The Extracellular Nature of Enamel in the Rat*

By MICHAEL L. WATSON, Ph.D.

(From The Departments of Pathology and Radiation Biology, The University of Rochester, School of Medicine and Dentistry, Rochester, New York)

PLATES 259 TO 261

(Received for publication, December 20, 1959)

ABSTRACT

Developing incisal enamel of the rat has been examined in sections with the electron microscope. Staining the sections with heavy metal and sandwiching them has revealed details hitherto unvisualized because of low contrast and destruction by the electron beam. In particular, it is seen that the cell membrane always lies between the ameloblast and the enamel and therefore that enamel is extracellular and not intracellular. Implications of this with regard to the possible keratinous nature of enamel matrix are discussed.

Although a number of electron microscope studies describing the fine structure of forming dental enamel have been published (1–3), none of these has shown clearly the precise relation of enamel matrix to the ameloblast. In the present communication, we will present evidence which shows that incisal enamel in the rat, like dentine, is extracellular.

Methods

Lower incisors were dissected from the mandibles of 200 gm.—Sprague-Dawley stock rats under ether anesthesia and placed in fixative. The blood supply was interrupted for about 5 minutes before fixation was started. Fixation was for 1 hour at 0–5°C. in 1 per cent OsO_4 buffered to pH 7.3 with veronal-acetate containing sucrose (4). The buffer strength was $\frac{1}{10}$ that recommended by Caulfield (4). Tissues were dehydrated in ethyl alcohol and embedded in butyl methacrylate. Sections were stained 30 minutes in lead hydroxide (5) and mounted on carbon-filmed grids and sandwiched (6) with evaporated carbon. Microscopy was done with the Siemens Elmiskop I operating at 80 kv. with a 50 μ objective aperture.

* These investigations were performed in part under contract with the United States Atomic Energy Commission at the University of Rochester, Atomic Energy Project, Rochester, New York, and in part with funds provided by Research Grant CY-3589 (C3) from the National Institutes of Health, United States Public Health Service.

RESULTS

The essential part of these observations lies in determining the position of the forming enamel with respect to the cell membrane. It was found that the cell membrane was difficult to see clearly unless the section was sandwiched, and it is for this reason, presumably, that earlier work has failed to reveal these details.

The rat incisor in cross-section is approximately triangular, one side of the triangle representing the labial aspect of the curved tooth. The enamel coats only this surface and is in the form of a ribbon which becomes much attenuated and finally disappears at its edges. In sections cut transversely to the axis of the tooth, it is possible to follow stages of enamel formation by proceeding medially from the edge of the enamel ribbon where no enamel has been deposited to the center of the ribbon where the enamel is thickest. A series of micrographs demonstrating this near the base of the tooth is shown in Figs. 1 to 4. At the extreme edge of the enamel (Fig. 1) essentially only dentine is present to represent the tooth substance. The surface of the ameloblasts is not in contact with the dentine, but follows a somewhat tortuous path, often approaching the dentine closely but in general leaving a well marked space between dentine and cell membrane. Within the ameloblasts, cytoplasmic constituents such as mitochondria and endoplasmic reticulum lie close

to the end of the cells toward the dentine. Adhesion plates or desmosomes are frequently seen between adjacent ameloblasts. The cells are not columnar and the nuclei are close to the dentine.

A short distance inwards from the edge of the enamel ribbon (Fig. 2) three changes are apparent. Nuclei are no longer close to the dentine, a zone containing little or no endoplasmic reticulum or mitochondria is present within the ameloblasts at the dentinal end, and the beginnings of enamel formation are discernible. Between the cell membrane and the dentine can be seen globular masses of finely stippled material as well as occasional areas of calcification which are recognizable as enamel by virtue of the length of the dense profiles of inorganic material. Both the globular masses and the early enamel are outside the cell membrane.

At a still more advanced stage in development (Fig. 3) forming enamel entirely covers the dentine. Dense, ribbon-shaped profiles can be seen embedded in finely stippled material like that described above. Extracellular, globular masses of this material are also seen between the ameloblasts at some distance from the enamel surface. The dense profiles are oriented approximately at right angles to the dentino-enamel junction which represents one point of their termination. The other point of termination of the profiles is at or close to the cell membrane. Various small, spherical globules are present in the clear zone of cytoplasm of the ameloblasts which are suggestive of the finely granular material outside. These may be precursors of enamel matrix present in the form of secretion granules.

Near the center of the enamel sheath (Fig. 4) the structure has taken on the characteristic appearance of enamel rods when viewed in sections (1). In this area only small amounts of the finely stippled material are present and are continuous with the areas of the enamel containing ribbon-shaped elements. Both the stippled material and the enamel proper are separated from the cytoplasm of the ameloblasts by the cell membrane. Numerous small, spherical globules of the type described above are present in parts of the clear zone of ameloblast cytoplasm close to the enamel.

Masses of the stippled material described above, although not always present, are a frequent finding in the rat incisor at later stages of inner enamel formation (Fig. 5). Whether their presence bears any systematic relation to enamel development we cannot say. This material does not represent enamel matrix, once calcified, but subsequently decalcified by the staining procedure, because it can also be found in unstained sections (7).

One further point of interest which we should like to emphasize is the large amount and high degree of development in the ameloblast of the rough surfaced endoplasmic reticulum, that is, endoplasmic reticulum bearing ribonucleoprotein particles. Greatly extended elements are oriented predominantly parallel to the long axis of the columnar cells (Fig. 6). The rough surfaced endoplasmic reticulum occupies a major portion of the cytoplasmic volume of the ameloblast. It is interesting to note that most of the ribonucleoprotein particles are arranged in circles or spirals similar to formations described by Palade (8) in other cells.

DISCUSSION

The stippled material which is present in variable amounts at the surface of forming inner enamel in the rat incisor appears to be continuous with the organic matrix in which the enamel crystallites are embedded. Sometimes large, globular masses of this material can be found; however, what relation it may bear to various granules and vacuoles (9) which have been described in light microscope studies is not clear. One may presume either that this represents the enamel matrix precursor in the process of deposition as suggested by Fearnhead (3) or that it is organic material lost from the enamel as calcification proceeds. Radioautographic studies by LeBlond, Bélanger, and Greulich (10) show that injected S^{35}-labeled sulfate is earliest deposited in greatest amounts at the surface of the enamel. This suggests, therefore, that this stippled material which is sometimes present in such large amounts has only recently been synthesized and that it is not organic matrix leaving the enamel. Whatever its role, it is continuous with and shares with the enamel a location which is extracellular.

The presence of large amounts of highly organized, rough surfaced endoplasmic reticulum gives the ameloblasts an outstanding morphological feature of secretory cells and in this respect puts them in a class with pancreatic acinar cells, and active fibroblasts. Cells in which the major synthetic product remains intracellular, such as normoblasts, the cortical cells of hair (11), or the cells which finally make up the cornified epithelium of the tongue, although containing many ribonucleoprotein particles possess relatively little

endoplasmic reticulum. Ameloblasts, though of ectodermal origin, thus differ from some other ectodermal cells in that morphologically they resemble strongly secretory cells. The product which is of a "structural" nature does not resemble hair and cornified epithelium in that the enamel is not made up of cornified cells but is wholly extracellular.

These observations disagree with earlier work of ours and of others (1–3) which suggested that enamel might be intracellular. The present finding that enamel is extracellular was reached because it was possible by sandwiching sections between high melting materials to prevent certain local distortions under the electron beam and thus clearly to observe the cell membrane near the enamel. It seems reasonable to presume that the secretory features of the ameloblast cytoplasm are connected with the elaboration of enamel matrix.

If, indeed, enamel is to be regarded as a eukeratin as has been reported in several studies (12–14), we have in this case the first example to the author's knowledge of a protein that may be found in either extra- or intracellular locations. In this connection, we should stress that by intracellular we mean present within the cytoplasm and not surrounded by a membrane. Thus, muscle protein and hemoglobin would properly be called intracellular, whereas pancreatic digestive enzymes while existing within the cell boundary as secretion granules, are surrounded by a membrane (15) and would, here, be considered as extracellular.

At the present time there appear to be two qualitatively different ways in which ribonucleoprotein (RNP) particles are associated morphologically with proteins undergoing synthesis. On the basis of present evidence, it is not unreasonable to assume that some proteins such as pancreatic digestive enzymes are formed within membranous vesicles which have on the outer surface of the membrane an investment of attached RNP particles. Other proteins such as hemoglobin or hair and epidermal keratins appear to form "naked" within the cytoplasm in regions close to RNP particles, but in the absence of any interposed membrane. Since the roles of RNA and of RNP particles in protein synthesis are far from clear, it would be fruitless to speculate on possible effects of a membrane enclosing the synthesizing product; however, these observations and considerations do suggest that enamel protein may well differ in important respects from other eukeratins. The presence in enamel of proline and hydroxyproline, much glycine, and little cystine is pointed out by Battistone and Burnett (14) as not typical of the keratins.

References

1. Watson, M. L., and Avery, J. K., *Am. J. Anat.*, 1954, **95,** 109.
2. Quigley, M., *J. Dent. Research,* 1959, **38,** 180.
3. Fearnhead, R. W., *in* Program of Fourth International Conference on Electron Microscopy, Berlin, 1958, (abstract).
4. Caulfield, J. B., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 827.
5. Watson, M. L., *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 727.
6. Watson, M. L., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 1017.
7. Watson, M. L., unpublished work.
8. Palade, G. E., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 59.
9. Saunders, J. B., Nuckolls, J., and Frisbie, H. E., *J. Am. Coll. Dent.*, 1942, **9,** 107.
10. LeBlond, C. P., Bélanger, L. F., and Greulich, R. C., *Ann. New York Acad. Sc.*, 1955, **60,** 631.
11. Birbeck, M. S. C., and Mercer, E. H., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 203, 215, and 223.
12. Stack, M. V., *J. Am. Dent. Assn.*, 1954, **48,** 297.
13. Stack, M. V., *Ann. New York Acad. Sc.*, 1955, **60,** 585.
14. Battistone, G. C., and Burnett, G. W., *J. Dent. Research,* 1956, **35,** 260.
15. Palade, G. E., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 417.

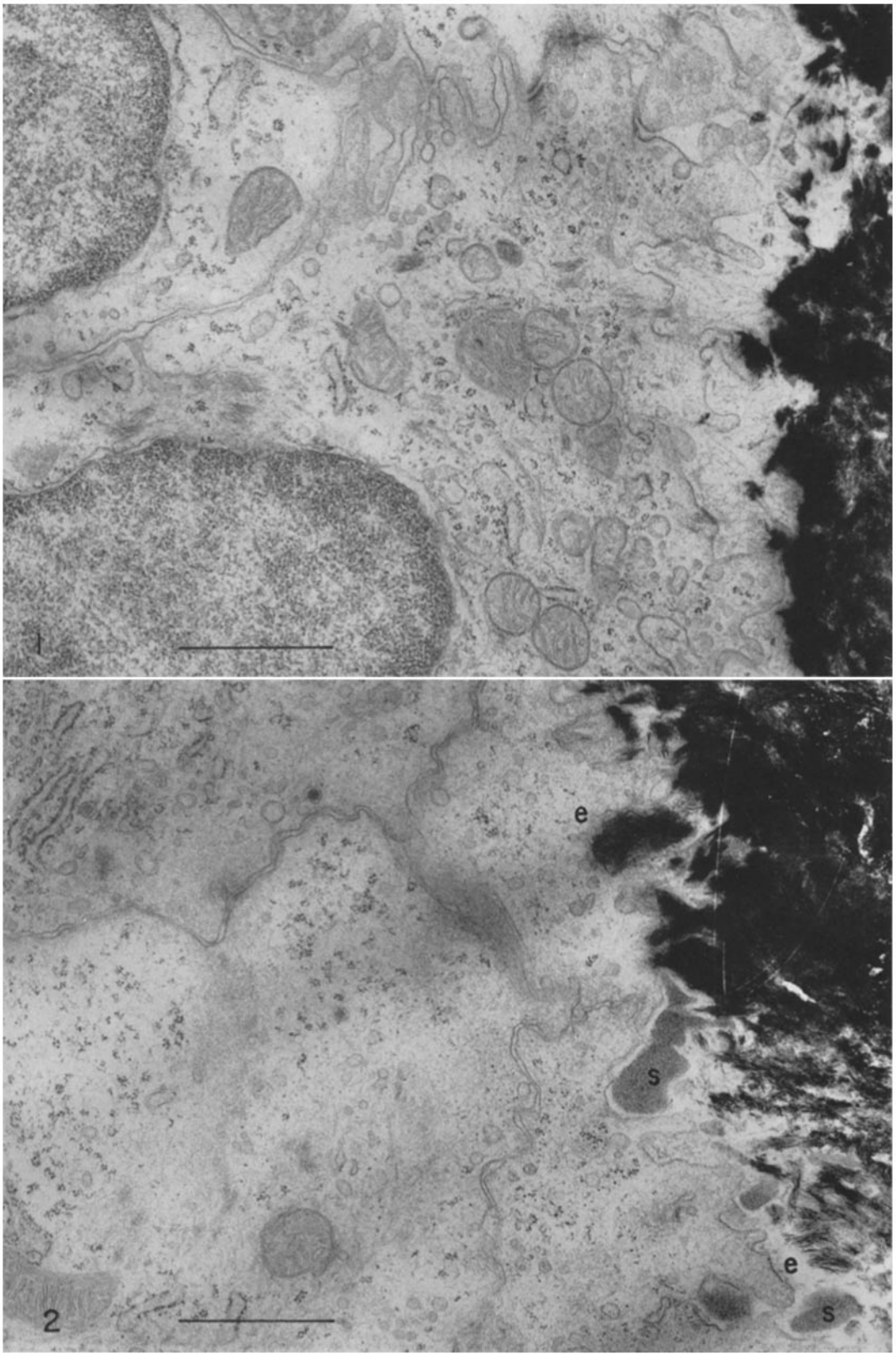
Explanation of Plates

Figs. 1 to 4. A series of sections cut transversely to the axis of the lower incisor of the rat near the base of tooth. Fig. 1 is taken at the edge of the ribbon of enamel which coats only the labial aspect of the incisor while Figs. 2, 3, and 4 are taken at points progressively more medial to this. All sections were stained with lead hydroxide for 30 minutes and were sandwiched with carbon. The calcified portion of the tooth appears at the right of each micrograph and the cytoplasm of the ameloblasts is on the left.

Plate 259

Fig. 1. The only calcified material appearing at the edge of the enamel ribbon is represented here by the dense image of dentine. No enamel has been deposited. Portions of two ameloblasts are shown. The ameloblast cell membrane follows a complicated path on the dentinal side of the cells and is spaced from the dentine by a region containing fine filaments and some uncalcified collagen. Nuclei are close to the dentine, and all parts of the cytoplasm contain the usual cell components such as mitochondria and endoplasmic reticulum. × 24,000.

Fig. 2. Very early stages in enamel formation are detectable. Masses of stippled material (*s*) are present between calcified dentine and the cell membranes of the ameloblasts. At one or two points (*e*) the beginnings of enamel calcification can be seen. Ameloblast nuclei are no longer close to the dentinal end of the cells, and the cytoplasm at that end now contains relatively few mitochondria and little endoplasmic reticulum. × 24,000.

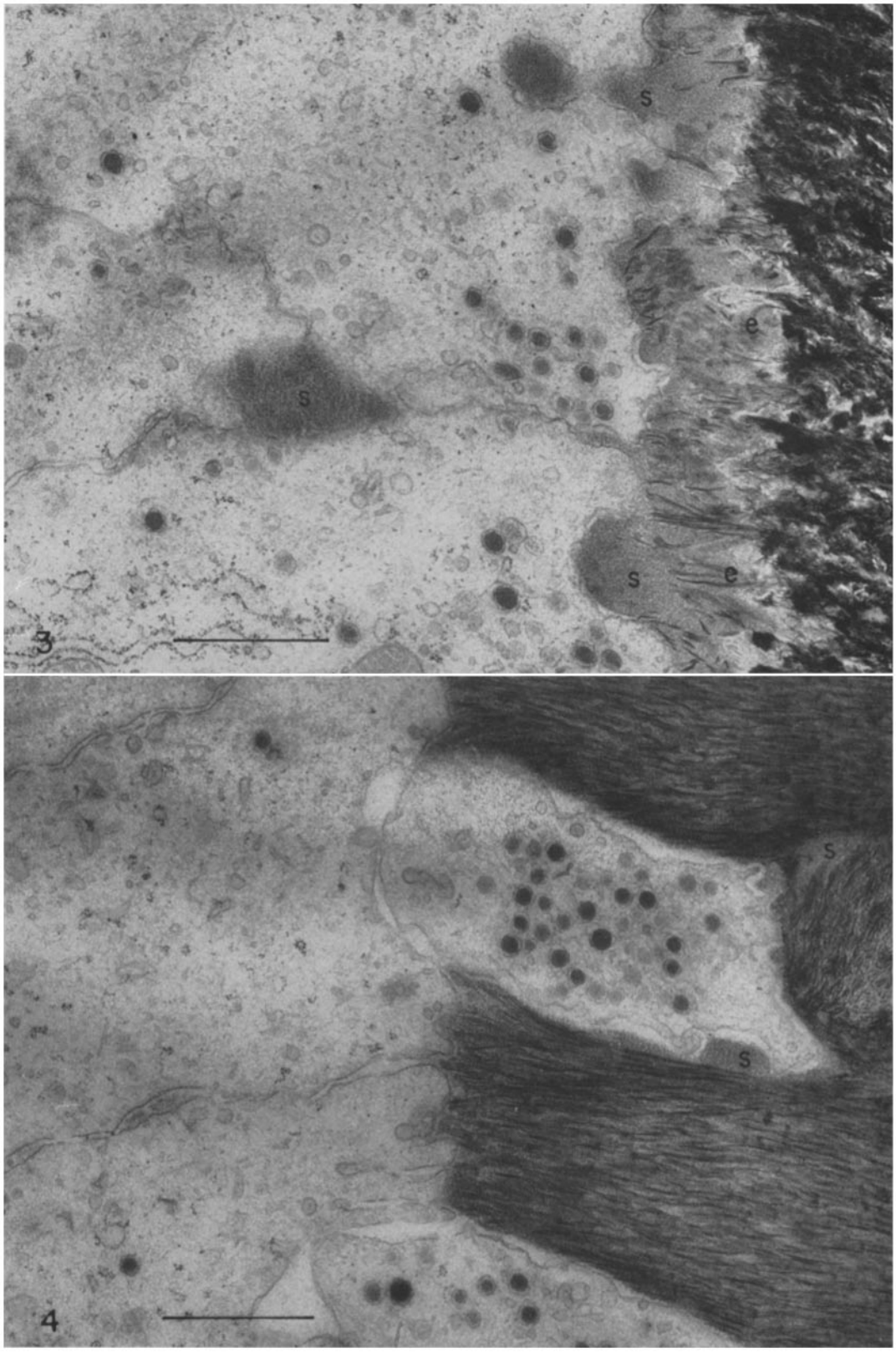


(Watson: Extracellular nature of enamel)

PLATE 260

FIG. 3. Dentine is now completely covered by a rudimentary layer of enamel (*e*). Large amounts of stippled material (*s*) are present with the lathe-shaped elements of calcifying enamel embedded within it. Other masses of stippled material are noticeable at some distance from the enamel. The enamel and stippled material are separated from the ameloblast cytoplasm by the cell membrane. Within the ameloblast are numerous, rather dense, membrane-enclosed globules which might represent granules of enamel matrix before secretion. × 24,000.

FIG. 4. Enamel is assuming the characteristic, highly ordered appearance of enamel rods in the inner enamel of the rat. Indentations in the serrated surface of the forming enamel are occupied by extensions of ameloblasts. The ameloblast cytoplasm is at all points covered by cell membrane and is not directly in contact with enamel. At some points stippled material (*s*) can be seen in contact with the enamel and apparently continuous with the matrix surrounding the dense, lathe-shaped elements. × 24,000.

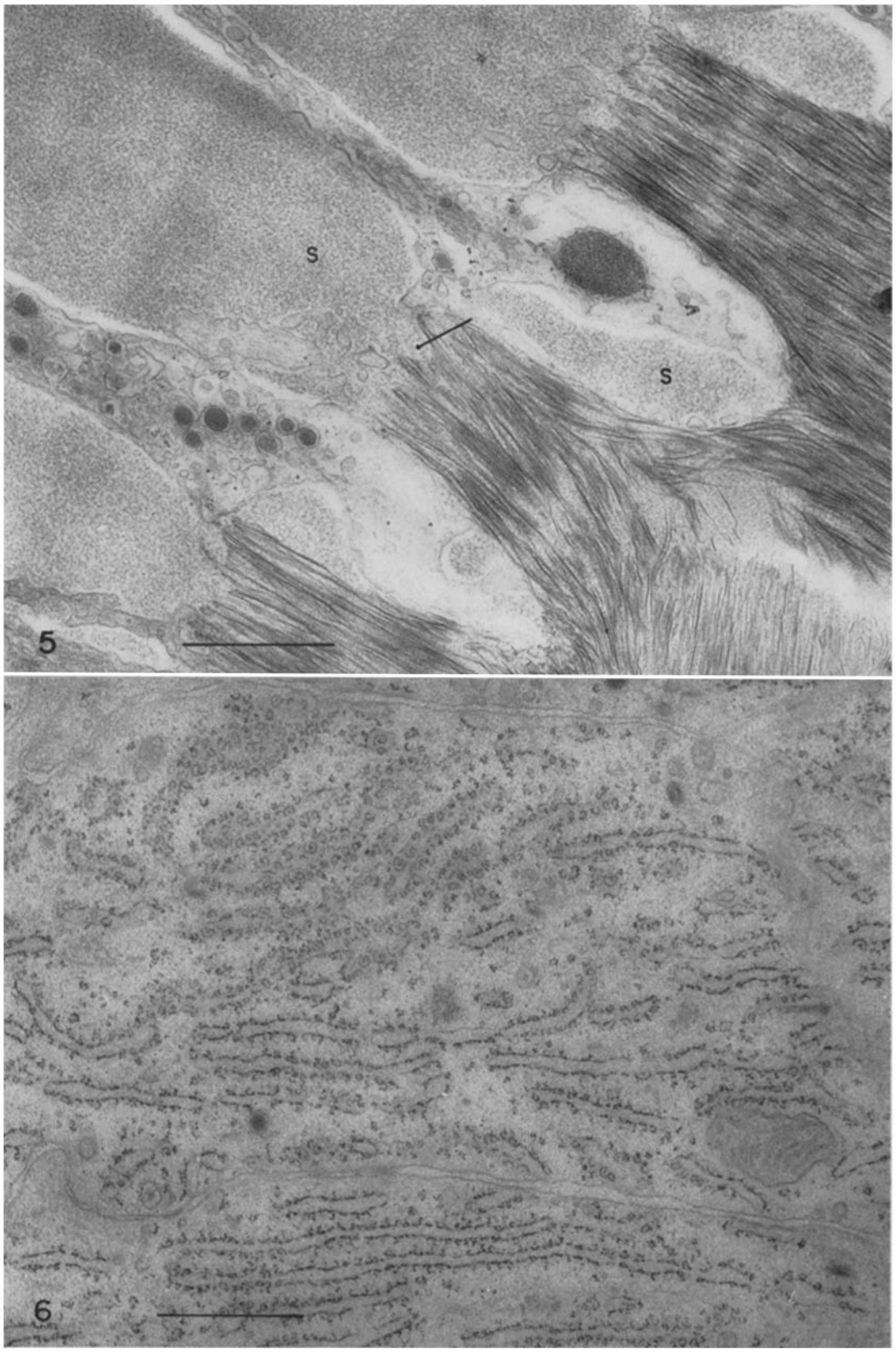


(Watson: Extracellular nature of enamel)

PLATE 261

FIG. 5. The surface of forming inner enamel at a much more advanced stage than that shown in the previous micrographs. At this stage, very large masses of stippled material (*s*) are present in the rat. This material is continuous with enamel matrix (arrow), but, like the enamel, is extracellular. Attenuated arms of ameloblast cytoplasm extend between the masses of stippled material and terminate within pockets in the surface of the forming enamel. × 24,000.

FIG. 6. Cytoplasm of the ameloblast at some distance from the forming enamel. Large amounts of endoplasmic reticulum are present having a dense investment of ribonucleoprotein particles. The particles are characteristically arranged in the ameloblast in short spirals. The high development of "rough-surfaced" endoplasmic reticulum suggests that the ameloblast is strongly secretory. × 24,000.



(Watson: Extracellular nature of enamel)