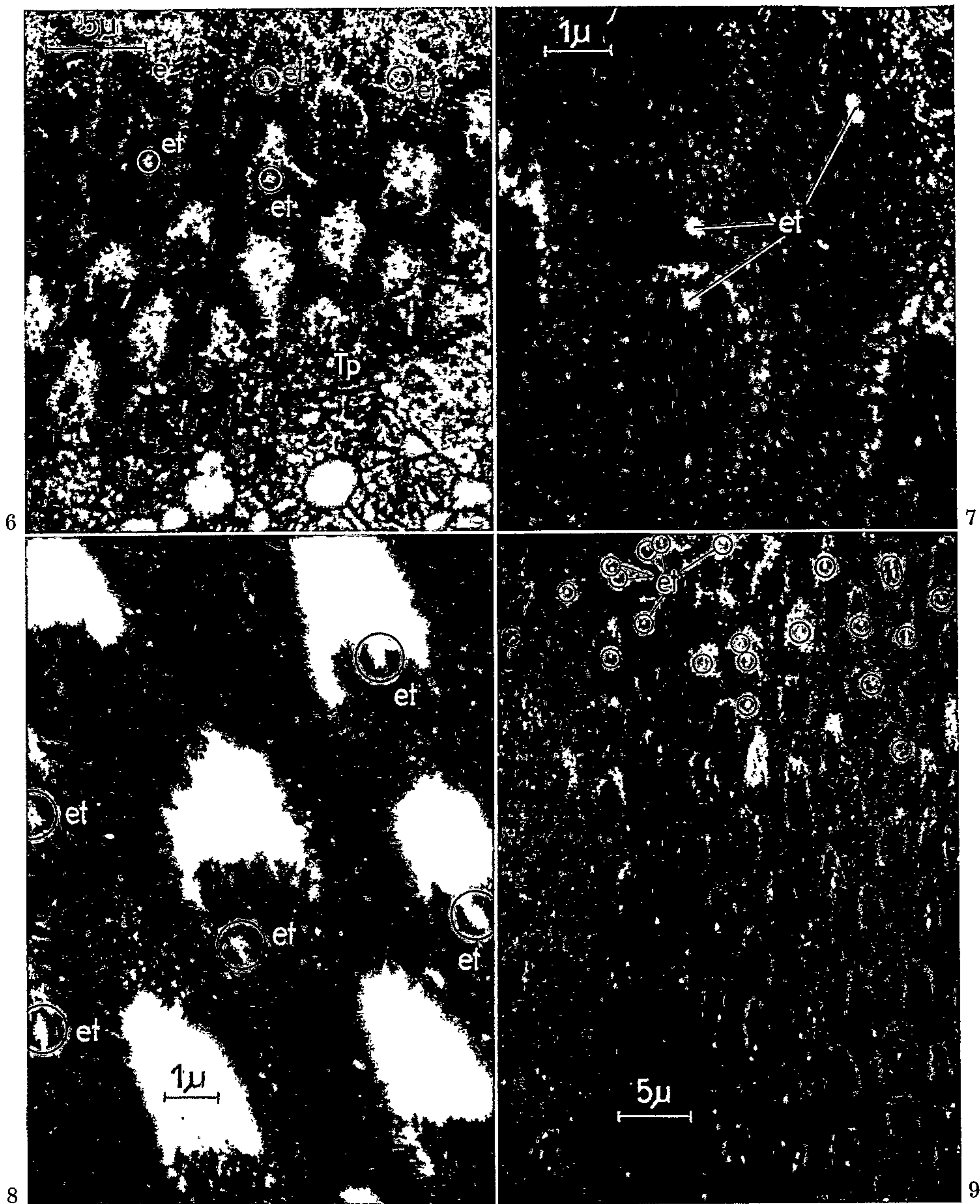


Fig. 6. Tangential section of developing enamel surface, one of a series of 35 in which the same pattern of tubules marked could be traced

Figs. 7 and 8. Transversely sectioned prisms near the developing front showing intraprismatic location of tubules

Fig. 9. Low power survey showing distribution of tubules, often several per prism

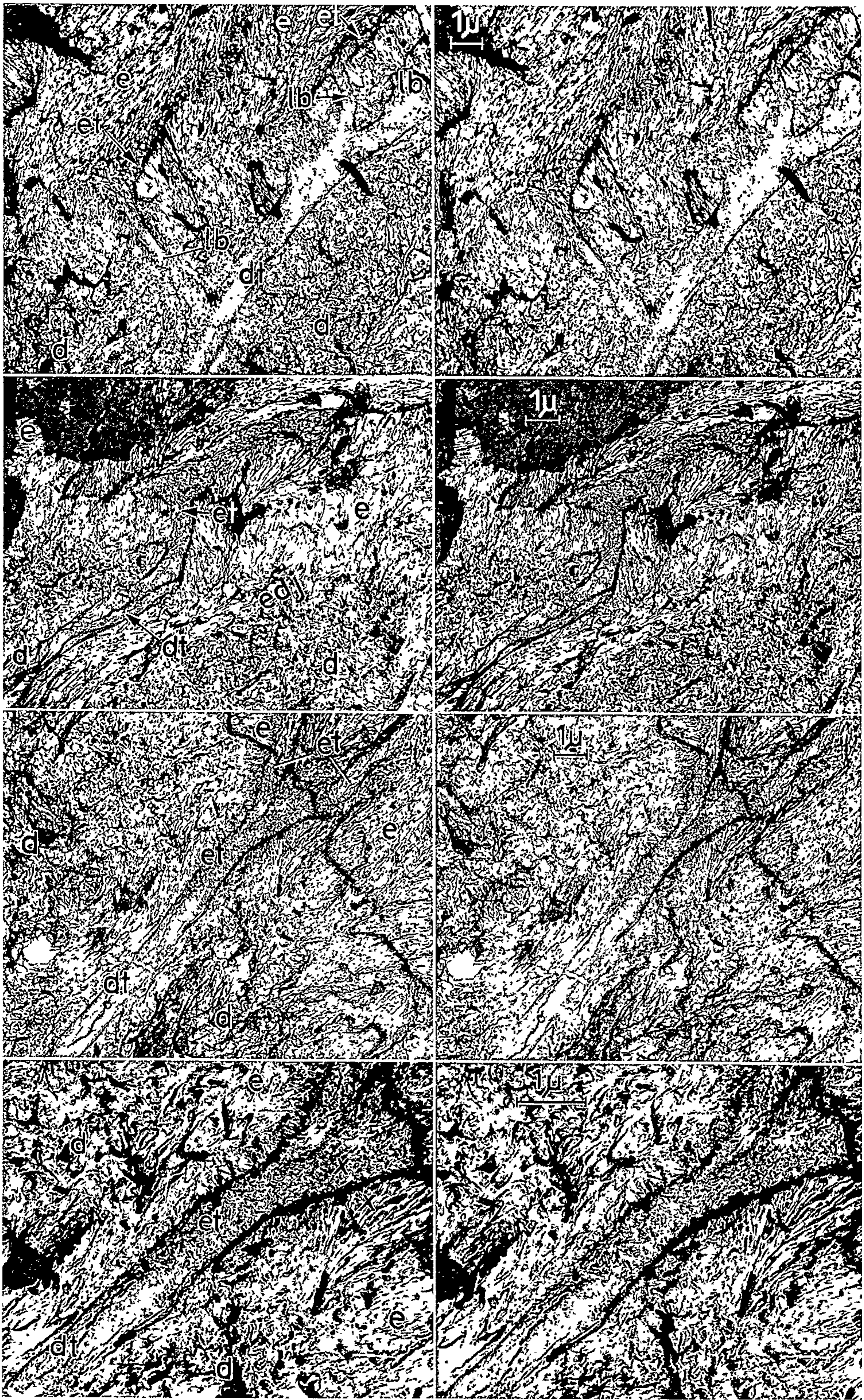
Figs. 10—15. Stereo-pair electron micrographs of carbon replicas of fractured enamel and dentine (10—14 *Macropus*, 15 *Sus*)

Fig. 10. Lateral branches of terminal part of dentinal tubule crossing enamel-dentine junction at right-angles and continuous with enamel tubule

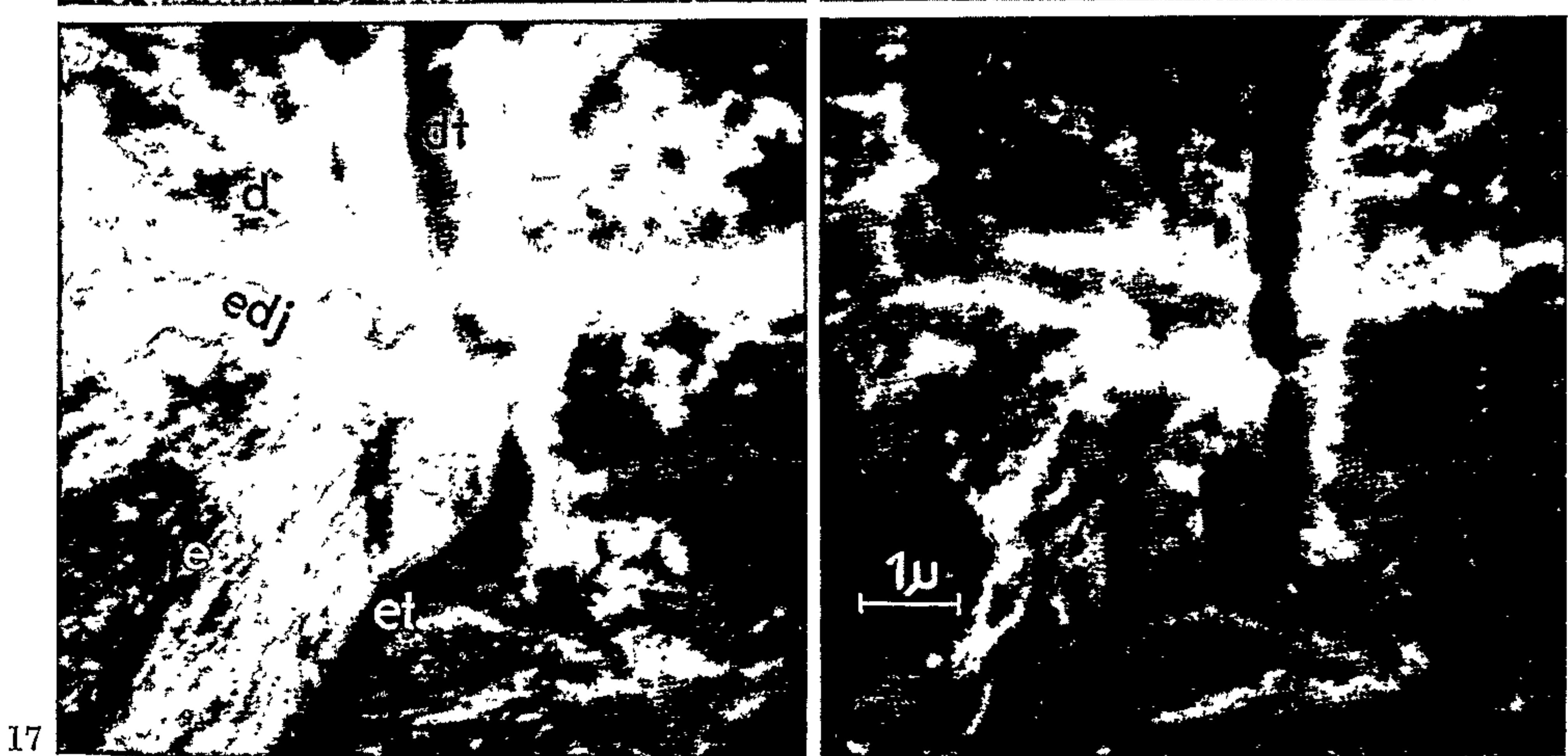
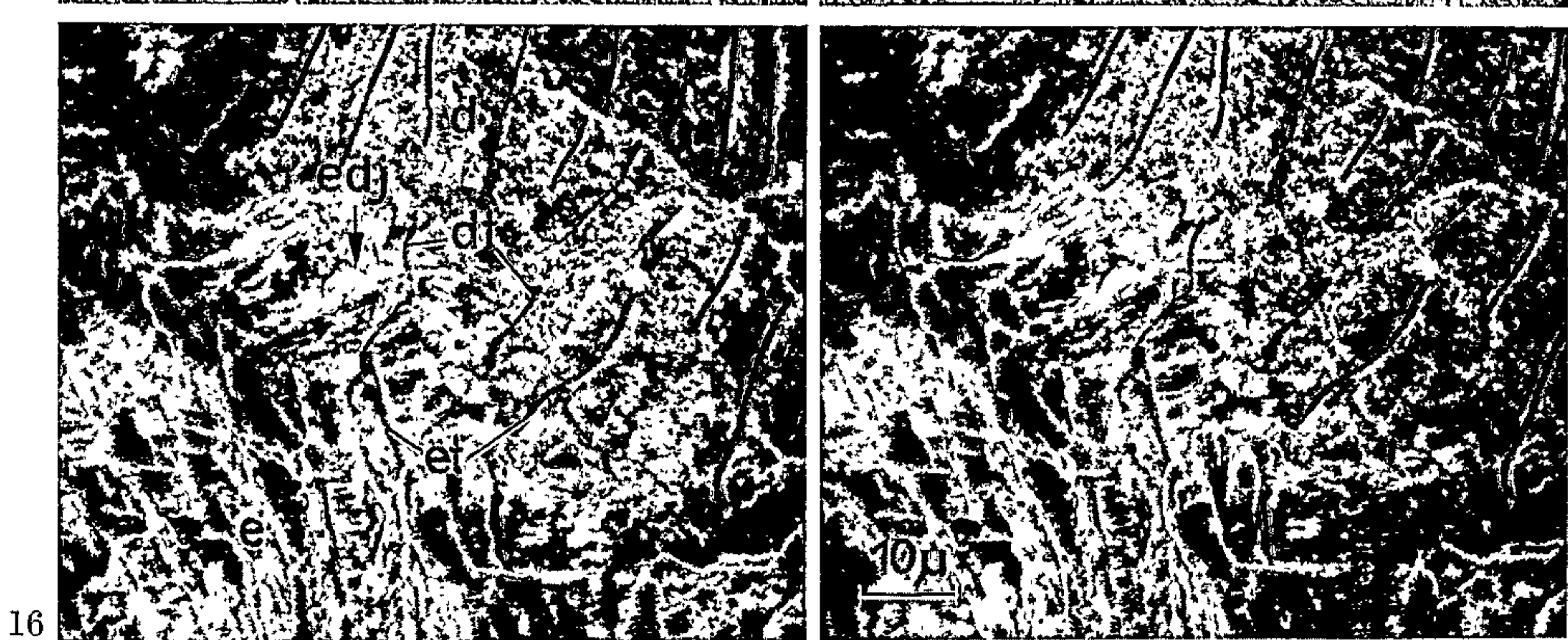
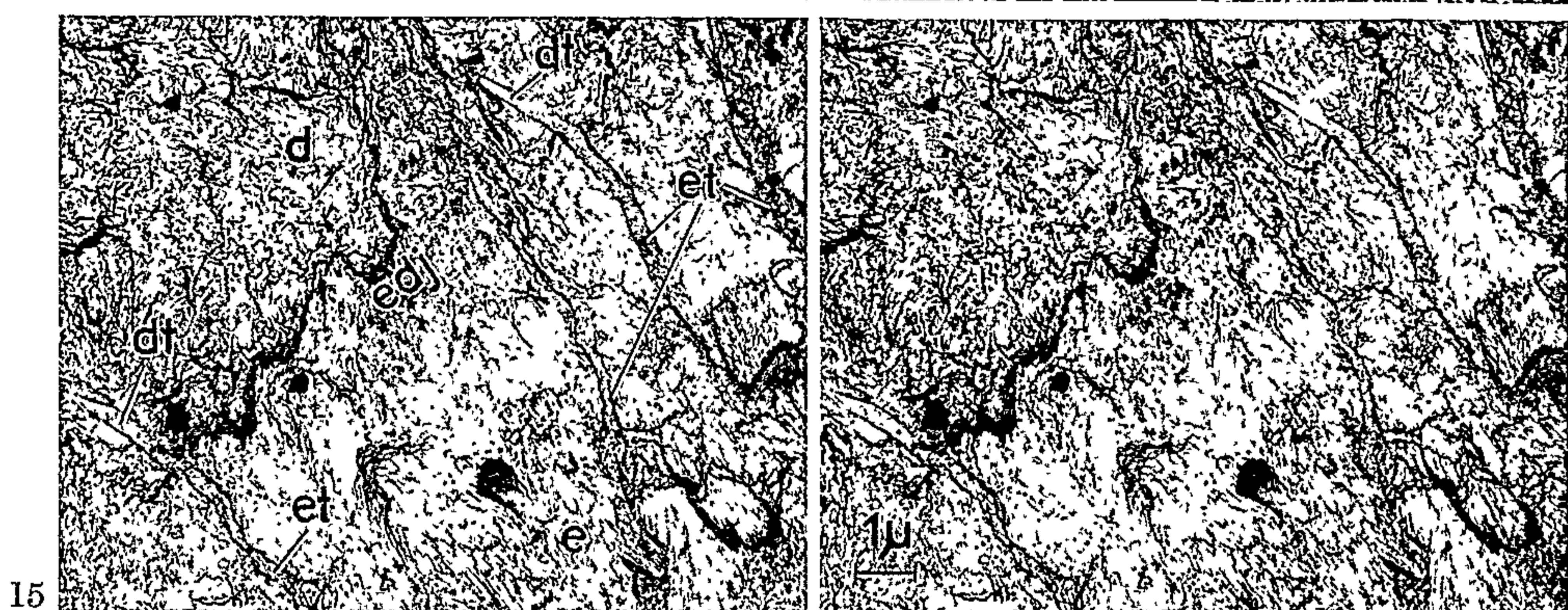
Fig. 11. Dentinal tubule bending abruptly to cross enamel-dentine junction at right-angles and the corresponding enamel tubule continuing parallel to its original course

Fig. 12. Single dentinal tubule continuous across enamel-dentine junction with two enamel tubules

Fig. 13. Enlargement of enamel-dentine junction region shown in Fig. 3 to show different nature of wall of dentinal and enamel tubule



Figs. 10—13 (for Legends see p. 564)



Figs. 14—17 (for Legends see p. 567)

not to have tubular enamel] where the dentinal tubules do not reach the enamel-dentine junction. This we have found to be associated with the presence of large "von Korff" fibre bundles oriented parallel to the enamel-dentine junction (LESTER and BOYDE, in press).

The majority of dentinal tubules reached the enamel-dentine junction without the branching and diminution of diameter normally a feature of tubules in this region. It was often observed in instances where there was terminal bifurcation of the dentinal tubule, that both the resulting branches were continuous across the enamel-dentine junction with tubules in the enamel. Single, unbranched dentinal tubules could often be traced in continuity with a tubule branching within the enamel.

The tubules always crossed perpendicular to the enamel-dentine junction, regardless of the course of the dentinal tubules and the enamel prisms away from the junction, the two usually lying in the same straight line. The tubules are thus approximately parallel with the enamel crystallites closest to the junction, since these first formed crystallites develop perpendicular to a relatively flat mineralising front: the ameloblasts have no Tomes' process projections into the enamel matrix at this stage. There was often a bulge in the enamel tubules where they bent until almost parallel with the enamel-dentine junction before straightening out to proceed with the prisms. This bulge, which was first described by TOMES (1849) but first figured by OWEN (1845), could be identified in many instances in the electron microscopic material. At the region of the bend and bulge the crystallites lay at a considerable angle to the tubules and even projected into the tubule "lumen" to some extent (Fig. 1, 2, 11 and 13). Thereafter, the tubules ran within clearly definable prism domains.

The enamel tubules could be distinguished from prism sheaths (with which they might possibly be confused in longitudinal sections or fracture profiles of the prisms) on the basis of the orientation of the crystallites surrounding them. Prism sheaths or boundaries are planes at which there is an abrupt change in crystallite orientation and they develop along lines at which there is a sharp change in the orientation of the mineralising front in the nature of a concavity towards the enamel. Enamel tubules on the other hand, were found to develop in the convex cervical floors of the depressions and, with one exception, were surrounded by crystallites having basically the same orientation even if this orientation was not parallel with the long axis of the tubule (Fig. 14). The one exception applied in the case of tubules which were confluent with the lateral part of a prism boundary, when the crystallites of the inter-row sheet forming one wall of the tubule obviously made a large angle with those in the other three-quarters of the wall and which belonged to a prism domain.

The structure of the walls of the dentinal tubules showed no differences from those of the placental mammals that we have studied (BOYDE and LESTER, 1967).

Fig. 14. Enamel tubule at some distance from the enamel-dentine junction showing parallel crystallites exposed as its wall

Fig. 15. Stereo-pair transmission electron micrograph of carbon replica of fractured tooth surface of *Sus domesticus*. Enamel-dentine junction region shows dentinal tubules continuous with minute enamel tubules

Fig. 16. Low power survey showing a number of tubules crossing the enamel-dentine junction

Fig. 17. Single tubule at junction



Fig. 18 (for Legends see p. 569)

Argon Ion Etching

The etch structure on argon ion-bombarded human enamel surfaces (BOYDE and STEWART, 1962) was mainly referable to the differences of crystallite orientation associated with its division into prisms. *Macropus* enamel, however, etched in a very different and characteristic way. Each enamel tubule was revealed by the formation of a deep etch pit (Figs. 22, 23, 24) surrounding its opening at the polished surface of the section: they could not be seen at all before bombardment.

The absence of differential etching in the surface zone enamel of *Macropus* (Fig. 25) first drew our attention to the thickness of this non-prismatic layer in this species.

The more rapid erosion of intertubular dentine led to a differential etching effect which left peritubular dentine standing proud of the surface. Fig. 23 shows that the peritubular dentine of *Macropus* reaches right up to the enamel-dentine junction.

Light Microscopic Observations

The enamel tubules were preserved as basophilic fibres in both the developing and mature decalcified enamel of all the marsupial species examined (Figs. 27, 28). In the mature enamel they were the only elements to be retained after decalcification and their relation to the original prism architecture could not be ascertained. In the "transitional" stage of maturation (CHASE, 1935) the basophilic fibres were well differentiated from the pale staining, acidophilic "matrix" (Fig. 26). The basophilic fibres were generally not distinguishable amidst the intense basophilia of the whole "matrix" in "young" enamel. It was not possible, therefore, to determine the relationship of the "fibres" to the honeycomb developing front of the enamel in this material.

Dye Diffusion Experiments

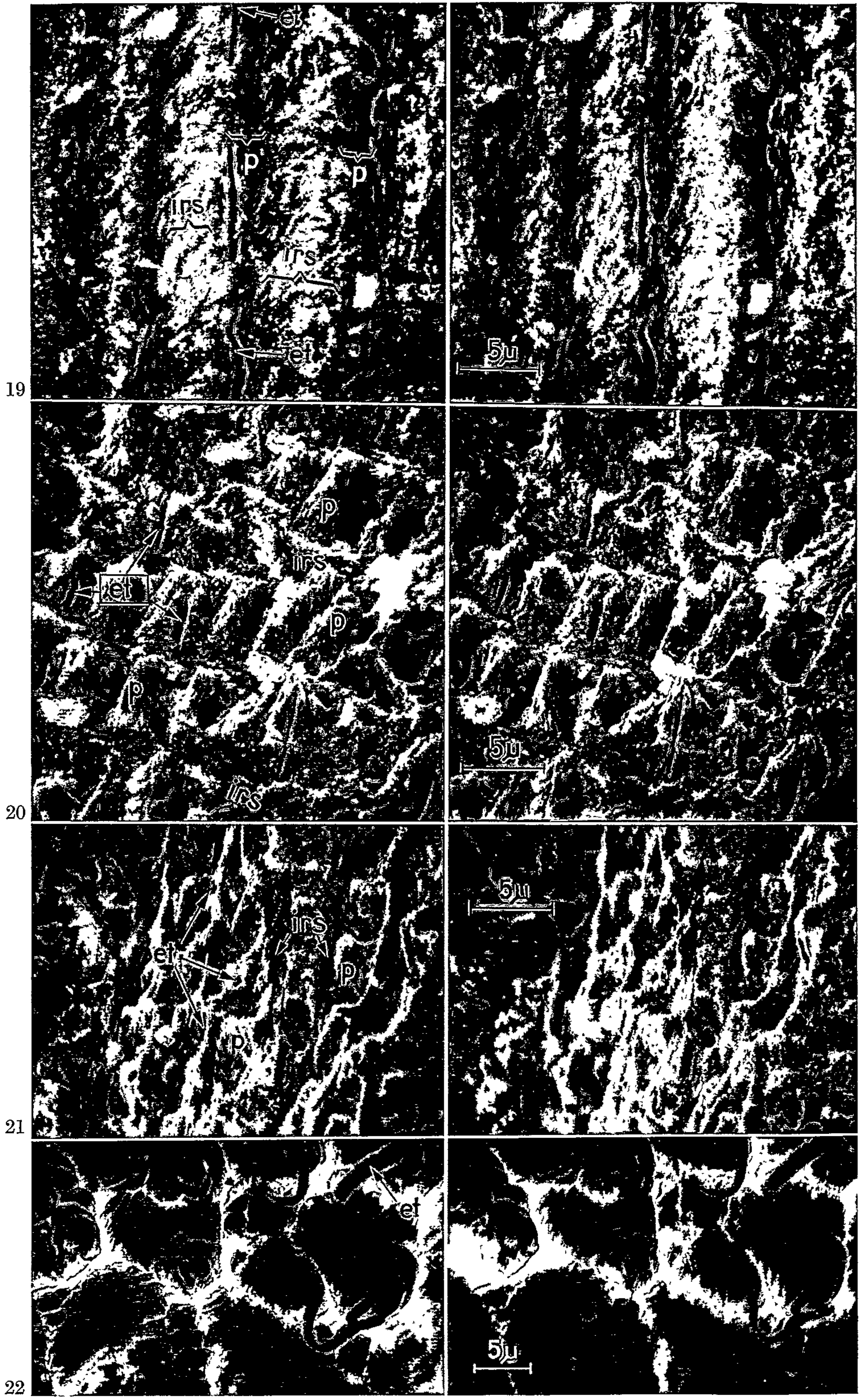
In vitro dye diffusion experiments on extracted *Macropus* molars showed that both the dyes used freely entered the dentinal tubules and thence the enamel tubules, to which they remained more or less confined. Some general diffusion of the dyes through the inner layers of the enamel did occur, but the enamel tubules could be seen more clearly because of their dye content.

No trace of penetration of trypan blue into either dentine or enamel was found in the opossum given vital injections of this dye six weeks previously and this in spite of the fact that the animal developed an overall blue colour which could be seen through the skin and oral mucous membrane.

The methyl blue dye inserted into the pulp of the cheek teeth 1 week beforehand had entered the dentinal tubules and penetrated some one-half to two thirds of the way towards the enamel-dentine junction. No trace of the dye could be found in enamel tubules. Tetracycline antibiotics were administered to some of these adult opossums, but no sign of the characteristic yellow fluorescence under ultraviolet irradiation was ever found in the enamel.

Figs. 18—21. Stereo-pair scanning electron micrographs of fractured *Macropus* enamel and dentine

Fig. 18. Part of montage showing one tubule crossing from the dentine and continuing into the enamel to the limit of the field. Inter-row sheet crystallites roof over the tubule in several places



Figs. 19—22 (for Legends see p. 571)

Discussion

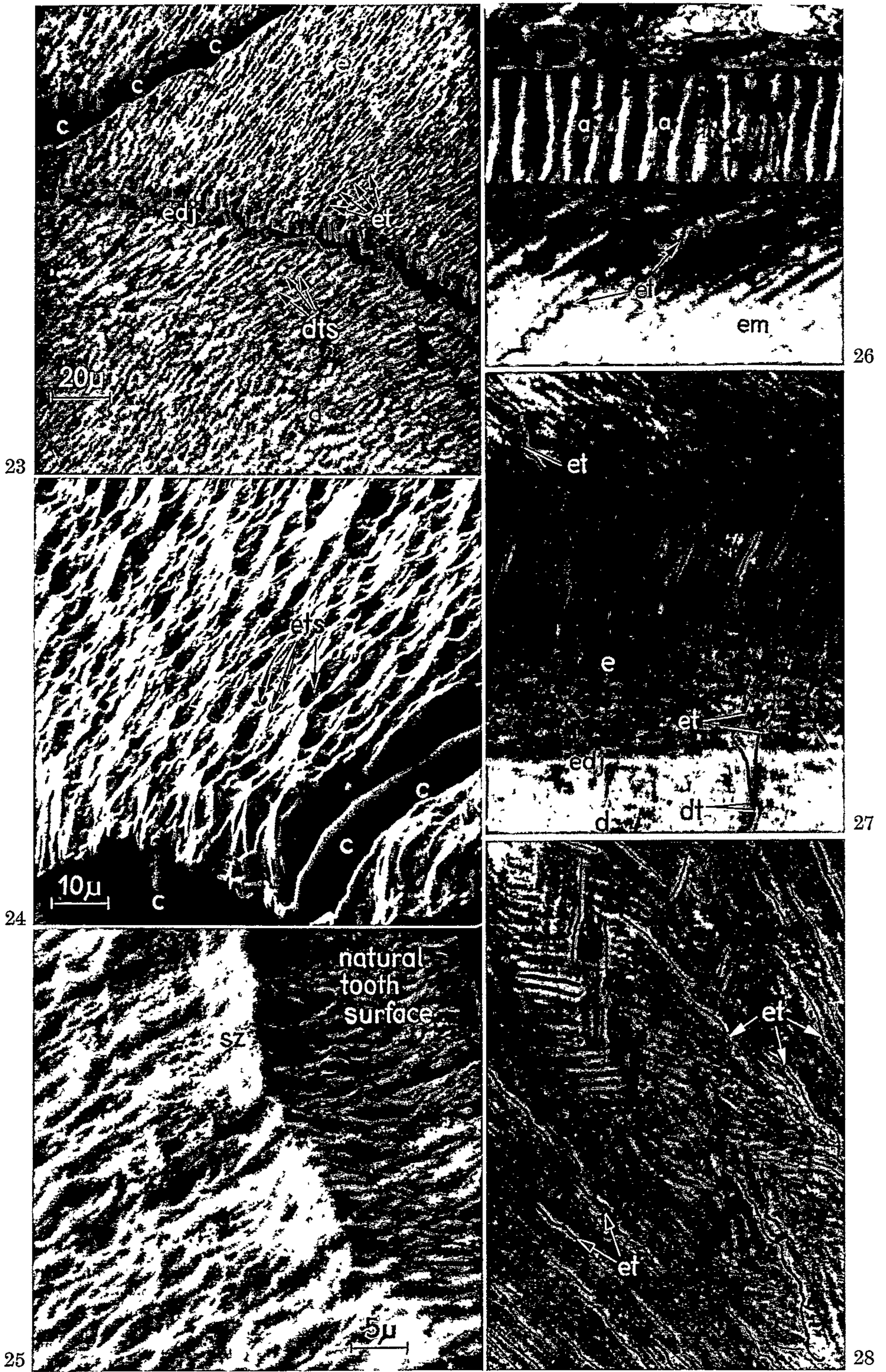
The present results lend support to both "tubular" and "fibrillar" concepts of the structure of the enamel "tubules". We have adopted the name "tubule" on the rather arbitrary basis that it is more suggestive of their nature as defects in a more or less continuous phase of "enamel". The tubules are "defects" in the sense that they contain fewer crystallites than the surrounding enamel. Use of the term defect is not meant to exclude the possibility that a factor causing the absence of the mineral component may reside in the organic matrix in the first place and it is not meant to imply that the genesis of the tubules is necessarily accidental. To state that the tubules are regions of the enamel which do not "mineralise" fully is only to summarise their nature and does not begin to provide an explanation of the mechanism of their development or of their adult function.

The present study provides no answer to the question of why the tubules develop in marsupial enamels. Features related to the enamel tubules have been described in rodent enamels: both actual tubules in the *Muridae*, *Sciuridae*, (VON EBNER, 1890) and more amorphous spaces in the *Hystricomorpha* (TOMES, 1850; KORVENKONTIO, 1934—35). These structures are continuous with neither the enamel-dentine junction nor the enamel surface. The fact that these rather similar regions in rodent enamels (i. e. the "cells" of TOMES, 1850) and the tubules in some marsupial enamels (e. g. in the cervical region of *Petaurus* molar enamel — TOMES, 1849; VON EBNER, 1890) are not continuous with the dentine tubules, would suggest that the *predisposing* cause of the development of these defects does not lie in connection with the surface of the dentine. In the development of those enamels in which the dentine and enamel tubules are continuous, we must consider that a predisposing cause is present at the earliest stage of formation of any given increment of enamel, that is to say when the first enamel crystallites develop on the surface of the dentinal collagen matrix. However, it is quite probable that some extra *initiating* factor in enamel tubule development resides at the future enamel-dentine junction in marsupials, and that this combined with a predisposition to the development of "defects" in crystallite formation determines the very high frequency of the tubules "crossing" the enamel-dentine junction in the teeth of marsupials.

The fact that a high proportion of the enamel tubules appear to be in direct continuity with the dentinal tubules might suggest that their presence may depend in some way on an "influence" from the dentinal tubules (the odontoblast processes). It must then be determined why the odontoblast processes persist so near to the enamel in the groups that possess tubular enamel with continuous enamel and dentine "tubules" and what the postulated "influence" is. There is no doubt that the so-called "enamel tubules" are purely enamel formations. There is no evidence for their containing a morphologically identifiable dentinal component, for example, collagen fibres. However, it is possible that the growth of the first enamel crystallites at the enamel-dentine junction depends, in some way, upon the presence of an underlying substrate of dentine, such as would exist if the odontoblast processes actually attain the amelo-dentinal contact plane.

Figs. 19—21. Fractures in various planes showing intraprismatic localisation of tubular defects

Fig. 22. Stereo-pair electron micrograph of formvar replica of argon ion etched *Macropus* enamel. — Scanning electron micrograph of formvar replica of ion etched *Macropus* molar showing long processes deriving from the tubules related to the bottom of ion etched pits



Figs. 23—28 (for Legends see p. 573)

It is conceivable that the initial nucleating factor which starts *enamel* crystallite formation at the enamel-dentine junction resides in the collagen fibres of the dentine matrix. Since these collagen fibres lie predominantly perpendicular to the surface of the dentine, and apatite crystallites are known to form on (or in) the surface of collagen fibres, an epitactic mechanism here might explain the degree of preferred orientation observed even in the first formed layer of enamel. Deficiencies in the surface of the dentine would result in "nucleation deficiencies" for the enamel crystallites. Defective territories in which crystallites did not commence to grow (i. e. the "tubules") could only be propagated in regions in which the crystallites were parallel and essentially at right angles to the dentine surface, that is to say mainly within the prisms.

The observation that the enamel "fibres" only appear as structures differentiated from the remainder of the decalcified enamel matrix in the "transitional" (CHASE, 1935) stage of maturation suggests that the "tubules" (fibres) acquire their high organic content as a result of "maturation". This would make their acquisition of an increased organic content analogous to the development of the "prism-sheaths", the organic matrix being forced into these regions during the growth in diameter of the enamel crystallites (BOYDE, 1964). The enamel "tubules" are thicker than prism sheaths and could contain a greater bulk of organic material: this would explain the greater mechanical stability of the content of the tubules (i. e. the "fibre" of TOMES, 1856) after acid decalcification.

It seems very probable that the development of marsupial enamel tubules and rodent enamel "cells" (TOMES, 1850) is associated with the extremely rapid deposition of these tissues; and is, as it were, an accidental effect of this cause. The rate of deposition of rodent incisor enamels at approximately 16 μ per day contrasts strongly with that in man, at an average of 4 μ per day (MASSLER and SCHOUR, 1946). CARTER (1917) stated that — "enamel of Higher Mammals is usually laid down slowly, and coincidentally undergoes almost complete calcification. In Marsupials, however, this is not the case, for the enamel matrix is laid down very rapidly, practically the whole thickness of the tissue being deposited, whilst but a slight amount of dentine has been formed". MOSS and APPLEBAUM (1963) also — "suggest that the apparently very rapid rate of enamel matrix formation may play a role in the production of this type of enamel". A surprising difference was found

Figs. 23—25. Scanning electron micrographs of ion-etched *Macropus* molar

Fig. 23. Enamel, showing ion etched pits related to the enamel tubules and, in dentine, the prominence of the peritubular dentine and that it extends to the enamel-dentine junction

Fig. 24. Enamel, showing more detail of the distribution of the etch pits

Fig. 25. Enamel, ground section surface to the left, showing lack of differential etching in the surface zone enamel

Fig. 26. Light micrograph of haematoxylin and eosin stained section of developing *Protemnodon rufrogrisea* enamel and ameloblasts. — Trichrome stained decalcified coronal section of *Protemnodon rufrogrisea* incisor. Close to final stage of amelogenesis. The enamel "fibres" are the dark spirals within the lighter staining enamel matrix

Fig. 27. Ground section of *Macropus* molar. — Longitudinal ground section of *Macropus* molar showing continuity of two air-filled tubules across enamel-dentine junction

Fig. 28. Decalcified section of developing *Protemnodon rufrogrisea* enamel. — Decalcified, trichrome stained section of *Protemnodon rufrogrisea* incisor showing decussation of enamel prisms together with their associated "fibres"

in the amount of enamel formed in the dentition of two sibling pouch young *Metachirus nudicaudatus* used in the present studies, the one killed 7 days after the other. If the length of the Tomes' process is in any way an indication of the secretory activity of the ameloblast then the greater length of the marsupial Tomes' process (C. S. TOMES, 1897, 1904) might indicate a greater secretion pressure, in the sense of a greater rate of activity, of the marsupial ameloblasts.

We cannot agree with the opinion of MOSS and APPLEBAUM (1963) that the enamel tubules in *Macropus* are in fact uncalcified enamel rods and that they are therefore equivalent to the tufts in other mammalian enamels, since tuft regions are only under-mineralised, not unmineralised, and are several prisms wide (BOYDE, 1964). Furthermore, enamel tubules are situated within perfectly normally mineralised enamel prisms.

MOSS and APPLEBAUM also believe that there is no continuity between the enamel and dentine "tubules". They state that ... — "considering the abundance of both dentinal tubules and of enamel fibers in the Marsupial, it is little wonder that the illusion of continuity between them is achieved with the use of relatively thick ground sections" and also (loc. cit) that — "the enamel "tubules" are an artifact of ground section preparations, and, further, that *in vivo* they contain uncalcified enamel matrix rather than the continuation of any odontoblastic process". There is certainly no justification for considering the tubules as artefacts in the sense in which we have used the term. Their conclusion that the tubules contain uncalcified enamel matrix is in agreement with our own findings.

The enamel spindles of other mammalian enamels cannot be regarded as structures analogous to the enamel tubules, since there is good evidence that the spindles are, or have, dentinal components (FRISBIE, 1952; SCHLACK, 1940). They are also much thicker, being greater in diameter than the prisms, and much shorter, reaching only some 50—100 μ from the dentine surface. The presence of spindles, or that of similarly shaped structures in a similar situation, has been noted between the ameloblasts just before the commencement of amelogenesis (LAMS, 1920; CHASE, 1948) whereas the tubules develop during enamel development as an absence of crystallite growth in limited territories within otherwise normal domains. FRANK (1965, personal communication) has studied spindles with the electron microscope and confirms that they may contain an odontoblast process.

Enamel "tubules", rather than the spindle-formed dilatations that are found in human enamel, are also found in certain members of the Orders *Rodentia* (e.g. the jerboa-TOMES, 1849; VON EBNER, 1890), *Insectivora* (TOMES, 1849 — e.g. hedgehog, mole, shrew), *Primates* (CARTER, 1922 — N. B. the *Lemuroidea*), and *Cheiroptera* (LÖHER, 1929). The marsupials are a special group in respect of their possessing "enamel tubules" in that only the one exception noted by TOMES (1849) has ever been reported (the wombat does not have enamel tubules).

We have examined the ground sections of cheiropteran teeth in the Tomes' collection of the Royal College of Surgeons of England and can confirm that tubules are present in some species, e.g. *Pteropus poliocephalus*, *Magaderma lyra*, and *Barbastellus communis*. Our preliminary electron-microscope studies of the teeth of a number of placental mammals — calf (*Bos bovis*), pig (*Sus domesticus*), dolphin (*Delphinus delphis*) and man (*Homo sapiens*) — have revealed the presence of very narrow structures in the enamel which are continuous across the enamel-dentine junction with dentinal tubules (Fig. 15). Although these structures almost

certainly do not extend through the entire thickness of the enamel, it is difficult to regard them as enamel spindles because they exhibit no extension of dentinal matrix, no dilatation along their length, and because their average diameter of ca. 0.4μ approaches the limit of resolution of the light microscope. Further, their ultrastructure is in every way similar to the enamel tubules of metatheria. Although the presence of these minute enamel tubules has only been observed in these four eutherian species to date, there seems little reason to doubt their more widespread existence¹.

Ion etching seems to have been a particularly suitable way of preparing the enamel surface for the purpose of visualising the distribution of enamel tubules. The tubules always arose from the bottom of an ion etched pit, and this is why they can be seen in the formvar replicas (Fig. 22), but not in the shadow of the bottom of the pits in the scanning electron micrographs (Figs. 23 and 24). STEWART (personal communication, 1967) has suggested that the formation of these etch pits may be related to the surrounding surface charging and focussing the ions into the openings of transversely sectioned enamel tubules, thereby leading to a locally increased rate of bombardment and sputtering.

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¹ A number of authors have purported to show either enamel "capillaries" or enamel tubes in human teeth by light microscopy (BEUST — *Dent. Cosmos*, 1914, 1924; KATO — *Dent. Cosmos*, 1929, 1930; ALLEN — *Dent. Cosmos*, 1930; FORHUFVUD — *D.Z.M.U.K.H.*, 1942; *Acta Odont. Scand.* 1946, 1947).

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FINE STRUCTURE OF 'FIBRILS' AND TUBULES IN DEVELOPING OPOSSUM ENAMEL

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The nature and mode of development of marsupial enamel tubules have been points of contention since the work of John Tomes (1849). Disagreement remains as to whether:—

- i. The tubule contains an organic 'fibril';
- ii. The 'fibril' (if present) is cellular or extracellular;
- iii. The tubule and 'fibril' (if present) originate from dentine or enamel. (For a review of the pertinent literature see Boyde, 1964; Boyde and Lester, 1967.)

Much of the early discussion concerning the development and content of enamel tubules is limited in its value by the resolution obtainable with the light microscope. This is unfortunate as the observations are extensive, the descriptions detailed, and the discussion sometimes entertaining in the frank expression of personal differences existing at the time (see, for example, Carter, 1917).

As might be expected, the application of electron microscopical techniques to the problem has provided indisputable evidence for a number of points (Boyde, 1964; Boyde and Lester, 1967). Thus, the reality of the hollow, cylindrical form of the tubules has been demonstrated and details of the tubule walls described. Likewise, continuity of enamel and dentine tubules across the enamel-dentine junction has been documented with the aid of stereoscopic images. In addition, the origin of tubules has been visualized in thin sections of developing enamel as localized regions in which crystallites fail to develop.

The present paper aims to characterize the enamel 'fibril' and to establish its relationship to the enamel tubule.

Two litters of pouch-young opossums (*Didelphis marsupialis*) (Morris, 1965) were sacrificed, the one litter at approximately 22 days and the other at approximately 28 days after birth. Tooth germs were dissected free from the developing jaws and placed in phosphate-buffered glutaraldehyde. The tooth germs were post-osmicated and routinely processed for the preparation of thin sections for examination by transmission electron microscopy.

Enamel tubules are easily identified on the basis of form (cylindrical or nearly so) and size (0.1–0.2 μm . in diameter). Tubule identification in the surface region of developing enamel was found to be complicated by the presence of Tomes' processes. In such instances, disputed tubules were followed in serial sections: transversely sectioned tubules remain relatively constant in form and size, whilst longitudinally sectioned tubules disappear within the space of one or two successive sections.

In this way, it is possible to identify enamel tubules positively at the mineralizing front of the enamel at the junction with Tomes' processes of the ameloblasts. In such

cases, the tubules were found to contain a cytoplasmic extension of the ameloblast. This cytoplasmic process we would identify as the enamel 'fibril' of the earlier workers (e.g., J. Tomes, 1849; C. S. Tomes, 1897; Williams, 1897; Carter, 1917; Skues, 1932). Fine filaments oriented parallel to the longitudinal axis of the 'fibril' represent the major structural component of the 'fibril'. The filamentous material passes directly, without interruption, from the ameloblast cytoplasm into the enamel 'fibril'. No organelles are observed in the cytoplasmic ground substance of the 'fibril'. An intact membrane (plasmalemma) cannot be discerned about all enamel 'fibrils' in our preparations or throughout the displayed length of any one 'fibril'.

Two components of the developing enamel are found at the 'fibril'/enamel interface (i.e., in the wall of the enamel tubule). The enamel crystallites represent one component; the crystallites having no preferred orientation with respect to the longitudinal axis of the tubule. The second component at the interface is in the form of small, roughly spherical particles (*ca.* 100 Å in diameter). These particles (stippled material?) are scattered throughout the enamel but often accumulate in crystallite-free areas along the tubule wall.

In summary, ultrastructural evidence is presented to support the view that enamel 'fibrils' in developing teeth of *Didelphis marsupialis* are a direct extension of ameloblast cytoplasm. The 'fibrils' originate at the junction of Tomes' process with the surface of the mineralizing enamel. As such the 'fibrils' are initially bounded by plasmalemma. The 'fibrils' are devoid of organelles, their main structural component being longitudinally oriented filaments continuous with those of the ameloblast cytoplasm. An enamel 'fibril' is enclosed by an enamel tubule, the wall of which is constituted, in developing enamel, by crystallites and by small (*ca.* 100–150 Å in diameter) roughly spherical particles.

Note on Terminology. The term enamel 'fibril' is used here because of its historical context. This simplifies presentation. 'Fibril' has, however, a specific connotation in present-day usage inconsistent with its application to a cytoplasmic process. It may be seen from the present study that developing marsupial enamel with its cellular processes and tubules is quite analogous to dentine, cementum, and bone. For these reasons, it is suggested that a more suitable term for enamel 'fibril' might be 'primary ameloblast process'. 'Primary' would serve to distinguish this particular ameloblast process from the better known 'Tomes' process', the latter making its initial appearance at a relatively later time during enamel development.

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DR. K. S. LESTER (New York): The further material I wish to present might be relevant to the consideration of passage of material through enamel. It concerns the nature and origin of 'fibrils' and tubules of enamel. Tubules are internal channels and the junction of their wall with their contents represents an interface. Historically, the story of enamel tubules begins in 1849 with John Tomes' work. If we follow the literature through, and there is much

controversy up until today, basically three questions remain. (i) Does the enamel tubule contain organic material? (ii) Is the tubule, if present, cellular or extracellular? (iii) Do tubule and 'fibril' originate from dentine or enamel? Finally, if one accepts the conclusions of Moss and Applebaum (1963) and Moss (1968), might the tubules be artefacts?

In a longitudinal section of developing opossum enamel (Lester, 1970), a regular deficiency in enamel crystallites, 0.2 μm . in diameter, can be seen. The wall of the tubule consists of enamel crystallites and we find some small spherical particles approximately 100–150 Å in diameter which resemble very closely the stippled material described by Fearnhead (1960) and Watson (1960) as occurring during enamel development.

In slightly higher magnifications of tubules and their contents, there is evidence of a double membrane, and there are certainly indications of filaments within the enamel tubules. We get a clue that the contents are cellular and a longitudinal section of the developing front (Lester, 1970) shows that enamel tubules are caused by cell processes left behind from the ameloblast, i.e., the tubule contains a slender cytoplasmic process of the ameloblast. The filamentous material in the 'fibril' ('fibril' is used here in a historical sense because light microscopists referred to the content of an enamel tubule as a 'fibril') is continuous with those in the ameloblast cytoplasm. There are no organelles in the 'fibril'. If we look at the junction between enamel and dentine prior to enamel formation, we find that there is a close juxtaposition and sometimes an interdigitation of odontoblast process and ameloblast cytoplasm. This does explain, and one can follow in subsequent sections an enamel tubule forming from this point, the continuity of the dentinal and enamel tubules across the enamel–dentine junction. We cannot demonstrate any cell attachments between these two cell types.

It should not be supposed that enamel tubules are restricted to marsupials. They occur in some Rodentia, Insectivora, Cheiroptera, Primates (they certainly occur in human material), and they occur in calf, pig, and kitten material (see Boyde and Lester, 1967). Some question exists as to the fate of the tubules. Obviously, the prognosis for the cellular element is not particularly good as the ameloblasts degenerate prior to the eruption of the tooth. There are further details, as to the location of the tubules within the prisms, the location of the tubules with respect to the developing front and, of course, the question of why the tubules should form at all.

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On the Nature of "Fibrils" and Tubules in Developing Enamel of the Opossum, *Didelphis marsupialis*¹

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The origin and nature of the contents of enamel tubules in developing teeth of the opossum, *Didelphis marsupialis*, are described and illustrated by electron microscopy. The tubules contain cell processes (the "fibrils" of earlier workers) which are extensions of ameloblast cytoplasm originating at the mineralizing front of the enamel. The "fibril" is bounded by plasma membrane initially, and is devoid of organelles; the main structural component being longitudinally oriented filaments. The wall of the surrounding tubule is composed of crystallites and of small (ca. 150 Å diameter) particles (stippled material?). Preliminary observations on tooth germs prior to enamel formation show there to be an overlapping or interdigitation of odontoblast processes and the cytoplasm of presumptive ameloblasts. This cytoplasmic relationship is maintained during subsequent enamel development and would account for the continuity of enamel and dentine tubules in the adult state.

Small tubules running essentially parallel with the prism direction have been described in the enamel of a variety of mammals, both eutherian and metatherian (4, 5, 8, 13, 18, 19, 27-30, 33). Of these mammals, members of the order *Marsupialia* are by far the best known for their tubular enamel which has been reported lacking in only one of their number, the wombat (11, 29). Tubules in marsupial enamel are generally more numerous and extensive than those in eutherian enamel and a high proportion are continuous with dentine tubules across the enamel-dentine junction.

The nature and mode of development of marsupial enamel tubules have been points of contention almost from the time of their original description by J. Tomes (29) in 1849 [for reviews see (1, 4)]. Some of the questions debated since that time have been: the location of tubules with respect to the prisms; the real or imagined continuity of enamel and dentine tubules across the enamel-dentine junction; the structure of the tubules and of their contents (if any); and the real or artifact nature of the tubules themselves.

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Much of the early discussion concerning the development and contents of enamel tubules is unfortunately limited in its value by the resolution obtainable with the light microscope. This is unavoidable for the diameter of the tubules is generally less than 0.5μ . As might be expected, the application of electron microscopical techniques to the problem (1, 4) has provided evidence on a number of points. Thus, the reality of the hollow, cylindrical form of the tubules has been demonstrated and details of the tubule walls have been described. Likewise, continuity of enamel and dentine tubules across the enamel-dentine junction has been documented with the aid of stereoscopic images. In addition, the origin of tubules has been visualized in thin sections of developing enamel as localized regions within prism domains in which crystallites fail to develop.

A number of questions remain to the present day. Among them are: whether the tubule contains an organic "fibril"¹; whether the "fibril" (if present) is cellular or extracellular; and whether the tubule and "fibril" (if present) originate from dentine or enamel.

The present paper offers evidence for the existence of enamel "fibrils" within tubules of developing opossum enamel. An attempt is made to characterize the "fibril," and its origin from ameloblast cytoplasm is demonstrated. In addition, the ameloblast-odontoblast relationship prior to amelogenesis is examined and early stages of enamel tubule development are described.

MATERIAL AND METHODS

A litter of three pouch-young of the opossum *Didelphis marsupialis* [terminology after Morris (16)] were sacrificed; one animal at 22 days, the others at 28 days after birth. Tooth germs were dissected free from the developing jaws and placed in phosphate-buffered glutaraldehyde (23). The individual teeth were postfixed in osmium and dehydrated and Epon-embedded after Luft (14). Thin sections were cut with a diamond knife mounted in a Cambridge-Huxley ultramicrotome. The thin sections, collected on distilled water, were stained with uranyl acetate (26) followed by lead citrate (22). Thin sections were examined in a Siemens Elmiskop I at 80 kV.

For the purposes outlined above, enamel tubules are best studied in sections the plane of which approximates the longitudinal axis of the enamel prisms. For ease of preparation, it is desirable to take sections close to the advancing edge of the mineralizing crown of the tooth germ before too great a bulk of enamel and dentine has formed. The typical appearance of such sections is shown in Fig. 1. The dentine is of greater bulk than the enamel which is intimately apposed to it. Irregular depressions in the surface of the developing enamel are occupied by Tomes' processes of the ameloblasts. The ameloblasts themselves are columnar

¹ The terms "fibril" and "fiber" have been used consistently in light microscope studies of marsupial and other enamel tubules (5, 15, 17-19, 24, 27, 28, 30, 32, 33).

in form but relatively short compared to those of other mammals at a similar stage of amelogenesis. Component cells of the enamel organ lie adjacent to the ameloblasts at their nuclear pole.

OBSERVATIONS

Thin, elongated areas, devoid of crystallites, are common in longitudinal sections of developing *Didelphis* enamel (Fig. 1). Such areas may be identified as enamel tubules on the basis of their form, size, and contents (see below and Figs. 2-4).

Form and content of enamel tubules. Enamel tubules are irregularly cylindrical, i.e., their walls are roughly parallel in longitudinal section (Figs. 2 and 3) and their outline is round or oval in transverse section (Fig. 4). Tubule diameter varies between 0.1 and 0.5 μ but is most commonly in the region of 0.2 μ [half that previously reported for *Macropus* material (4)]. No tubule could be followed throughout the entire thickness of developing enamel in any one section; the tubules most often running a sinuous course within the boundaries of any one prism domain [see also (4)].

The enamel tubules contain a membrane-bounded structure (Figs. 3 and 4) which we would identify as the enamel "fibril" or "fiber" of earlier workers (e.g., 5, 15, 18, 19, 24, 27, 28, 30, 32, 33). Fine filaments oriented parallel with its longitudinal axis represent the only visualized structural component within the "fibril". These filaments show no preferred clumping within the "fibril" substance (Figs. 2-4).

Continuity of "fibril" and ameloblast cytoplasm. Tubule identification in the surface region of developing enamel is complicated by the presence of Tomes' processes.

Key to abbreviations

<i>a</i>	ameloblast	<i>od</i>	odontoblast
<i>cv</i>	coated vesicle	<i>op</i>	odontoblast process
<i>d</i>	dentine	<i>pd</i>	predentinal collagen
<i>dg</i>	dense granule	<i>si</i>	stratum intermedium
<i>e</i>	enamel	<i>sm</i>	stippled material
<i>et</i>	enamel tubule	<i>Tp</i>	Tomes' process
<i>f</i>	"fibril" of earlier workers (primary ameloblast process)	<i>x</i>	enamel crystallites

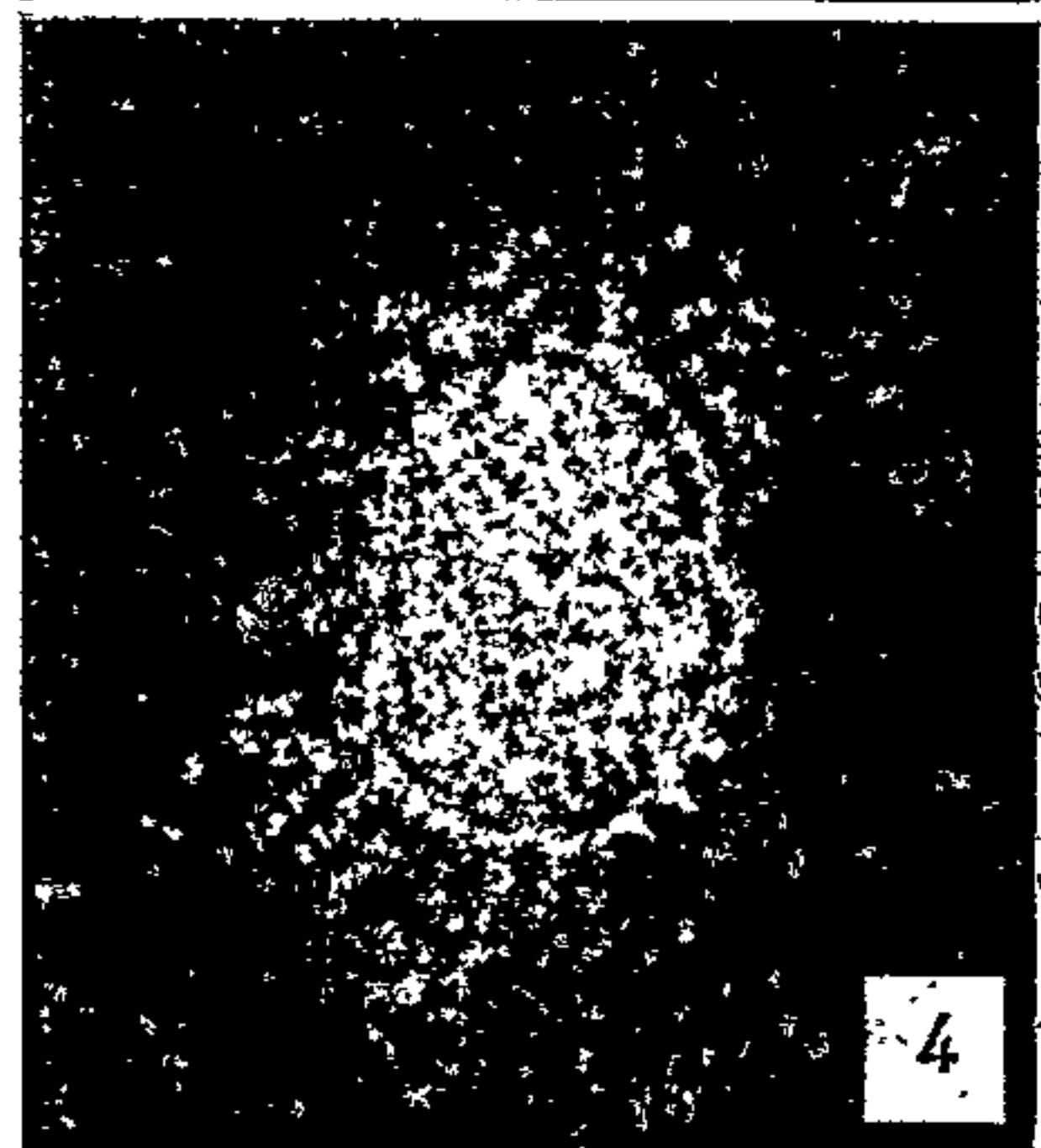
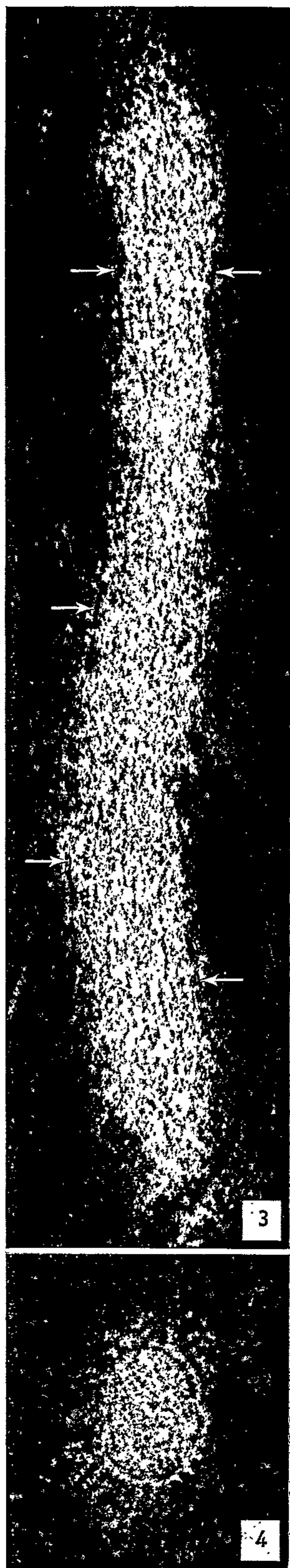
All figures are electron micrographs of oblique longitudinal sections of the developing edge of the crown of opossum (*Didelphis marsupialis*) tooth germs.

FIG. 1. Survey picture showing disposition of tissues about the developing enamel. Note the two thin, elongated, crystallite-deficient areas in the body of the enamel identified as enamel tubules. Part of the tubule at left is seen at higher magnification in Fig. 2. $\times 1920$.

FIG. 2. Higher magnification of part of longitudinally sectioned tubule and "fibril" shown in Fig. 1. Note the small, roughly spherical particles (stippled material?) constituting part of the tubule wall (at arrows). $\times 38,500$.

FIG. 3. Longitudinal section of part of an enamel tubule and contained "fibril" in the body of the developing enamel. The longitudinal filaments are seen as evidence of a surrounding membrane (at arrows). $\times 89,600$.

FIG. 4. Transversely sectioned enamel tubule and its "fibril". Note the surrounding membrane and the transversely sectioned filaments. $\times 80,000$.



Disputed tubules in this area were followed in serial section: transversely sectioned tubules remain relatively constant in form and size whilst longitudinally sectioned tubules disappear within the space of one or two successive sections.

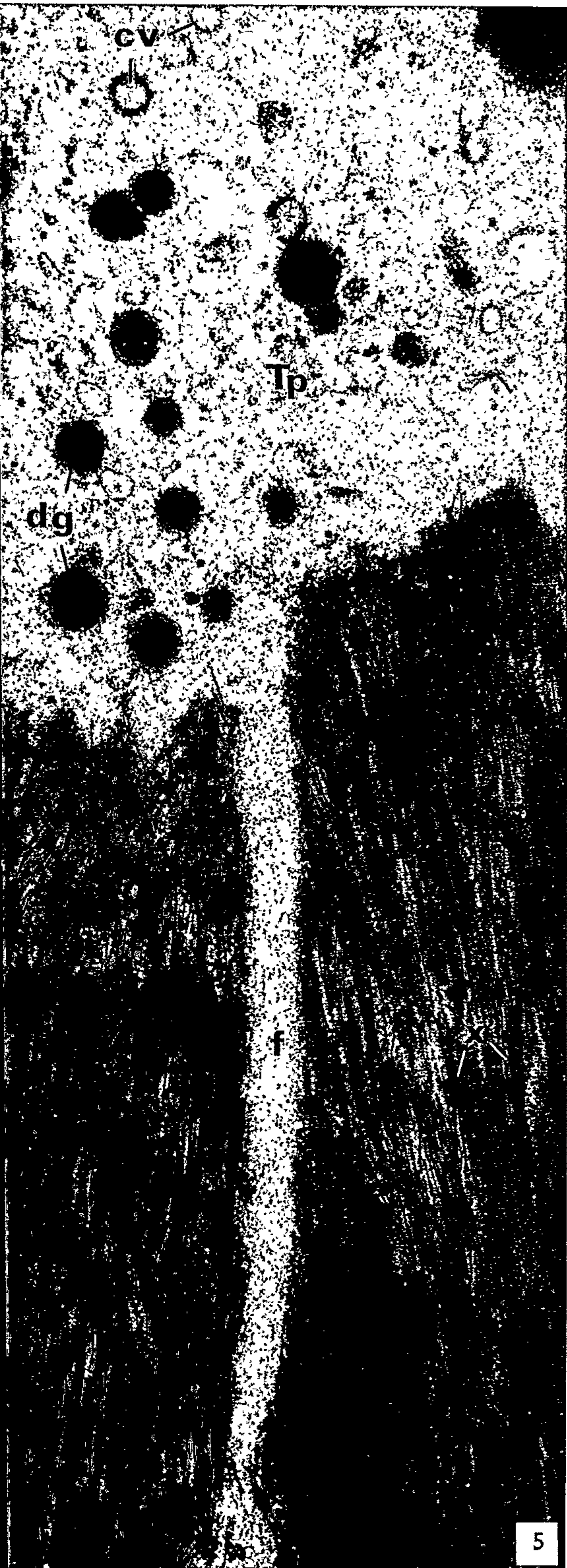
In this way, it is possible to positively identify enamel tubules and their "fibrils" at the junction between the mineralizing front and Tomes' processes of the ameloblasts. In such cases, an enamel "fibril" is seen to represent a cytoplasmic extension of an ameloblast (Figs. 5 and 6). The filamentous material of the Tomes' process cytoplasm passes directly into the enamel "fibril". The cytoplasmic process ("fibril") does not always originate from that cytoplasm immediately apposed to the mineralizing front (as in Fig. 5). Rather, the plasma membrane of Tomes' process is often folded back on itself and the "fibril" takes origin from within the substance of Tomes' process (Fig. 6).

Tubule wall. The tubule, in which the "fibril" lies, is an internal channel in the enamel. The tubule "wall" has no special structural modifications and is merely the "fibril"-enamel interface. Two components of the developing enamel are found in the wall of the enamel tubule. One of the components, the enamel crystallites, are preferentially oriented in a majority of tubules so that their longitudinal axis subtends an acute angle at the tubule wall from the direction of the enamel-dentine junction (Figs. 2, 3, 5, and 6). The second component of the tubule wall is in the form of small, roughly spherical particles (ca. 150 Å in diameter) (Figs. 2 and 6). These particles [stippled material? (9, 31)] are scattered throughout the developing enamel but often accumulate (or are more easily discerned) in crystallite-free areas along the tubule wall.

"Fibril" development. Some preliminary observations were made on opossum tooth germs at stages prior to enamel formation. Fig. 7 shows the appearance of a longitudinal section of the ameloblast-predentine interface prior to mineralization of the collagen. The odontoblasts have withdrawn and the dentinal collagen abuts directly against the distal surfaces of the ameloblasts, the basement lamina having disappeared by this time [see (21)]. Odontoblast processes project into the dentinal collagen and, in favourable sections, may be seen to contact the distal cytoplasm of an ameloblast (Fig. 7). There is an overlapping (Fig. 8) and often an interdigitation (Fig. 9) of odontoblast and ameloblast cytoplasm in such regions of cell contact. No specialized cell attachments could be resolved between the cell processes. Ameloblast processes,

FIG. 5. Developing front of enamel showing longitudinal section of a "fibril" in continuity with ameloblast cytoplasm. $\times 33,000$.

FIG. 6. Developing front of enamel showing origin of a "fibril" from within a Tomes' process. The "fibril", part of which lies in an adjacent section, contains longitudinally oriented filaments continuous with the ameloblast cytoplasm. $\times 58,000$.



apparently unassociated with odontoblast processes, were also found projecting into the predentine collagen (Fig. 7) and, at later stages, into mineralized dentine.

Juxtaposition of ameloblast and odontoblast cytoplasm is also found in later stages of tooth development where enamel formation has begun. Figs. 10 and 12 represent alternate serial sections of such an area. Fig. 11 is a higher magnification of part of Fig. 10 and shows the continuity of cytoplasm of an enamel "fibril" from the newly formed Tomes' process to the enamel-dentine junction region. The area of contact at the enamel-dentine junction between the same ameloblast and an odontoblast process is seen in Figs. 12 and 13. Images such as these allow one to reconstruct the relationships of a developing enamel "fibril" with respect to both ameloblast and odontoblast cytoplasm.

DISCUSSION

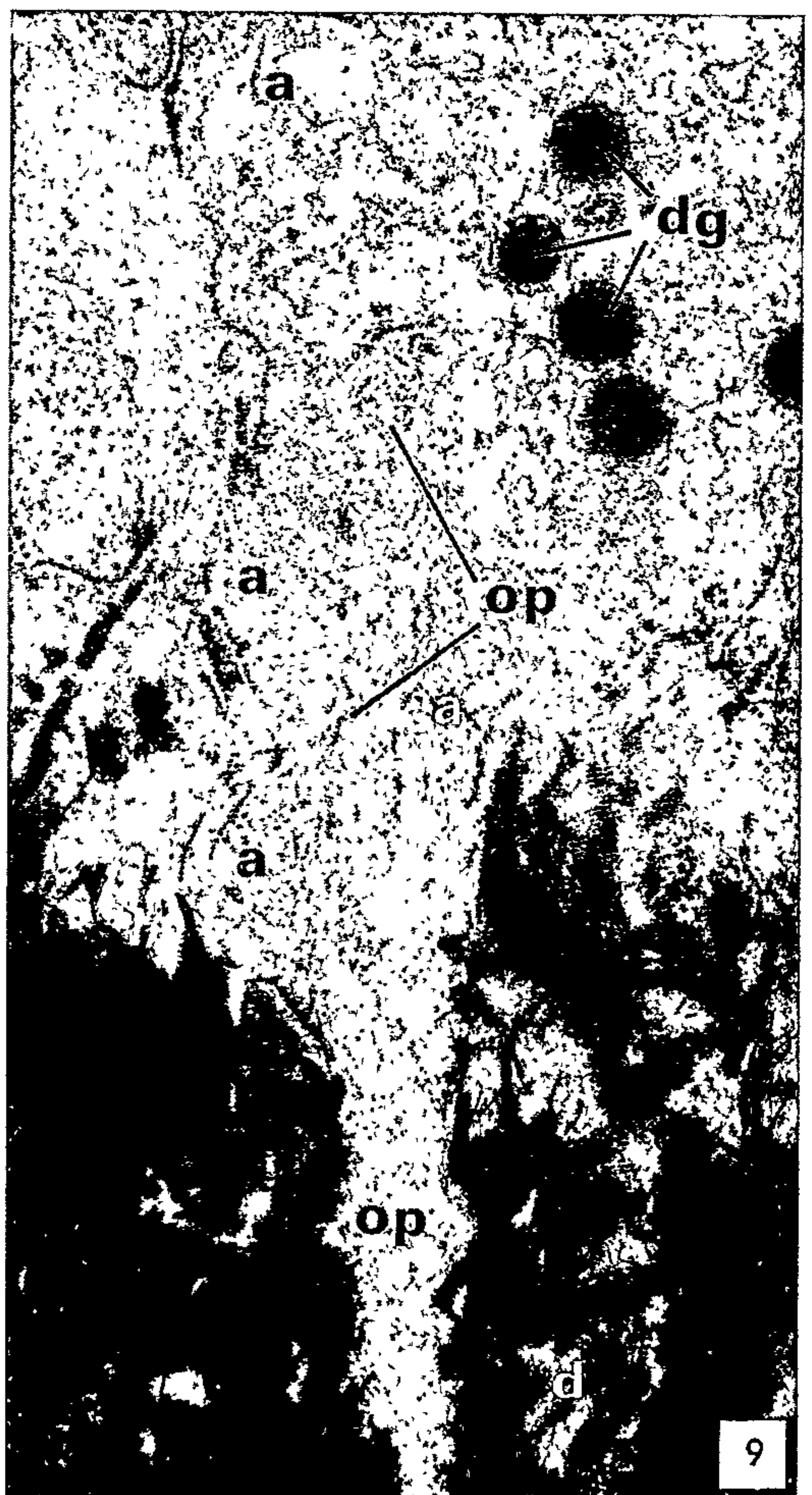
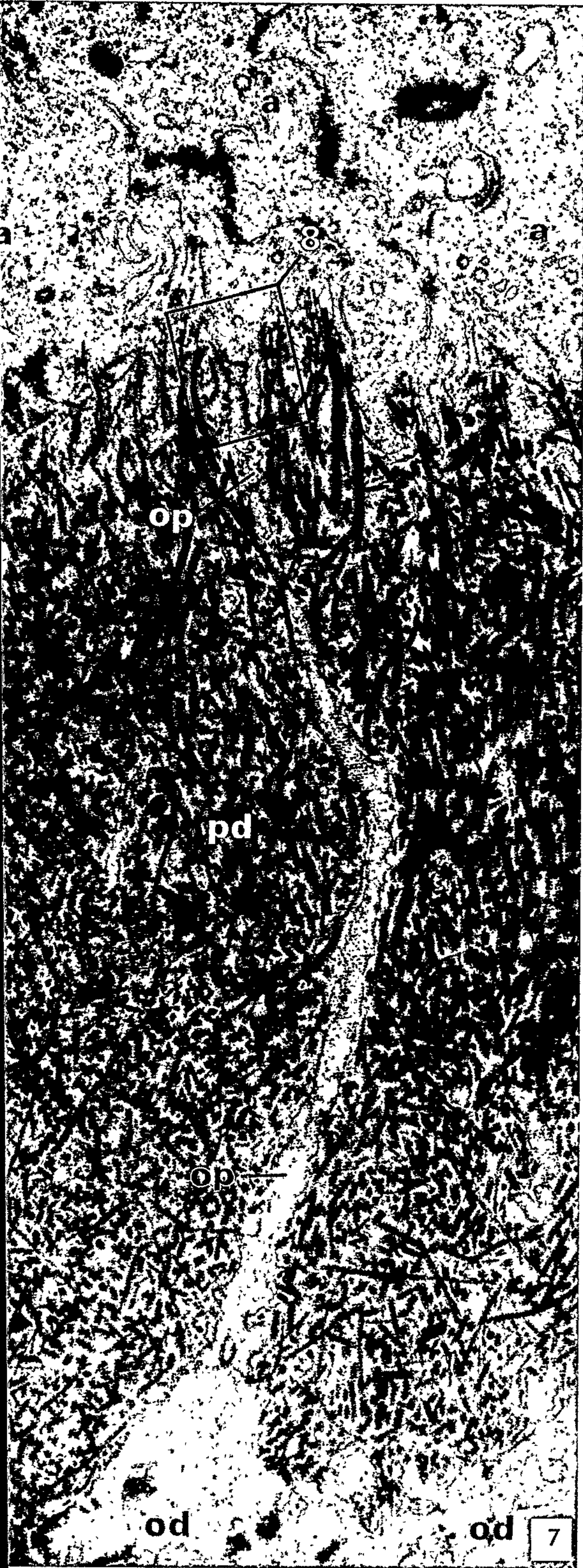
It is concluded on the basis of the present study that tubules in developing enamel of the opossum, *Didelphis marsupialis*, contain cytoplasmic processes. These processes originate at the mineralizing front of the enamel as direct extensions of ameloblast cytoplasm (Figs. 5 and 6). As such, the processes are bounded by a plasma membrane; their major structural component being longitudinally oriented filaments continuous with those of the ameloblast cytoplasm. We would identify these cytoplasmic processes with the "fibrils" or "fibers" of light microscope studies (e.g., 5, 15, 18, 19, 24, 27, 28, 30, 32, 33).

It has been stated previously that enamel tubules are restricted to prism domains (1, 4). This intraprismatic location was said to be determined by the site of development of the tubules in the "cervical floor" [for terminology see Boyde (1-3)] of the depressions in the developing enamel surface (see also Fig. 14). In the early stages of enamel formation observed here, however, some tubules (Fig. 15) are located in sheets of crystallites between prisms—the inter-row sheets or interprismatic regions. Further, a high proportion of tubules are located in peripheral areas of prism domains adjacent to the inter-row sheets—as may be judged by the angulation of the crystallites on either side (Figs. 2, 5, and 6). Variation is also encountered in the relationship between Tomes' processes and the depressions in the mineralizing front: thin extensions of one ameloblast may occupy a peripheral position in the depression exten-

FIG. 7. Ameloblast-predentine interface prior to mineralization of predentine collagen. An odontoblast process overlaps a projection from the distal surface of an ameloblast. Note similar projections of ameloblast cytoplasm apparently unassociated with odontoblast processes. $\times 12,000$.

FIG. 8. Higher magnification of the area outlined in Fig. 7 to show detail of overlapping cell processes and the lack of organized cell attachment. $\times 64,000$.

FIG. 9. Ameloblast-dentine interface showing an odontoblast process interdigitating with the cytoplasm of an ameloblast. This stage of development is intermediate between that represented in Figs. 7 and 10, i.e., after dentine mineralization but before enamel deposition. $\times 37,500$.



sively occupied by another ameloblast (Fig. 14). In other words, cytoplasmic units of two ameloblasts project into a single depression in the developing front. Conversely, in some sections, a projection (convexity) of the developing enamel surface is related to the cytoplasm of only one ameloblast (*cf.* 1-3). These questions need further study. The possibility exists that the extensive formation of microvillous processes from the ameloblast on the enamel side of the distal terminal bar apparatus is related to these variations both in tubule location and in the relationship between ameloblasts and the mineralizing front.

The present observations on "fibril" and tubule development show that odontoblast processes and ameloblast cytoplasm contact and overlap or interdigitate prior to enamel formation (Figs. 7-9). There is evidence (Figs. 10-13) that this cytoplasmic juxtaposition remains after enamel formation has begun and that the contribution from the ameloblast represents the first part of an enamel "fibril". This would account for the continuity of dentine and enamel tubules across the enamel-dentine junction. Moreover, the bulk formed by the juxtaposition of odontoblast and ameloblast processes could explain the swelling which occurs (e.g., 4, 6, 20, 29) at the point of continuity between dentine and enamel tubules. Further investigation of the nature of the initial ameloblast-odontoblast (ectodermal-mesodermal) contact is required to establish the morphological basis for its permanence. The fact that there are more cytoplasmic processes of ameloblasts than there are of odontoblasts at the mineralizing enamel-dentine junction suggests that an odontoblast process *per se* is not the prime causal factor in the subsequent elaboration of an enamel "fibril" [see also (4)]. The causal factor is unknown but is thought to be related to the rapid rate of formation of marsupial enamel (4, 5, 17, 27). Taking the mineralized tissues overall, there does appear to be a direct relationship between incorporation of parent cell cytoplasm and the rate of apposition of the tissue.

It should be noted that a surrounding membrane and inner filaments are not clearly visible throughout the displayed length of all the "fibrils" examined. Some "fibrils" show evidence of what might be interpreted as cytoplasmic degeneration: areas of dense, homogeneous material (Fig. 14) some of which show myelin figures. It is uncertain from the present study whether this lack of clearly visualized structure in some enamel tubules is a result of poor penetration of fixative or whether it is a reflection of their true condition. In any event, degeneration of "fibril" cytoplasm

FIGS. 10-13. These figures enable the reconstruction of the relationship of a developing enamel "fibril" with respect to the ameloblast and odontoblast. Figs. 10 and 12 represent alternate serial sections of the same area at the enamel-dentine junction. Figs. 11 and 13 are higher magnifications of the areas outlined in Figs. 10 and 12, respectively.

FIGS. 10 and 11. An early stage of the development of an enamel "fibril" from Tomes' process. Note the branching of the "fibril". Both $\times 7200$.

FIGS. 12 and 13. Area of contact between ameloblast and odontoblast cytoplasm. Both $\times 44,000$.



must accompany termination of tubules in the enamel surface zone. If tubules do attain the outer enamel surface during development [see, for example, (33)], "fibril" degeneration would accompany the inevitable degeneration of the parent cell occurring after enamel maturation is complete. Tubules of adult enamel are thus unlikely to contain anything other than the remnants of a cytoplasmic "fibril". The status of the contents of adult enamel tubules represents a problem similar to that for adult dentinal tubules [see, for example, (10, 12)]. The main differences between the two would seem to be the time for which the respective tubules are likely to be occupied by a viable cytoplasmic process and the potential for deposition of new material. It is possible that during enamel maturation, protein matrix material could be redistributed [see (7)] with respect to the tubule walls. The claim by Sprawson (25) that a partial obliteration of enamel tubules occurs with age is an interesting one in this context and should be re-examined.¹

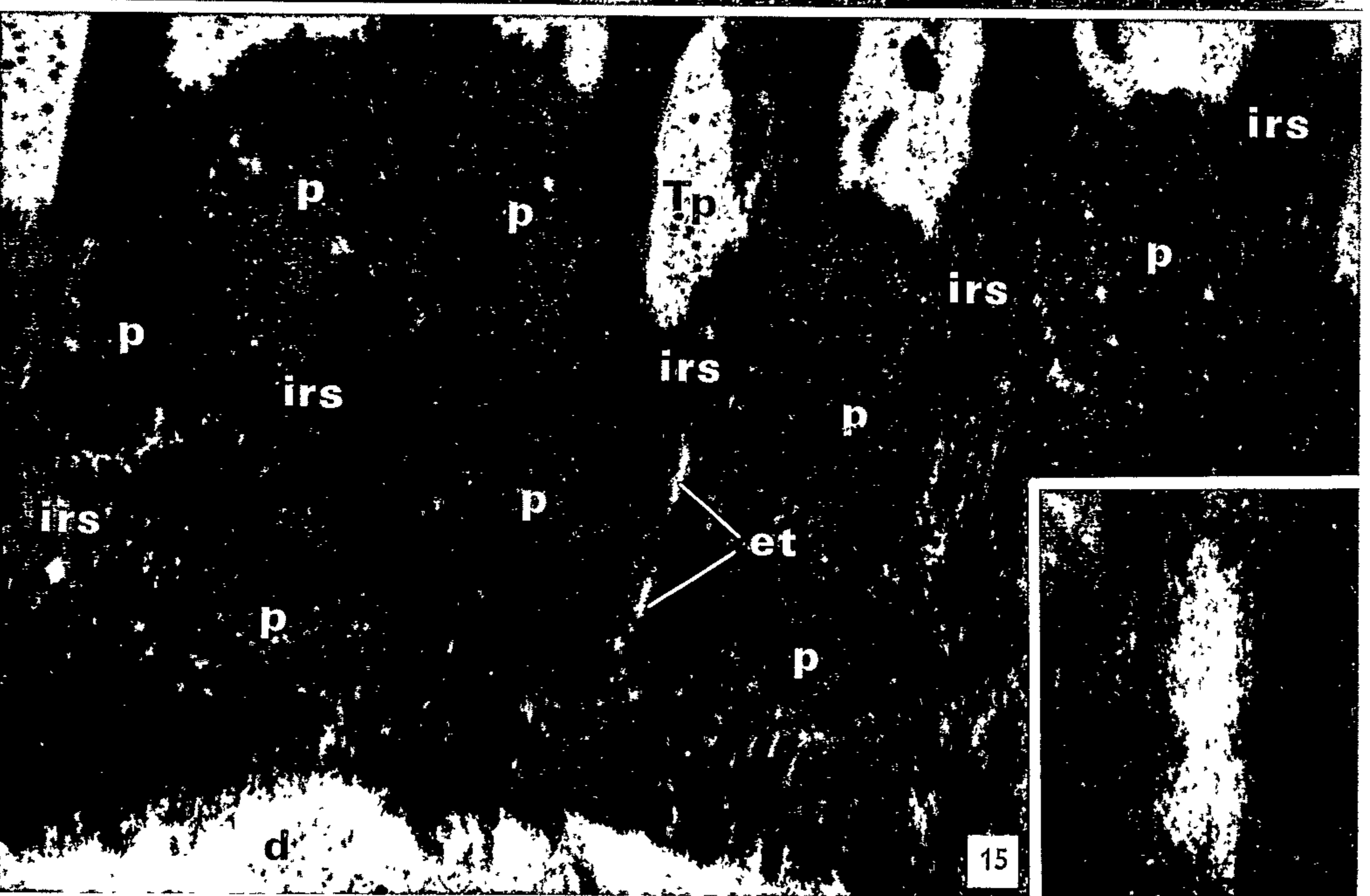
Many of the very early descriptions of enamel "fibrils" are inextricably bound up with concepts of enamel formation held at that time. Of these, the observations of C. Tomes published in 1897 (27) and 1904 (28) are probably the most complete, remarkably so, in terms of what we now know of the nature and origin of enamel tubules and "fibrils" by electron microscopy. C. Tomes was not, and could not have been, aware of the extracellular nature of enamel formation which has since been demonstrated (9, 31). More recent descriptions by light microscopy (17 and Moss, 1968²) find the enamel tubules to be an "artifact of ground section preparation." We must disagree with this and also with the contention of Moss and Applebaum (17) that J. Tomes' 1849 interpretation of enamel tubules is "totally incorrect". It may be seen from the present findings that this dismissal of J. Tomes' detailed observations on the basis of interpretative data is at best ill-founded.

Note on terminology. The term "enamel fibril" has been used here because of its historical context. This past usage simplifies presentation. "Fibril" has, however, a specific connotation in the present day which is inconsistent with its application to a

¹ Partial occlusion of enamel tubules by mineralized material has been noted in anorganic, adult *Macropus* enamel examined by scanning electron microscopy (BOYDE, A.—personal communication).

² MOSS, M. L., in PERSON, P. (Ed.), *Biology of the Mouth*, p. 37. Amer. Assoc. Adv. Sci., G. King, Baltimore, 1968.

FIG. 14. A Tomes' process and part of the developing enamel front in "battlements" plane of section [see Boyde 1-3]. Note origin of enamel "fibril" from Tomes' process (Tp_1) and the continuity of filamentous material between the two. Dense material in "fibril" (at single arrow) may represent cytoplasmic degeneration. A cytoplasmic process (at double arrow) of an adjacent ameloblast (Tp_2 at left) projects into the depression in the mineralizing front extensively occupied by Tp_2 . $\times 30,000$.
 FIG. 15. Survey picture showing location of prisms (p) and inter-row sheets (irs) or interprismatic regions. Note two portions of an obliquely sectioned enamel tubule in the inter-row sheet. $\times 4500$. Inset shows the lower portion of tubule and contained "fibril" at higher magnification. $\times 36,000$.



cytoplasmic process. It may be seen from this study that developing marsupial enamel is analagous to dentine, cementum, and bone with respect to the incorporated processes of parent cells and the tubules containing them. For these reasons, it is suggested that a more suitable term for enamel "fibril" might be "primary ameloblast process". The "primary" would serve to distinguish this particular ameloblast process from the other, better known ameloblast process, "Tomes' process", the latter making its initial appearance at a relatively later time in marsupial enamel development.

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