SOME ASPECTS OF THE GROWTH AND
DIFFERENTIATION OF THE MOLAR
TOOTH GERM IN THE MOUSE
(Mus musculus)

By

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ABSTRACT

The present work is concerned with two aspects:

I. A description is given of the distribution of mitoses in the inner enamel epithelium of the mouse molar teeth during ontogeny, from the aspect of the establishment of the crown pattern. This distribution is correlated with cusp formation.

Within limits of experimental error during reconstruction and plotting the mitoses, it has been shown that mitosis ceases at an early stage in areas which will eventually lie at the cusp tips, but continues between them, so that the valleys grow downwards leaving the cusps in a more elevated position. In other words, the relative positions of the cusps (mitoses-free areas), and the valleys (areas of intense mitoses) on the crown have been shown to be mapped out (predetermined) early in development. The theory of "insinuation" of the stratum intermedium among the inner enamel epithelium has been rejected.

II. A description of the cytology of the ameloblasts and odontoblasts is given with the aim of adding more detail to earlier accounts. In particular, it is shown that the Golgi apparatus changes polarity and varies in form during the life cycle of the ameloblasts, and an attempt is made to
arrive at a more concrete idea of its role during the life of ameloblasts.

The Aoyama method is used for the first time for demonstrating the Golgi apparatus in teeth. Also, Powers' method for nerve fibres, is used for the first time to show the Golgi apparatus, a method which has not been used for this purpose in teeth or any other tissue. The cytologic activity of the formative cells has been correlated with the morphologic changes of the Golgi apparatus. This correlation has been used to illustrate important phases of amelogenesis and dentinogenesis. The presence of the "kionoblasts" among the ameloblasts and the "radial cells" among the odontoblasts is denied.
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It was not until 1835 that the compound microscope was perfected and dental histology had its beginning about 1840. The reader is referred to a thesis by Norberg (1929) for a masterly historical survey of early work. The odontological literature of the last century contains many accounts of investigations on the development of mammalian teeth, and the great majority of these accounts are concerned with histology of the established enamel organ and particularly with the formation of enamel and dentine. In spite of the numerous papers that appeared, there is still considerable diversity of opinion on many points.

Teeth are composite structures, consisting of hard and soft tissues. The hard tissues are of organic and inorganic content, produced at first in a "plastic" condition, which later becomes stabilized by a process of calcification. This is a secretory process, and therefore,
the cells responsible for the production of the hard tissues (ameloblasts and odontoblasts) are specialized and differentiated.

Teeth, therefore, grow partly by proliferation of the soft tissue layers, and also later by accretion, i.e., deposition of the hard tissues.

During growth, the soft tissue layers "fold" to map out the crown pattern. In other words, the basic crown pattern is formed in soft tissue as a result of mitotic proliferation. Once the deposition of the hard tissues commences the basic features of the crown pattern are stabilized and no further growth by cell multiplication can take place in these areas.

The determination of the crown pattern as a result of mitotic proliferation is a point of special investigation in this thesis.

Secretion of the hard tissues, as a result of the activity of the highly specialized cells of the odontoblasts and ameloblasts, makes it reasonable to suppose that the cytological features of these specialized cells might be correlated with their metabolic activity. Hence, a detailed investigation of this cytology during ontogeny is presented here.
Growth can comprise increase in mass, volume, area or length of cells, tissues or organisms, and any of these can take place without cell division contributing at all. However, mitosis is considered as one of the events contributing to growth. As Weiss (1949) says: "The relation between cell division and growth is by no means as close as the widespread habit of treating them interchangeably would make it appear."

Proliferation, or the occurrence of cell division in the enamel organ of tooth germs of limited growth was first reported by Canalis (1886), whose results were confirmed by Rose (1891), Sachse (1895), von Ebner (1902-22), Kolliker (1902) and Schaper and Cohn (1905). More recently, Marsland (1951), Lefkowitz et al. (1953) and Gaunt (1955) have shown that proliferation takes place, but ceases before enamel matrix production. Also, Ten Cate, (1959), has shown that one of the three factors involved in the growth of human molars is mitotic proliferation of the inner enamel epithelium. All these authors reported that growth takes place by proliferation (cell division) of the inner enamel epithelium. This is against the assertion of Baume et al. (1954) that such cells are too specialized to proliferate. However, they agreed with the above
mentioned workers in that proliferation takes place only at the cervical loop. None of these investigators described the distribution of these mitotic figures in a quantitative manner. The results reached by these workers led Butler (1956) to put forward the hypothesis that the folding of the inner enamel epithelium which creates the crown pattern of the tooth is controlled by its intrinsic growth pattern.

Most observations on cell division in teeth have been made on permanently growing rodent teeth. The occurrence of mitoses in the odontogenic zone of the rat incisor was studied by Sicher (1942a). Ness and Smale (1959) investigated the distribution of mitoses in the developing incisors of the rabbit. However, the equivalent process in the rodent cheek teeth has not so far been studied. In the latter case, the problem is more complex and more interesting in that cusp formation is involved. From the above, it is evident that no report was found in the literature which described in detail the growth of the molar teeth in mice in terms of the distribution of mitoses in the inner enamel epithelium by the method adopted in this study.

2 - GOLGI APPARATUS AND MITOCHONDRIA

No cellular components have aroused more controversy than the Golgi apparatus and mitochondria.
Their association with physiological reactions within the cells makes it important to consider them in any cytological study. This might explain why these two cell organelles are studied side by side in cells, i.e., there is hardly any work done on the Golgi apparatus which does not include a study of the mitochondria or vice versa.

However, the investigations on these cytoplasmic organelles in the ameloblasts are limited in number. The Golgi apparatus and mitochondria of the ameloblasts have been observed with the light microscope by Jasswoin (1924), Shibata (1927), and Beams and King (1933). Recently, Nylen and Scott (1960), using the electron microscope, gave a detailed description of the ameloblasts with beautiful and accurate demonstrations of the Golgi apparatus and mitochondria in these cells. Reith (1960) made a similar interpretation. Other electron microscope studies mentioning the presence of the Golgi apparatus have appeared (Fearnhead, 1960).

The work of Duthie (1933) and that of Hirsch (1932) have thrown light on the relationship of mitochondria and Golgi apparatus to the origin and growth of secretion granules.

Bearing in mind the fact that one of the two classical methods used for demonstrating the Golgi apparatus, namely the osmium impregnation technique,
has already been studied in a wide variety of vertebrate cells by electron microscopy (Dalton, 1952), Lacy and Challice (1955) used the other classical method of demonstrating the Golgi apparatus, namely the silver impregnation technique, in a trial to see exactly where the silver is deposited. So, beside using the osmium method, they applied the Aoyama's silver technique for the Golgi apparatus on mice pancreatic tissue.

It seems that they were the only investigators who have used the Aoyama method in an electron microscope study of the Golgi apparatus. This is mentioned here to remind the reader that in all the very few studies on the Golgi apparatus in teeth, made either by the ordinary light or by electron microscope, the material was fixed in a fluid containing osmic acid (Nassonow) in the first case, and pure osmic acid in the second case. It would be probable that the present work is the first attempt to demonstrate the Golgi apparatus in teeth with the ordinary microscope, using the Aoyama silver impregnation technique, which proved to be the best one adopted by pioneer cytologists such as Gatenby (1950).

In an approach to the subject of the Golgi apparatus and mitochondria, I should like to mention some few points characteristic of these cytoplasmic organelles, reached through long work on other vertebrate...
tissue. Some of the information given in this section is quoted from an M.Sc. by the writer (Anga Ramadan, 1957).

A. The Golgi Apparatus

The "internal reticular apparatus", more commonly known as the "Golgi apparatus", was first observed and accurately described by the Italian neurologist Golgi (1898) in nerve cells after treatment by a silver impregnation method.

Although the priority for the discovery of the Golgi apparatus has become varyingly attributed to La Vallette St. George (1874), Nansen (1887), and Ramón y Cajal (1890), there is no doubt that the descriptions prior to Golgi (1898) are concerned with elements not exactly identifiable with the genuine Golgi apparatus, or else they imply very incomplete observations.

Knowledge of the Golgi apparatus in animal tissue is based almost exclusively on the methods of impregnation with silver nitrate and osmic acid.

Gatenby (1919b) stated that the Golgi apparatus is present in every cell of vertebrates and invertebrates, with the single exception of non-nucleated red blood cells. It has been seen in its classical network form in the living cells by the ordinary microscope by Bensley (1911), O'Leary (1930), Beams (1930), and others; also by the phasecontrast microscope by Gatenby (1949).
and others.

Recent work with the electron microscope has shown that double parallel membranes, vacuoles, and minute granules are the three elements always present in the Golgi zone of a variety of cells of vertebrate animals (Dalton and Felix, 1953, 1954; Gatenby, 1954; Lacy and Challice, 1955, 1956; Lacy, 1956; Rinehart and Fargner, 1953; Sjostrand and Hanzon, 1954; Gresson, 1956; and Burgos and Fawcetts, 1955).

In view of the above facts, it was concluded that the Golgi apparatus is a distinct type of organelle, morphologically well defined, and which in all probability, is a universal constituent of all animal cells.

It is a known fact that it is never of the same shape even in the neighbouring cells of the same kind. In other words, it varies considerably in form from one cell to another, and even in one and the same cell according to its physiological condition.

In general, the morphology of the Golgi apparatus, in spite of its variability, is characteristic of each type of cell. For example, its shape in acinus cells of the pancreas is quite different from that of polynuclear leucocytes (Cowdry, 1921). Further if the pancreas is examined in several different groups of animals, the same shape of Golgi apparatus is encountered. This fact gives a clear indication that variations in
its morphology are closely related to variations in cellular organization and function.

There is a general agreement that on cell division, the networks are broken up into smaller masses which are distributed approximately equally to the daughter cells, in which the networks are again reconstituted. However, Dalton (1951) found that the Golgi apparatus disappears entirely in hepatoma cells during mitosis.

The Golgi apparatus appears in two principal forms, the "localized" and the "diffuse", which may be converted into one another in changing phases of cell activity, and are, therefore, regarded as merely different phases of the same structural element. Sosa (1949), after an extensive study of normal and experimental material, described the Golgi apparatus as a cytological element that must not be taken as a dynamic entity, that is to say, the conceptions of the network or of the isolated filaments or granular golgiosomes may be changed by the concept of a "form variation cycle", according to the state of normal or reactional activity.

The position of the Golgi apparatus in the cell has been considered significant by many investigators, and is relatively fixed for each cell type. In young cells, and often in old ones, it lies most
commonly at one side of the nucleus, but in certain cases may completely surround it.

In exocrine gland cells (as acinus cells of the pancreas and salivary glands), where the secretory polarity is fixed, the Golgi apparatus has been observed by all investigators to be located between the nucleus and the secretory pole (lumen) (Beams, 1930a; Gatenby, 1931b).

In the endocrine glands, on the other hand, the situation is complicated. The Golgi apparatus, here, has a variable polarity. Many authors have recorded reversal of polarity (Cajal, 1915; Cowdry, 1922; Bowen, 1926; Cramer and Judford, 1926). Beams and King (1933) reported this change of polarity of the Golgi apparatus in the inner enamel epithelium (pre-ameloblasts), before the secretory phase is reached.

In many cases, the Golgi apparatus may lose its reticular form and fragment into coarse granules which become dispersed in the cytoplasm. This takes place in certain functional stages. Usually, the Golgi apparatus is well developed in active stages of cytomorphosis becoming progressively smaller as the cell ages, until it finally disappears with senility and death.

Very little is known of the chemical composition and physiochemical properties of the Golgi apparatus. This is due to two reasons: (1) The complete
failure to isolate the Golgi apparatus, which leads to the impossibility of carrying out any quantitative analysis respecting its composition. (2) The total dependence of cytologists on its selective impregnation with silver and osmic acid, because both compounds may be reduced by other substances, for example the impregnation of fats is generally much more rapid than that of the Golgi apparatus. Nevertheless, the chemical composition of the Golgi apparatus varies to some extent in different cells. The majority of cytologists believe that the Golgi apparatus contains lipoids, in a wide sense, or a lipoprotein compound (Ciaccio, 1910; Gatenby, 1920b; Parat, 1928; Owen and Bensley, 1929; Nath, 1933; Tarao, 1940; Baker, 1944, 1949; Sosa, 1949). Moreover, Gersh (1949), using the freezing and drying technique, and staining with leucofuchsin (McManus-Hotchkiss method) concluded that the Golgi apparatus contains a carbohydrate protein complex.

Recent studies have shown that there is a relationship between the Golgi apparatus and vitamin C, and that there is a topographical coincidence between the two elements (Bourne, 1950, 1951, 1955).

In spite of the extensive research done on the Golgi apparatus, the problem of its functional significance is not yet exactly known. One of the main functions attributed to this organelle is secretion.

That the Golgi apparatus takes part in secretion
had been suggested by Saguchi (1920b). Bowen (1926) showed the association of the Golgi apparatus with the production of secretion droplets in many types of exocrine glands.

Kirkman and Severinghaus (1938), Bourne (1942-51), and Hibbard (1945) have reviewed the literature dealing with the different theories regarding its origin, identification, structure, polarity and function.

The most convincing evidence of the secretory function of the Golgi apparatus has been provided by a study with both the light and electron microscope of the cycle of secretion in the mammalian pancreas (Hirsch, 1958, 1959).

B. The Mitochondria

Although it is customary to trace back our knowledge of mitochondria to the researches of Altmann (1880-1890) (hence they were called Altmann’s granules), they were seen and imperfectly described by other workers before him, notably by Flemming and Kolliker (Cowdry, 1924). Benda (1897) was able to demonstrate them not only in fixed and stained cells, but also in living cells and gave them the name mitochondria. They are very soluble in alcohol, ether, chloroform, acetic acid and other similar reagents. In other words, they are destroyed or imperfectly preserved with routine fixatives. For example, in the classical mixture of Zenker, the 5% acetic acid which it contains helps to give a uniform presentation, with sharp nuclear
detail, but its solvent action is usually sufficient to remove all traces of the mitochondria normally present within the cells. However, the replacement of acetic acid by formalin, in "Zenker-formol", usually called "Helly's fluid", is a better preservative of mitochondria. The mitochondria are rendered relatively insoluble by chromatization. For this reason, all the methods of fixation of the mitochondria are based essentially on preserving and stabilizing them by prolonged action of oxidizing agents such as osmic acid, chromic acid, and potassium dichromate, i.e., Regaud's or Champy's fluids. These fixatives render lipoids, in general, insoluble in fat solvents such as xylol.

The form of the mitochondria is quite variable, but in general, it is either filamentous or granular. In the liver, pancreas, and kidney, there have been described cyclic morphological changes in the mitochondria. Thus, in the pancreas, for example, they are believed to break up and form granules which eventually metamorphose into zymogen granules. In most animals, however, the shape of the mitochondria is constant and characteristic for any particular organ. The mitochondria are usually distributed uniformly throughout the cytoplasm of the cell. There are, however, many exceptions to this rule. For example, in certain phases of cellular activity, they may be collected into clumps, or they may be particularly numerous in
certain regions of the cytoplasm. Thus, in the cells of the kidney, they are aggregated particularly in the basal region near the blood capillaries.

As regards the function of the mitochondria, there has always been a general agreement among cytologists concerning the importance of mitochondria in the physiology of the cell. It is a well known fact that mitochondria play an important role in the metabolic processes of the cell. It is very difficult to determine the mitochondrial content of a cell, but in general, it can be said that it varies with the cell type and the functional condition of the cell. For example, in glands of active secretion, there is an increase in the number and size of mitochondria. On the other hand, in the cells where an increase in the quantity of fat or glycogen occurs, there is a diminution of the mitochondria.

A reciprocal relationship appears to exist between the amount of mitochondria and the amount of fat; in general where there are many drops of fat in a cell there are few mitochondria and vice versa (De Robertis, Nowinski and Saez, 1954).

In erythrocytes the mitochondria diminish as the quantity of haemoglobin increases; in mature erythrocytes the mitochondria usually disappear completely (Rojas and De Robertis, 1935).

Mitochondria are concerned in histogenesis. A
list of 80 substances in the formation of which mitochondria are said to be concerned was published by Cowdry (1918). They comprise materials of the most diverse character, including granular secretions, pigments, fibrillar substances of different kinds, fat, protein, glycogen, etc. In brief, there is a definite topographic relationship between mitochondria and many products of cellular activity.

The mitochondria also play an important part in cellular oxidations. Kingsbury (1912) was the first to suggest that they function in protoplasmic respiration. He pointed out that the osmic acid, potassium dichromate and formalin are the chief ingredients of mitochondrial fixatives and that their value depends upon the presence of reducing substances in the cytoplasm. These he believed to be the mitochondria on account of their lipoidal properties.

More detailed evidence was given by Meyer, Rathery and Schaffer (1914b). These investigators stated (1) that mitochondria are phosphatids containing unsaturated fatty acids, with ethylidene groups and, therefore, chemically adapted to function in oxidation and reduction, (2) that the agents which attack lipoids (like alcohol, ether, chloroform among anaethetics) at the same time cut down respiratory oxidations. Isolated mitochondria were found to be bound up with the uptake of oxygen (Bensley
and Hoerr, 1934). Moreover, it was proved that the mitochondria contain a number of oxidative enzymes such as cytochrome oxidase, succinic oxidase, and fatty acid oxidase. In fact, the presence of the latter enzyme provides direct evidence of the relationship of mitochondria to fat metabolism.

As to their chemical composition, Bensley and Gersh (1955) using the freeze-drying method on liver cells, proved that proteins constitute the most important component of mitochondria. Bensley (1957), using the technique of "cell fractionation", found that mitochondria have a lipoprotein composition in which the protein part predominates. By means of adequate histochemical techniques, it has been possible to demonstrate the presence of various enzymes and vitamins in the mitochondria (Bourne, 1950).

3. DIFFERENTIATION OF AMELIOBLASTS AND ODONTOBLASTS

On reviewing the literature on the histology of teeth, one is impressed with the lack of agreement among the views expressed by various writers. One cannot select a single tissue entering into the formation of the tooth whose structure or function is not, even today, interpreted in several ways.

Rodent teeth, incisors and molars, particularly
those of rats and mice, have received great attention in the field of dental research. The dentition is monophyodont. The incisors are continuously growing, while the molars are teeth of limited growth. One of the pioneers in dental research who worked on the whole order of Rodentia was J. Tomes (1845) whose name was given to the processes of the formative end of the ameloblasts. Since the time of Tomes' valuable publications, many papers appeared on this subject, each investigator confirming or correcting the views of his predecessor, or adding new facts to those already recorded.

The early differentiation and the histologic changes taking place in the ameloblasts and odontoblasts in the rat during development have been described by several workers: von Brunn (1887-1891); Addison and Appleton (1921); Santone (1935); Glasstone (1936, 1938); Hahn (1941); Weinmann (1943); Shapiro et al. (1944); Lefkowitz et al. (1953); and Marsland (1951, 1952). Mahn (1890) briefly described the ameloblasts and odontoblasts in the developing teeth of mice. Bhaskar (1948) recorded the time of first formation of dentine and enamel in teeth of mice, but gave no histologic details.

Gaunt (1955) described the cells of the different layers of the tooth germ of the mouse in relation to the establishment of the crown pattern. In 1961 he modified his previous statement on the crown pattern of the cheek
teeth of the mouse. In 1956 he reported the presence of the apical pits and enamel-free areas on the upper molars, and in 1959 he showed that these structures are also present on the lower molars. Moreover, the mouse teeth have been investigated by many workers, who employed histochemical methods to prove the presence of glycogen, polysaccharides, alkaline and acid phosphatases, nucleic acids and other substances in the dental tissue (Johnson and Bevelander, 1954; Symons, 1956; Glasstone, 1958; Pourtois, 1961; Ten Cate, 1962). Dentine and enamel are products of odontoblasts and ameloblasts of ectomesenchymal (De Beer, 1947; Platt, 1893-7, cited in Horstadius, 1950 - The neural crest) and ectodermal origin respectively.

A. **Ameloblasts and Enamel**

Although it is the undifferentiated cell which enters into the formation of the dentine-enamel limiting membrane, or membrana praeformativa, full maturity of the cell is required for the formation of the organic enamel rod.

1. **Ameloblasts**

The ameloblasts, or enamel forming cells, differentiate from the inner enamel epithelium which in its primitive form is derived from the stratum germinativum of the oral epithelium. They undergo further differentiation to form tall columnar cells, or ameloblasts. This histo-
differentiation is accompanied by a change in the cell polarity of both the nucleus and the Golgi apparatus (Jasswoin, 1924; Beams and King, 1933). The nucleus moves away from the dentine enamel membrane while the Golgi apparatus moves towards this membrane. These changes in cellular structure which occur prior to the functional period of the cells, might be related to specific phases in extracellular matrix formation. Although most of the present day knowledge of the ultrastructure of the cell has been gained from the investigation of other tissues, these features of the ameloblasts made them most interesting test objects for cytological studies. In recent years, accounts of cellular ultrastructure during tooth formation have appeared (Lenz, 1957; Nylen and Scott, 1958ab; Quigley, 1959; Watson and Avery, 1954).

The differentiation and change of polarity first occur at the apices of the cusps, and at this stage of development the enamel-forming cell begins to deposit the organic matrix of the enamel at the dentine-enamel junction. According to many reliable authorities, the first changes which follow reversal of polarity and which can be associated with amelogenesis, are the appearance of "Tomes' processes" and also the "terminal bar apparatus". The latter is apparently a condensation of stainable material between the ameloblasts in a position which marks the boundaries between the cells and its "Tomes process".
Tomes' processes seem to be intimately connected with the formation of the matrix. The two most popular views are that either these processes secrete the matrix, or, that they are directly converted into the matrix as they grow out from the formative end of the ameloblasts. Mummery (1924), Wassermann (1944), Orban et al. (1943), Nuckolls et al. (1943) and Marsland (1951) believe in the second view. The first view is supported by Fearnhead (1960). The ameloblast now has become a highly specialized structure whose function is the formation of an organic matrix which is keratinous in nature (Nuckolls et al., 1943). It has been shown (Schour, 1953) that each ameloblast is responsible for the deposition of one unit of enamel, which in its final form, is called an enamel prism or rod. These prisms are separated by an organic interprismatic matrix which later becomes mineralized.

Thus, the adult enamel rod is regarded as a product of metabolic activities of an ameloblast, secreted in the form of an organic matrix which undergoes mineralization (Marsland, 1951, 1952; Nuckolls et al., 1943). Although the process of matrix formation is distinct from that of mineralization, both may be regarded as a function of cell metabolism. It has been suggested that the inter-rod matrix might originate from a special group of cells called "kionoblasts", the rods being formed from the ameloblasts (Symons, 1955). Another view, held by some
electron microscopists (Fearnhead, 1960), is that both rod and inter-rod matrix are formed by the ameloblasts.

When the formation of enamel is complete, the ameloblast undergoes a striking reduction in size and a change in its cytological characteristics, followed by degeneration.

2. Enamel matrix, structure and formation:

It has been noted that the inter-rod matrix is formed before the rod matrix (Watson and Avery, 1954; Quigley, 1959) and contains some minerals (Nylen and Scott, 1960). However, the rod matrix becomes completely calcified before the inter-rod matrix calcifies. The finding of organic inter-rod substance after decalcification of enamel has been noted by many dental histologists (e.g. Avery et al., 1961). When the mineral component of enamel is removed, the organic residue at the periphery of the rods appears to be in a more condensed form than that of the rod and inter-rod regions, a difference which has led to the use of the term prism or rod-sheath to denote this structure. There are some workers who deny the presence of the rod-sheath (Watson and Avery, 1954), and others who deny the existence of the inter-rod region (Lenz, 1958).

With a sudanophil reaction and a toluidine blue stain Sognnaes (1955) demonstrated the formation of the prism-sheaths and the interprismatic structure. Gustafson (1957) described the interior of the prism sheaths of
enamel rods and showed an organic framework divided or arranged in regular spaces within the sheaths. Helmcke (1955) stated that prism-sheath boundaries do not actually exist. This is in agreement with Lams (1921) who stated that there were no definite limiting membranes between the enamel prisms. He also stated that the limiting membranes of the ameloblasts were very delicate and scarcely stainable, being much more like condensations of peripheral material.

The hard adult enamel, in most cases, is decalcified before preparing histological sections. This may explain the basis of such controversy, which would seem to rest in the nature of treatment necessary for the preparation of dental tissues prior to histologic observations. It is obvious that the use of strong acids for purposes of decalcification may not only distort and macerate the finer cellular elements but may also alter the reactions of the tissues on which histological, cytological and histochemical determinations depend. Under these conditions, interpretation is difficult and misleading and, in consequence, has given rise to schools of opinion directly opposed to one another, and made the process of enamel formation and its calcification one of the most difficult problems of dental research.

The layer next to the ameloblasts previously called "honey comb" by Mummery (1924) is known as "pre-
enamel matrix" by Orban, Sicher and Weinmann (1943), and Nuckolls, Saunders and Frisbie (1943). Very many accounts of mammalian enamel formation are to be found in dental and zoological literature; summaries of the most important details are given by Kvam (1946) and Marsland (1951, 1952).

There is perhaps no more confused and controversial field in dental histology than that which concerns the precise elaboration of the enamel. The manner whereby enamel is formed has been a subject of considerable importance through the years. Various interpretations of the process of matrix formation have been put forward (Chase, 1932; Marsland, 1951; Orban, Sicher and Weinmann, 1943; Saunders et al., 1942). Enamel matrix formation begins at the dentine enamel junction at the tips of the cusps as soon as the first increments of dentine begin to mineralize, gradually moving down the sides of the crown towards the cervical region. Enamel formation of the cuspal region is thus always in a more advanced stage than that of the cervical region during development. The enamel matrix is soft and can be cut without the use of decalcifying agents. Protein elements have been known to exist in enamel matrix, but their structure and role in the final formation of enamel have not always been agreed upon (Marsland, 1951; Mummery, 1924; Nuckolls et al., 1943; Pincus, 1936; Rosebury, 1930; Scott, 1955; C. Tomes,
Mature enamel structure:

When enamel is viewed within the resolving power of the standard light microscope the enamel rod is the smallest distinct building block unit. The rods, sometimes called prisms, are separated from each other by inter-rod or inter-prisms. These rods are segmented by cross striations reflecting the rhythm of appositional development. Retzius (1837) considered that enamel was deposited in a rhythmical manner in calcified layers, the same view being upheld by Hoppe (1862) and Schmidt (1925), who proposed that in all stages of developing enamel, the inorganic components were laid down in the matrix in the crystalline form. This view remained unchallenged until von Ebner (1906) and Kitchin (1933) using polarized light, came to the opposite conclusion, that the calcium salt in the young enamel matrix is in an amorphous non-crystalline form. Kitchin described a "ladder like" structure in newly formed enamel in the mandibular incisors of rats. Applebaum (1938), working with the Grenz rays, provided further confirmation that calcification is a process secondary to matrix formation. Beams and King (1933), Gustafson (1955) and Wellings (1940), have shown that a protein pattern is formed and arranged in an orderly fashion inside and between each prism, and that afterwards, the inorganic parts of enamel are laid down in the form of crystallites within
these organic meshes or spaces. Also, von Beust (1928) experimenting on pig molars, said that enamel was not laid down in calcified layers and that the inorganic salt entering into its structure was secondarily deposited. He concluded that enamel is calcified "en masse". Chase (1929-32, 1935, 1940) agreed with von Beust that the rhythmic deposition of enamel, represented by the striae of Retzius is incorrect in so far as the ameloblasts do not deposit calcified layers of enamel.

4. Maturation

In 1940, Chase gave the name "maturation" to the transformation which takes place after the enamel matrix has been deposited by the ameloblasts. However, he opposed von Beust in regarding maturation as a rhythmically progressive process paralleling the incremental lines. Chase divided enamel formation into four stages which correspond closely with the zones of immature enamel which von Ebner (1906) described. Marsland (1951, 1952) adopted the same nomenclature, namely: matrix formation and maturation, the latter being the process by which the acid insoluble organic matrix is changed into the acid soluble product of the highly calcified adult enamel. During maturation (mineralization), enamel matrix undergoes certain changes which include an influx of calcium salts and a decrease in water (Deakins, 1942); the final product is the hard mature adult enamel. According to Stack (1955),
adult enamel largely consists of hydroxyapatite crystals in intimate association with very little organic matter (2.5%) and water (1.5%).

Mammalian enamel is prismatic in nature. The organic framework of enamel, when viewed in stained sections, is markedly different from that of mesenchymal hard tissues such as dentine, cementum, and bone. With routine stains, the young enamel matrix reacts like other cornifying epithelial derivatives (such as hair), Nuckolls et al. (1947). Poole (1957) stated that during its development, the properties of the matrix change many times. At first it is acidophil and is known as pre-enamel, then an influx of calcium salts produces a change to intense basophil properties. According to Weinmann, Wessinger and Reed (1942), the enamel matrix remains in this condition with a mineral content not greater than 35% until it has reached the final thickness of the future enamel. Immediately before the heavy influx of mineral, a return to the acidophil properties is shown, but as calcification proceeds, there is a withdrawal of organic material and water, the residual matrix becoming soluble in acid.

Some authors believe that the calcium enters the enamel via the odontoblasts (Jasswoin, 1924; Kato, 1930; Lefkowitz et al., 1943, 1947; Wassermann, 1944; Marsland, 1952), while others suggest that the calcium required for
maturation of enamel is derived from the enamel organ (Adams, 1962; Reith and Cotty, 1962; Orban, 1957; Schour, 1960).

Voluminous literature on the subject of enamel maturation has been reviewed, but the writer will not attempt to cite again the numerous individual contributions of the many investigators in this field. An evaluation of the past studies leads to the conclusion that at least two general concepts of enamel mineralization have been developed. One concept postulates that mineralization of the enamel follows the pattern of formation of the enamel matrix from the dentine enamel junction peripherally (Chase, 1935; Nuckolls et al., 1947; Allan, 1957; Crabb, 1958, 1959). The second concept suggests that the enamel matrix formation and final mineralization are separate processes, the latter not occurring until after the former is complete (Marsland, 1953; Applebaum, 1943; Weinmann et al., 1942; Kitchin, 1933). This latter concept indicates that calcification of enamel matrix begins at the dentine enamel junction of the cusp tips and proceeds cervically. Text books (Schour, 1953, 1960; and Orban, 1957) have adhered to this latter concept. It is, however, generally accepted that the process consists of two phases: matrix formation and subsequent maturation.

5. Structure of the rodent enamel

The complicated architecture of rodent enamel
was first described over a century ago by J. Tomes (1849), but its structure and histogenesis still remain to be adequately studied. However, it has been re-emphasized by recent optical microscopy (Marsland, 1951, 1952; Fleming, 1958) as well as by electron microscopy (Watson and Avery, 1954; Lenz, 1957, 1958; Nylen and Scott, 1958; Quigley, 1959; Frank and Sognnaes, 1959, 1960).

The contribution of the electron microscopy to tooth biology up to 1953 have been reviewed by Scott (1953), from which one can notice that the bulk of this early work dealt with the structure of enamel and dentine.

B. Odontoblasts

The odontoblast cells associated with the development of dentine, differentiate from the surface cells of the dental papilla. The process of differentiation is observed with the light microscope as an increase in size and an alteration in the shape of the cells involved. The differentiation takes the following sequence:

First, a noticeable condensation of mesenchyme cells is always seen in association with the inner enamel epithelium in its early development. Tonge (1952, 1953) discussed the distribution and arrangement, in human embryos, of the condensations of the mesenchyme cells that are related to the development of teeth and bones of the jaws. However, the peripheral mesenchyme cells of the tooth germ have no particular arrangement until the enamel
organ assumes the cap or bell stage, when they become arranged in a layer facing the inner enamel epithelium. These cells undergo further differentiation, becoming more elongated than the other mesenchyme cells, and finally assume the characteristic fully differentiated cells or odontoblasts.

Full differentiation of the odontoblasts occurs in sequence, commencing at the highest part of the papilla (cusp tips) and extends progressively to the cervical loop. The most recent work on the differentiation of the odontoblasts is an electron microscope study by Noble et al. (1962); Gibbins (1962); Reith (1960); and Nylen and Scott (1958).

There are two opposing views: one considers that the odontoblasts differentiate under the organizing influence of the inner enamel epithelium (Noble et al., 1962; Glasstone, 1955; Schour, 1953, 1960; James, 1957; James and Wellings, 1943); the other view which considers that the inner enamel epithelium differentiates under the influence of the mesenchyme, is held by Turner (1961).

There is a general agreement that the "fibrils of Tomes" in the dentinal tubules are cytoplasmic processes from the odontoblasts (Orban, 1957; Schour, 1953, 1960). However, Klein (1879), Mummery (1892) and James (1957) seem to be the only authors who expressed doubt of the presence of such cytoplasmic processes from the odontoblasts.
into the dentinal tubules.

Von Korff's fibres (1928) arise from the layer lying more deeply in the pulp and extend between the odontoblasts in the form of cork-screw structures. There is a general belief that von Korff's fibres constitute the collagenous matrix of dentine. This problem has been discussed by Symons (1956), Nylen and Scott (1958), and Noble et al. (1962).

Immediately beneath the odontoblasts there is the "Weils" layer, in which the cells are very scarce. This layer is indistinct or absent during early dentine formation, and contains many fine nerve fibres which can be revealed by special methods. One of the features of particular interest is the innervation of dentine (J. Tomes (1856); Mummery (1912, 1918, 1924); Wellings (1940); Cocker and Hatton (1955); Bradlaw (1939); Powers (1951, 1952); Fearnhead (1960)). The point of controversy is whether nerve fibres enter the dentinal tubules or not.

4. AIM OF WORK

The work here presented is concerned with two related aspects of the growth and differentiation of the cheek teeth of the albino mouse.

The first aspect deals with the distribution and frequency of mitoses in the different cell layers laying emphasis on the inner enamel epithelium of the tooth germ.
during ontogeny. The purpose of plotting the distribution of mitoses in the inner enamel epithelium in this quantitative manner was to test Butler's hypothesis (1956), that the unequal growth of the inner enamel epithelium played a major part in moulding the crown pattern, and to see how far this distribution has a bearing on the interpretation of the mouse molar pattern in terms of growth. It was thought that this might throw some light on the process of cusp formation.

Thus, the distribution of mitoses in the inner enamel epithelium in the pre- and post-natal embryos of known age was plotted, with an attempt to correlate mitotic activity with cusp formation. These observations have been extended to the stratum intermedium, a layer considered to be involved in cuspal development, and to other layers such as the stellate reticulum, the outer enamel epithelium and the pre-odontoblasts.

Because of the presence of enamel-free areas on the crowns of the mouse cheek teeth, the writer made a comparative study on teeth which do not possess this feature, such as those of the cat and of a bat, to see if the mitotic distribution pattern in the stages studied remain the same whether these enamel-free areas are present or absent.

Thus, the mitotic distribution has been investigated in the "bud" and "cap" stages of the cat, and in
the "cap" stage of the bat *Hipposideros beatus*, to ascertain whether or not the pattern of the distribution in the mouse might not be due entirely to the presence of enamel-free areas on the crown. Particular attention was paid to the enamel pulp in order to make some contribution to the knowledge of its significance and function. Some consideration has also been given to those transitory formations which fall under the name of enamel knot and cord, and which are still objects of controversy.

The second aspect is essentially cytological with particular attention to the location of the Golgi apparatus and the mitochondria. The role of these organelles in the formation of the hard tissues of the teeth is discussed. Also, note is made of the presence and distribution of other cytoplasmic inclusions of a fatty and polysaccharide nature. The change of polarity of the Golgi apparatus in the ameloblasts has been re-investigated, so that the history of this type of cell can be traced. This was undertaken to clarify and supplement the information provided in earlier studies.

For this purpose, the Aoyama silver impregnation method was used for demonstrating the Golgi apparatus. This method has not been used before for showing this organelle in the cells of the dental tissue.

Another method, originally devised for tracing the nerve fibres in teeth, was applied to demonstrate the
Golgi apparatus. This method has not been used before for this purpose in any tissue.
II - MATERIAL AND METHODS

A. Material

This investigation is based upon a study of the first and second upper and lower molars. The material used consisted of the heads of developing albino mouse embryos of the pure-bred strain Tuck's No. I, fed on a standard diet B 41 pellets. The animals were kept in special cages.

In order to reduce any possible genetical variation, brother-sister crosses were maintained throughout.

The cheek teeth of the mouse were selected not only on account of availability of material, but also for the following reasons:-

(1) The experimental animal has a monophyodont dentition.
(2) The molars are of limited growth - brachyodont.
(3) The ontogeny of the crown pattern has been firmly established (Gaunt, 1955, 1961).
(4) The small size of the tooth row allows for thin sections of manageable numbers.
(5) A relatively short time is taken for the teeth to pass through all the accepted developmental stages.
(6) The rate of enamel formation is fairly rapid, and this enables observations to be made at daily intervals.
(7) Added interest is presented by the presence of enamel-free areas.

(8) The presence of cusps, ridges and intervening clefts on the crown present features more in keeping with "typical" mammalian teeth than do the incisor teeth.

Pre-natal stages ranging in age from 13 to 21 days in utero were used. The time of fertilization was arbitrarily established the morning following mating. Twenty-four hours later was considered as the first day of embryonic development. Both sexes of post-natal stages ranging from 1 to 14 days were also used.

Heads were cut at the occipital condyle region, and fixed immediately. No dissection of the teeth was done, in order to minimize the time between decapitation and fixation. It is also known that the Golgi apparatus and mitochondria are sensitive indicators to cellular injury, thus pinching the tissue with the forceps is liable to make these organelles change their form and position (Ludford, 1945).

Decapitation was preferred to gassing, since the latter might alter the cytoplasmic configuration, by acting as a poisonous substance. The effect of anaesthetics on the Golgi apparatus was studied by various authors. Bancroft and Richter (1931), in their experiments on the physiological action of anaesthesia on tissues, suggested that narcotics such as morphine cause an aggregation of the
cell colloids. Horning (1934) studied the changes that the Golgi apparatus and mitochondria undergo during morphine poisoning.

Another important point is that care must be taken not to excite the animal up to the moment of decapitation, for it has been shown (Zaklarov, 1956; Alov Trud, 1955) that substances, such as adrenaline, might appear in the blood circulation which affects cell division. Utkin and Kosichenko (1955, 1956) in an investigation of the role of nervous regulation of cell division and the effect of the surroundings on mitoses, concluded that cell division is directly regulated by the nervous system, and that the phase disturbances can act as symptoms of neurodystrophic processes in the tissues. In this case also, both the Golgi apparatus and the mitochondria are the most sensitive indicators of such cellular physiological disturbances (Bourne, 1945).

B. General Remarks on Methods
I. Fixation

Rapid fixation of all tissue elements, which are of great importance in critical cytological studies, is difficult when calcified oral tissues are involved, because the penetration of the fixing fluid through the dense alveolar bone, enamel and dentine is a slow process.

The choice of a cytological fixative is dependent
upon the tissue itself and the purpose for which it is to be preserved. Special reagents prepare the tissue for the study of specified elements or reactions, and chemically prepare for the application of selective stains.

The amount of fixing solution should be at least 20 times the volume of the tissue. Time of fixation should be long (at least 24 hours) to ensure good penetration.

After five days from birth, the skulls were skinned and fixed. It was noticed that, if not skinned, the material floats and the skin acts as a leathery insulator preventing good penetration of the fixative to the tissues.

It has also been found that cutting the head into two halves, right and left, with a sharp razor blade, two to three hours before the time of fixation is finished, then leaving in the fixative for a few hours, had the following advantages:— (1) a flat surface is provided for easier embedding, (2) a larger area is exposed to the action of the subsequent reagents. Splitting the head before fixation spoilt the tissues. Sometimes this process was carried out during dehydration in stages which did not need decalcification.

Several fixatives were tried, including: (1) 10% formalin saline, which was recommended by Norberg (1929) and used by Gaunt (1955) and Rowles and Brain (1959). (2) Formalin chloral hydrate mixture — a
solution recommended by Powers (1952) (formaldehyde 40% 10 c.c., chloral hydrate 10 gms, distilled water 90 c.c.).
(3) Serra fixative (absolute alcohol 6 parts, formaldehyde 40%, 3 parts, glacial acetic acid, 1 part). (4) Helly's fluid (distilled water 100 c.c., mercuric chloride 5 gms, potassium dichromate 2.5 gms, sodium sulphate 1 gm - to each 100 c.c. of this solution add 5 c.c. of neutral formalin immediately before use). (5) Regaud's fluid (3% potassium dichromate, 80 c.c., neutral formalin, 20 c.c.). (6) Champy's fluid (7 c.c. of 3% potassium dichromate, 7 c.c. of 1% chromic acid, 4 c.c. of 2% osmic acid).
(7) Modified Champy (2 c.c. of 6% potassium dichromate, 2 c.c. of 1% chromic acid, 2 c.c. of 2% osmic acid).
(8) Aoyama's fluid (1 gm cadmium chloride, 15 c.c. neutral formalin, 85 c.c. distilled water). (9) A 2% osmic acid solution.

Although several methods of fixation were utilized, only the most successful ones will be mentioned in detail, later when each topic of the subject is dealt with separately.

Useful technical methods as well as considerable treatment of all topics can be found in Lee's "Microtomist's Vade-Mecum" (1950). Other references are: Pantin (1948), Gray (1954), Baker (1950), Darlington and La Cour (1960).

In general, fixatives should be prepared just before use, especially those containing osmic acid or formalin.
Following fixation, tissues were washed in running water to remove as much of the fixing fluid as possible.

II. Decalcification

Decalcification is most important in the preparation of teeth for microscopic examination. It is perhaps desirable to mention some disadvantages of teeth as a subject of cytology or histology. One of these is that sections of teeth with their surrounding bony tissues are difficult to obtain without removal of the calcium, so that decalcification of hard tissues is a necessity prior to microscopic study of the soft tissues, and this complicates the preparation of sections and their staining.

Decalcifying fluids may be organic or inorganic acids. Of the organic acids, trichloroacetic acid and trifluoroacetic acids are most commonly used, and of the inorganic acids, nitric acid is the most common decalcifying reagent used to decalcify oral tissues. They are used in aqueous or alcoholic solutions.

The choice of this fluid is very important, for, the effects of various chemical decalcifying fluids upon the tissue components differ. This is the greatest problem of the whole technique. The primary fixation picture can be changed by subsequent treatment, and sometimes only a generalized non-specific staining results
(this point will be discussed later), and sections must be carefully studied so that correct conclusions are drawn as to the effect of the decalcifying fluid upon the cellular elements. If staining in bulk is used, as in the Aoyama method of Golgi apparatus, one must find out the suitable time for decalcification whether it is carried out after fixation or after fixation and impregnation. This is very important and will be discussed later.

The length of the time a specimen remains in the decalcifying agent depends upon the choice and concentration of the acid and the size of the specimen.

A large quantity of decalcifying fluid should be used. Heating of the acid to hasten the process of decalcification as mentioned by Morse (1945) should be avoided, particularly in cytological studies, as it leads in most cases to some complications; for example, hot trichloroacetic acid extracts all the DNA, in addition to causing remarkable shrinkage of the tissue, and the dissolution of the fat content of the cells as well as the mitochondria.

Prolonged action to bring about complete decalcification very often results in distortion, destruction and maceration of the tissue. However, short periods of action by the decalcifying fluid upon the tissue do not result in these changes, and the shorter the time, the
better is the staining.

After fixation and washing, decalcification was carried out. Only stages starting from three days (after birth) required decalcifying. The following fluids were tried:

(1) Formic acid sodium citrate mixture, suggested by Evans and Krajian (1930) - equal parts of 20% Na citrate and 50% aqueous formic acid. Morse (1945) recommended a reagent made up of equal parts of 20% aqueous Na citrate and 45% aqueous formic acid. This method was also recommended by Powers (1952). She believes that Glickman and Wood (1942) were the first to report the use of this mixture upon oral calcified tissues, and she suggests that the citrate counteracts the swelling tendency of the formic acid. Kristenson (1948) preferred to use a sodium formate formic acid solution.

Another formic acid solution, which was also used, is that suggested by Brain (1949, 1951), consisting of 5% formic acid saturated with calcium phosphate.

All these formic acid mixtures gave nearly the same results.

(2) 5% trichloroacetic acid in 50% ethyl alcohol or 5% aqueous trichloroacetic acid; a solution used by Powers (1952). Schneider (1945) reported that trichloroacetic acid extracts DNA, especially when heated. This was confirmed in the present study, and, consequently, trichloroacetic acid was not used for decalcification of
tissues to be treated according to Feulgen's technique, for the study of the distribution of mitoses.

The method of Sognnaes (1949) which also implies the use of trichloroacetic acid, was tried. This consists of decalcifying in a solution of 100 c.c. of 5% trichloroacetic acid, 2.5 gms of potassium dichromate, 10 c.c. formalin, for three days, changing the fluid daily; further, the teeth were decalcified for another two days in a solution recommended by Brain (1951), i.e., 5% formic saturated with Ca phosphate. Butcher (1955) found that both enamel and dentine were better decalcified by this treatment than by either solution alone.

(3) Trifluoroacetic acid. A fluid which is said to give complete decalcification of teeth in five or six days without loss of cytological detail was reported in Peter Gray (1954) to be used by Rosback and Leavitt (1952). Its formula is: 95 c.c. water and 5 c.c. trifluoroacetic acid.

This solution was tried and gave satisfactory results. However, it has some drawbacks, especially with material fixed in Aoyama fluid to show the Golgi apparatus after impregnation with silver. Normally, silver impregnation is achieved just after fixation. With calcified teeth where calcification is involved, one has to find out whether to use the decalcifying fluid before or after the impregnation. In this case, two heads were fixed; one
was decalcified and then impregnated, the other was fixed, impregnated and then decalcified. The results obtained were as follows:-

Usually, in an Aoyama silver impregnation preparation, the position of the nucleus is empty, i.e., it does not take any stain, and the Golgi apparatus is stained dark brown.

In the first case, where decalcification preceded impregnation, it was noticed that the nuclei were stained dark brown and the cytoplasm was empty; no Golgi apparatus could be demonstrated.

In the second case, where decalcification followed impregnation, the preparations showed the normal reaction, i.e., the nuclei were colourless and the Golgi apparatus only was demonstrated in its usual form as dark brown bodies. This might be explained on the basis that the Golgi apparatus being composed of lipoids, the application of this decalcifying fluid containing acetic acid, which is a fat solvent, changed its chemical composition and thus impregnation failed to give the characteristic picture of the Golgi apparatus.

(4) Nitric acid mixtures were also tried: (a) To 100 c.c. of 5% formaldehyde aqueous solution, from 7.5 to 15 c.c. nitric acid was added (Carleton and Leach, 1947, page 211). The formaldehyde is said to inhibit any tendency towards maceration on the part of the nitric acid.
Decalcification of young animals is complete in 24 - 48 hours, provided a large amount of this fluid is used and changed once or twice a day.

The acid is removed by treatment with 5% aqueous solution of sodium sulphate, lethium sulphate or potash alum, for 12 - 24 hours. (b) Nitric acid and alcohol: 5% nitric acid in 90% alcohol (Meyer), 5% nitric acid in 70% alcohol (Gaunt), 3% nitric acid in 70% alcohol, (the writer adopted this solution mostly). (c) Nitric acid and alum: A saturated aqueous solution of alum is diluted with an equal volume of water and to each 100 c.c. of the dilute solution is added 5 c.c. of strong nitric acid. As cited in Vade-Mecum (1946, page 252), this is said to be perhaps a better decalcifier, for teeth, than the alcohol mixture.

I found that the nitric-alum solution has also a drawback, with the Aoyama fixed material. It was noted that if this decalcifying fluid was applied after impregnation with silver, the alum replaces the silver, resulting in a non-specific reaction. This is true, because, in silver preparations, when Heidenhain haematoxylin is used as a counterstain to show the mitochondria, side by side in the same cell, care must be taken not to leave the sections in the mordant (iron alum), which removes the Ag.

3% nitric acid in 70% alcohol, as a decalcifying
fluid was found to be particularly effective in retaining the normal relationship of the tissues and in giving reliable staining reactions of all cytoplasmic organelles and inclusions studied. So it was employed nearly throughout this investigation.

An electric pump was used for bubbling air to agitate the material and decalcifying fluid. The time required for decalcifying tissues varies with the age of the animal (from 1 to 5 days).

Because an X-ray test was not possible, testing chemically for the end point of decalcification was preferred to methods of needle testing and flexibility as they are not very accurate.

This took the following steps:
To 5 c.c. of the used decalcifying reagent, add 0.1 c.c. of a concentrated ammonium hydroxide, mix thoroughly, then add 0.1 c.c. of a saturated aqueous solution of ammonium oxalate. A precipitate will form when calcium is present. Repeat the additions of 0.1 c.c. amounts of ammonium oxalate at intervals of 15-20 minutes until a total of 0.4 c.c. has been added. If a precipitate fails to form after the addition of 0.4 c.c. of ammonium oxalate, the tissue has to be left in the same decalcifying reagent for about two days, and repeat the test. When the test remains negative proceed to dehydration and infiltration.

This chemical test was published by Arnim (1935),
and adopted by Morse (1945). When the test for the presence of calcium gave a negative result, the tissue was washed in running water for 24 hours. Usually, the tissue was transferred from the decalcifying fluid to alcohol (as in the case of nitric alcohol fluids). Both methods, washing and direct transfer to alcohol, seem to have no difference in their effect upon the subsequent cytological picture. However, washing for several hours eliminates from the tissue any reagent which tends to interfere with the subsequent staining.

III. Dehydration

Slow dehydration of the oral tissues is recommended (not more than three days) using changes of 30%, 50%, 70%, 90%, and 95% ethyl alcohol for 12 hours each; then through two changes of absolute ethyl alcohol, followed by clearing in xylol, benzene or terpeneol (using a mixture of absolute alcohol and the clearing agent before pure clearing agent). Zirkle (1930) and Lang (1937) considered n. butyl alcohol the most satisfactory of all dehydrating agents, for it permits slow and gradual dehydration without the serious hardening effects of ethyl alcohol. In addition, butyl alcohol is also a clearing agent which eliminates the necessity of clearing tissues in other clearing agents, which all have a tendency to further hardening effects.

The Schedule in Vade-Mecum (1946, page 632), using butyl alcohol, was followed. Satisfactory results
were obtained. It was found that slight traces of butyl alcohol in the paraffin blocks did not render them crumbly, as does xylol or benzene.

IV. Embedding

The recognized method of paraffin embedding was followed (Gatenby and Beams, 1950; Baker, 1950), in a vacuum-oven. Vacuum-embedding has the advantage of reducing the time of embedding and any air present in the tissue is extracted. Wax of M.P. 56, 58 or 60° C was used according to the time of year. The addition of 1% ceresin to the wax prevented crystallization of the wax and facilitated section cutting. Infiltration with wax took about 8 hours (in 3 change wax). Longer periods of time of embedding lead to the hardening of the tissues.

Also, the Peterfi rapid double embedding (paraffin-wax-celloidin) method (Pantin, 1948, page 29) was tried.

It was noticed that in spite of carefully sharpening the microtome knife each time a block was cut, and checking that all the screws were tightened to prevent any vibration of the knife, carrier, or block, during sectioning, difficulty was sometimes found in obtaining a good series. This was of common occurrence in material impregnated with silver, and as is known, metallic silver is deposited which made it hard to cut, even after descalcification. In such cases, all possible remedies, such
as changing the knife angle, critical embedding, resharpening the knife, etc., were tried with no success. Soaking the cut end of the block in water (David, 1935; Grains, 1944) allowed for only some good sections. The same applies to soaking the cut surface of the block in a solution as suggested by Baker (1941) (water 36 c.c., 95% alcohol 54 c.c., glycerine 10 c.c.) (Gray, 1954, page 667). All these trials were only temporary and resulted in an incomplete series. Finally, it was concluded that if no success was met with from the beginning, the block should be discarded, especially when a series is required.

Tropical Ester Wax 1960 (Steadman, 1960, page 48) has been used for embedding calcified teeth up to 14 days (after birth). Ester wax blocks were cut using a heavy sliding microtome with a special knife, following the sellotape method.

The aim of using the ester wax for embedding calcified tissues was mainly to compare sections of calcified with sections of decalcified material, to see if the decalcifying fluid had any effect on either the mitochondria or the Golgi apparatus.

This sellotape method was abandoned because in its procedure one has to remove the sellotape by using ether and chloroform, which are very destructive to these organoids, in addition to the fact that it removed the
silver from the tissues which had been impregnated in bulk.

So, normal cutting with the ordinary knife and
the rotary Beck microtome, as well as the sliding micro-
tome, was tried to avoid the use of these fat solvents,
and I was lucky, even more than with the decalcified
tissues, in obtaining serial sections with no, or very
little, distortion.

This leads to the conclusion that this wax,
being hard, holds the tissues more firmly in a normal
position and, therefore, it is recommended to ordinary
paraffin wax. Serial transverse sections were cut at
5 \( \mu \) thick throughout this investigation. It should be
noticed that most of the work done on the histology of
teeth was on material cut at 8 - 10 \( \mu \) wax sections or
30 - 100\%/celloidin sections.

Sections were sometimes coated with 0.5\% or 1\%
celloidin before staining and also before mordanting to
prevent loss of sections and precipitation.
III - DISTRIBUTION OF MITOSES

A. METHODS FOR THE STUDY OF THE DISTRIBUTION OF MITOSES

1. Method used for demonstrating the mitotic figures

The material consisted of pre-natal stages ranging in age from 13 to 21 days in utero and post-natal stages ranging in age from 1 to 5 days after birth.

The material used was fixed in each case for 24 hours in Regaud's fluid (20 c.c. neutral formalin, 80 c.c. of 3% $K_2Cr_2O_7$).

Only embryos of 3 to 5 days old needed decalcification. The decalcifying fluid used was 3% nitric acid in 70% alcohol. Trifluoroacetic acid was also used, as well as trichloroacetic acid. The former gave good results showing clear mitotic figures. While the latter extracted DNA from the nuclei and thus the Feulgen reaction was negative. This has been shown by Schneider (1945).

The last two fluids were used only for comparison.

Serial transverse sections were cut at 5 μ. It should be noticed that thick sections often lead to overlapping of nuclei, obliterating mitotic figures.

The "Feulgen Method" (Darlington and La Cour, 1960; Danielli, 1958; Gatenby and Beams, 1950).

The Feulgen method which is specific for the localization of DNA on chromosomes, was used throughout
This investigation.

This took the following steps:

1. Bring sections down to water.
2. Rinse in N/HCl at room temperature.
3. Hydrolyse in N/HCl at 60°C for 15 minutes (after formalin and dichromate fixation, for the time of this bath depends on the fixative used).
4. Stain in leucobasic fuchsin for two hours.
5. Transfer to fresh sulphurous water (SO₂) in stoppered jar, 3 changes, 10 minutes each.
   (Rinsing in running tap water 2-3 minutes increases the intensity of the stain).
6. Rinse in distilled water.
7. Dehydrate in the alcohol series.
8. If a counterstain is desired, after dehydrating up to 95% alcohol, stain 30 to 60 seconds in a highly dilute solution of light green, followed by three changes of absolute alcohol.
9. Clear in xylol and mount in balsam.

The reagents used in this method were as follows:

**The hydrolyzing solution:**

This is merely a normal solution of hydrochloric acid, which is prepared as follows:

Hydrochloric acid (sp. gr. 1.19) .............. 82.5 c.c.
Distilled water ........................................ 1000 c.c.

This dilute solution is placed in a staining jar
and brought to a temperature of 60°C, a temperature which should be maintained constant throughout hydrolysis. A water bath is used for this purpose.

The Staining solution:

Leuco-basic fuchsin is prepared according to a modified formula after De Tomasi (1936) and Coleman (1938) as follows:-

Dissolve one gram basic fuchsin by pouring over it 200 c.c. boiling distilled water. Shake well, and cool to 50°C. Filter and add 30 c.c. N/HCl to filtrate. Add 3 gms of potassium metabisulphite. Allow the solution to bleach for 24 hours in the dark. Add 0.5 gm of de-colourizing carbon (vegetable carbon). Shake well for about a minute, and filter rapidly through coarse filter paper. Store in a tightly stoppered bottle in the dark.

The Washing solution (sulphurous water):

This is prepared as follows:-
10 c.c. N/HCl; 10 c.c. of 10% solution of an hydrous sodium metabisulphite; and 200 c.c. of distilled water.

It is advisable to avoid HCl acid in decalcification because it enters in the procedure of Feulgen's method, for hydrolysis. Also trichloroacetic acid, as mentioned before, because it extracts DNA (Schneider, 1945).

The reagents used in all the developmental stages were highly chemically pure. The same brands were always
used. This is especially important to ensure qualitative and quantitative reproducibility of the Feulgen reaction.

The Feulgen reaction itself was carried out according to a rigidly kept time schedule; this includes exposure to the Schiff's reagent (leuco-basic fuchsin) and to the bleaching solution (SO₂ water).

2. **Method of Wax model Reconstructions**

The well known wax-plate method devised by Born (1888) was adopted in preparing three-dimensional wax-model reconstructions. Reconstructions of the crown patterns of the developing teeth have been used by many workers since Born described his technique. Rose (1891), Peter (1899), Sicher (1917) and Green (1938) adopted this method. Glasstone (1938) used this method in investigating the early crown features in the rat and rabbit. Gaunt (1955, 1956) used this method in the investigation of the crown pattern of the mouse.

With the aid of a standard microprojector, each section in the series of a tooth germ was projected on paper at constant magnification (X 120). The outline of the dentine enamel junction was traced. The sections were then examined under the microscope, and the positions of the dividing cells in the inner enamel epithelial layer were plotted on the microprojector tracings, each dot corresponding to a dividing cell. These tracings with the dots were then transferred to the wax plates, which were
then finally stuck together with a hot needle, and the three-dimensional wax model reconstructed.

Dental toughened wax which contains plastic "K₂" for improved toughness was used in the reconstruction of the models.

Models of right and left teeth of the same head were made, to see if both give the same distribution pattern. In this work anything recognizable as a mitosis was considered, i.e., all stages of mitosis from prophase to telophase were included in the plotting, even when it could be seen that only one part of the nucleus (one daughter cell) was included in the section under observation. To avoid including the same mitosis twice, mitotic figures were plotted in alternate serial sections.

Slides containing the third molar of a bat (Hipposideros beatus) and slides containing the third milk molar of the cat were borrowed from Mr. Gaunt, and wax models showing the distribution of mitoses in the bud and cap stages were made. These models represent teeth which do not have enamel-free areas and were used for comparison with the mouse teeth which show this feature. This was done to test whether or not the pattern of distribution was due to the presence of the enamel-free areas on the crown of the mouse teeth.

One of the difficulties is that the models distorted easily, unless stored in a cool place maintained
at a more or less constant temperature.

This was overcome by coating the models with a resin (Vinalak 5909).

The Feulgen method, mainly used for plotting the mitoses, was also used for demonstrating the localization of polysaccharides.

B. OBSERVATIONS

Certain features of a more general nature have been observed with regard to the nucleus and the morphology of the cells during the active mitotic phase and the interphase. The visible changes have been observed in the different layers, but were most obvious in the inner enamel epithelium. The interphase nucleus enlarges before entering the mitotic phase. This can be clearly seen in the early prophase, and when the nuclear membrane disappears at the end of this phase, the cell loses its characteristic shape and becomes rounded. The distribution of mitoses will now be described and it must be emphasized that the dots representing the mitoses on the wax models refer only to those mitoses in the inner enamel epithelium.

I. Dental lamina and Bud Stage

The epithelial thickening, produced by the invagination of the germinal layer of the oral epithelium along the axis of the jaw is the first indication of the
dental lamina from which arise the tooth buds.

Thus the tooth primordia develop from groups of cells which originate from the basal layer of the oral epithelium at regular intervals along the lamina. Simultaneously, mesenchyme cells are aggregated around the dental lamina. Further development proceeds by invagination of the oral epithelium into the mesenchyme. This results in a widening of the lamina and thus the future tooth germ resembles a flask with the neck towards the oral epithelium.

This is the first stage of tooth development, the bud stage, and is seen at the thirteenth to fourteenth day in utero for the first molars, and the fifteenth to sixteenth day for the second molars. This means that at a particular age, the development of the first molars are at a more advanced stage than the second molars. For example, at 15 to 16 days in utero the first molars will be in the cap stage, while the second molars will be in the bud stage.

The bud consists of a peripheral layer of radially arranged cells, which are continuous with the Malpighian layer of the oral epithelium, surrounding a core of haphazardly arranged cells.

The dividing cells in this bud are as seen in Fig. (1), confined to the peripheral layer in the "neck" region of the bud, together with some mitoses in the cells of the central core. But the cells of the peripheral layer at the deepest part of the bud reveal no mitoses at
this stage, though mitoses occur in the layer immediately above them. This distribution is reflected in the wax reconstructions shown in Figs. (2 and 3).

The peripheral cells of the bud are of low columnar form, while the central cells of the bud are rounded. Both possess large nuclei nearly filling the cells, i.e., the amount of cytoplasm is very small.

Condensation of the mesenchyme cells around the flask-like bud can be seen in Fig. (1).

Mitotic figures are observed in the mesenchyme cells adjacent to the peripheral layer of the bud.

II. Early Cap stage

From the bud stage transition accors to the early cap stage by further proliferation, and there is a tendency to an asymmetrical development such that the lingual cervical region of the cap dips more deeply in the mesenchyme than its buccal counterpart. As development proceeds, the deepest portion of the bud becomes concave, and as a result, the crown has a dominating ridge. This is the cap stage and is seen at the fifteenth to sixteenth day in utero for the first molars and the seventeenth to eighteenth day in utero for the second molars.

According to the distribution of mitoses, the lingual aspect of the enamel organ from the neck to the cervical loop seems to be growing more rapidly than the
buccal side.

With progressive differentiation, the enamel organ becomes formed of three layers, namely: the outer enamel epithelium, the stellate reticulum, and the inner enamel epithelium. The stratum intermedium layer at this stage is indistinguishable from the stellate reticulum. The region where the outer and inner enamel epithelia meet will be called the cervical loop (proliferative zone), and it is proposed to describe them in turn.

(1) **The Outer enamel epithelium:**

There is no visible morphological difference between the outer enamel epithelium and those in the bud stage. The frequency of mitoses on the lingual side exceeds that on the buccal side. While mitoses occur sporadically in the outer enamel epithelium they are always to be observed in this layer in the vicinity of the cervical loop, though those on the lingual side dominate (Fig. 4a).

(2) **The Stellate Reticulum:**

It is found that active cell division occurs in this layer from the cap stage right through to the completion of the crown (up to five days after birth). The central cells, or the cells of the stellate reticulum, possess large nuclei, many of them show mitotic figures. Those adjacent to the inner enamel epithelium are crowded, and more differentiated with large nuclei. These will
eventually form the stratum intermedium layer, whose destiny is closely bound to the pre-ameloblasts. They also show mitotic figures. The "heaping up" of these cells above the inner enamel epithelium resembles the so-called "enamel knot", and it should be emphasized that this crowding of cells is not in the form of concentric layers.

(3) The Inner Enamel Epithelium:

The cells of the inner enamel epithelium, lining the concave base, are slightly taller than those of the outer enamel epithelium and more differentiated, with large centrally located nuclei occupying most of the cell. These are called the pre-ameloblasts and they will differentiate into ameloblasts.

It is of interest that in the early cap stage, no mitoses occur in the inner enamel epithelium except at the cervical loop (Figs. 4a and b).

On examining the wax model reconstructions of the early cap stage (Figs. 5a and b, 6, 7, 8), one can see a mitoses-free area which represents the inner enamel epithelium of the successive sections, surrounded by mitotic figures at the periphery, so that the part occupied by the dots (mitotic figures) correspond to the cervical loop. This is the first mitoses-free area on the tooth germ.

(4) Dental Papilla and Follicle:
Condensation of the mesenchyme cells continues to form the dental papilla and dental follicle. The latter is the marginal condensation in the mesenchyme surrounding the enamel organ and dental papilla.

The pulp primordium and the tooth follicle show only randomly distributed mitotic figures; moreover, they are much fewer than those of the enamel organ at this stage. Blood capillaries are widely spread in the pulp tissues. The cells adjacent to the inner enamel epithelium enlarge and later differentiate into odontoblasts.

There is a visible similarity between upper and lower molars at this stage, although the latter are smaller than the former. Also, the second molars show a striking resemblance to the first in development.

Continuation of histodifferentiation leads to the stage commonly known as:

III. The Late Cap Stage

The enamel organ grows towards its predestined morphology by further proliferation of the cells of the cervical loop into the mesenchyme, so that the late cap stage is reached by the seventeenth to eighteenth day in utero for the first molars and the twentieth to twenty-first day in utero for the second molars.

The following changes seen in the late cap stage are preparatory to those in the subsequent bell stage.
(1) **The Outer Enamel Epithelium:**

Near the cervical loop, this layer consists of a single row of short cuboidal cells which continue to produce additional cells by mitosis. The rest of the outer enamel epithelium from the neck region to the base of the tooth germ shows gradations from columnar to cuboidal cells. These also show mitotic figures. However, they are less frequent than the previous stage. In some sections, the cells of the outer enamel epithelium from the neck region to the base of the tooth are columnar to cuboidal to flat on one side, while on the other side they are all cuboidal, showing no such gradation in shape.

(2) **The Stellate Reticulum:**

It was observed that the cells of the enamel pulp in the neck region and towards the periphery, i.e., towards the buccal and lingual sides, become more spaced, and show less mitoses. There are, however, certain zones in the interior of the enamel organ in which this spacing occurs very late or not at all. The cells of this interior zone possess large rounded nuclei and show many mitotic figures. The stellate reticulum cells overlying the cusp tip are still somewhat crowded.

(3) **The Stratum Intermedium:**

This layer consists of 1 to 3 cells thick, those in immediate contact with the inner enamel epithelium remain crowded and possess large nuclei, some of which are
actively dividing. The "heaping up" of these cells above the inner enamel epithelium, which resembles the so-called "enamel knot", still persists from the previous stage. However, an enamel knot, in the form of concentric layers, does not exist.

(4) The Inner Enamel Epithelium:

This consists of taller cells of more than one layer, each layer being difficult to distinguish from the others. Cells on the future lingual cusp are more columnar, i.e., more differentiated. Mitotic figures have been observed in this layer in certain regions. This can be explained as follows:

When the sections are examined serially along the whole tooth germ at this stage, mitoses are absent from the inner enamel epithelium over the greater part of its central portion. Further back, however, mitoses are to be seen along the whole length of the inner enamel epithelium, and further back again, they become absent, save in the immediate vicinity of the cervical loop. Hence the wax models of the teeth at this stage, viewed in an occlusal direction (Figs. 9, 10, 11) show the anterior and posterior parts of the crown devoid of mitoses, with a central transverse zone of mitoses between them. In other words, when these figures are compared with those of the previous stage (early cap stage, Figs. 5, 6, 7, 8), one can observe that a second mitoses-free area appeared
separated from the first by a band of intense mitoses across the tooth. As will be shown later, the anterior and posterior mitoses-free regions are equivalent to the cusps and associated enamel-free areas, while the bands of mitoses correspond to the main transverse valleys.

On the first molars a third mitoses-free area is found. This is because the anterior central cusp (cusp 1) on the first upper molar is missing on the second upper molar, and the anterior paired cusps (L 1, B 1) on the first lower molar are missing on the second lower molar. This might explain why the first molars have more mitoses-free areas than the second molars. I adopted the cusp terminology in Gaunt’s paper (1955), see Figs. (36 a, b).

The future enamel-free areas, especially that associated with cusp 2 on the first upper molar (middle mitoses-free area, Fig. 11a) can be detected at this stage by the visible difference in the cell morphology of the inner enamel epithelium in this region and by the frequency of mitoses.

Sections in this region show the cells of the inner enamel epithelium to be very low columnar, short cuboidal, or flat. No mitotic figures are to be seen in the inner enamel epithelium in this region.

As to the other layers of the enamel organ in the enamel-free area, the stratum intermedium cells are flat and the stellate reticulum cells are small and poorly
developed. Very few mitotic figures are found in these two layers in this region.

From figs. (2a, b; 5a, b; 6; 10; 11 a, b) one can observe the close similarity of the distribution pattern exhibited by right and left teeth of the same embryo. However, it has been found that the embryos in the same uterus differ in the degree of embryonic development.

(5) The Pre-odontoblast layer:

The mesenchyme cells destined to form the odontoblasts possess large nuclei more or less arranged in a layer facing the inner enamel epithelium. Mitotic figures are observed among these cells. They are more frequent than in the previous stage. It has been observed that the dividing cells in the different layers, i.e., in the inner enamel epithelium, in the stratum intermedium and in the pre-odontoblast layer (Fig. 12 a, b, c) show intense Feulgen-positive granules on the faintly Feulgen-positive threads of the chromonemata.

(6) Tooth Follicle:

Many cells of the surrounding tooth follicle show mitotic figures (Fig. 13). Blood capillaries are numerous in the tooth follicle, but not within the enamel organ.

By the seventeenth to the eighteenth day stage, the crown topography of the first molars, and to a lesser degree, the second molars, is more clearly defined.
The distribution of mitoses in the cap stage was checked statistically by applying the chi-square and the Poisson tests. This was undertaken to prove that the observed distribution pattern is not a matter of chance or a random distribution. The statistical evidence which proved that this distribution is non-random will be presented at the end of this chapter.

IV. The Bell Stage; Early and late:

As proliferation continues, the cap deepens, and its margins (i.e., the cervical loop on each side) continue to grow by showing mitotic divisions. Finally, the enamel organ assumes the bell stage. This stage is reached by the twentieth to twenty-first day in utero for the first molars, and one day after birth for the second molars. At this age the first molars represent the late bell stage, while the second molars represent the early bell stage. These are the significant observations at the bell stage:

1. The Outer Enamel Epithelium:

The outer enamel epithelium which formed a continuous layer in the previous stages, is now incomplete due to the invasion of blood capillaries from the highly vascular follicle, into the enamel pulp organ to provide nutritional supply for the following activity of the inner enamel epithelium. These cells show a gradation from low columnar near the oral epithelium of the enamel organ to cuboidal as we go towards the cervical loop. The oral
mucosa is continuous over the neck of the enamel organ. The outer enamel epithelium shows many mitotic figures, but not as many as in the cervical loop region. The cervical loop, which is the region where the outer and inner enamel epithelial layers are continuous and reflected into one another, is the zone of transition between the cuboidal cells of the outer enamel epithelium and the columnar of the inner enamel epithelium. It is an active part and shows numerous mitotic figures during all the stages of development.

(2) The Stellate Reticulum:

The cells of this layer assume the same picture as is presented in the late cap stage: some of them have the appearance of star-shaped cells, with large nuclei which are active and show mitotic figures.

(3) The Stratum Intermedium:

This layer lies between the stellate reticulum and the inner enamel epithelium. It is composed of several layers (1 to 3) of flat to cuboidal cells. Mitotic figures are frequent in this layer.

(4) The Inner Enamel Epithelium:

This consists of a layer of cells which are derived from the peripheral cell layer of the oral epithelium, the nuclei of which are arranged at different levels. Above the future cusps, the cells are taller and the nuclei are away from the basement membrane. These will
differentiate prior to amelogenesis into tall columnar ameloblasts. At this stage, the inner enamel epithelium (or the pre-ameloblasts) shows the general distribution pattern described in the late cap stage, with the bands of intense mitoses separating areas free of mitoses, more clearly seen (Figs. 14, 15b, 16b, 17b, 18b). By this stage, the tooth germ has undergone additional growth, especially in the cervical region.

Although the dentine enamel junction, when the tooth is more advanced, exhibits cusp outline, the crown of the tooth germ at the early bell stage is flat with no cusp elevations. This flatness is evident in wax model reconstructions seen in a lateral view of the second molars, either buccal or lingual (Figs. 15c, d; 16c, d). However, the presence of high and low parts on the crown, i.e., cusp elevations, is apparent in the late bell stage (Fig. 18c, d).

The dots on the buccal and lingual views of the models presented in Figs. (16, 17, 18) show mitotic figures in the inner enamel epithelium on the sides of the enamel organ to the cervical loop (i.e., base of the tooth germ). Figs. (15a, 16a, 17a, 18a) are all basal views of the tooth germ. The dots on these figures represent the position of mitotic figures in the inner enamel epithelium where the outer enamel epithelium is turned into the inner enamel epithelium. In other words, they represent the
same parts seen in the previous figures (Figs. 15c, d; 16c, d; 18c, d).

(5) **Pre-odontoblasts:**

The peripheral mesenchymal cells of the dental pulp, i.e., those cells adjacent to the inner enamel epithelium, undergo gradual histodifferentiation into odontoblasts. They assume a columnar form, and prepare to produce dentine. This differentiation starts first at the cusp tips and spreads to the cervical loop. In other words, the pre-odontoblasts are not all of the same developmental stage, and consequently they are not all columnar. Therefore, the odontoblasts at the cusp tips have stopped mitoses while the cells of this layer, particularly those adjacent to the inner enamel epithelium in the cervical loop and the deep lying cells of the papilla, show many mitotic figures.

The basement membrane, or, the membrana praeformativa, separating the inner enamel epithelium from the pre-odontoblasts at the time just preceding dentine formation, might be called now the dentine enamel membrane.

(6) **Tooth Follicle:**

Its cells show a circular arrangement and resemble a capsular structure. Mitotic figures are still observed in this layer. Bony encapsulation surrounds the tooth germ except at the occlusal portion.
V. One day after birth:

The general appearance of the tooth germ of the first molars and, to a lesser degree, that of the second molars, resembles the finished crown of the tooth.

(1) Outer Enamel Epithelium:

The vascular invasion of the follicular blood vessels through the outer enamel epithelium has increased. From the neck region to the cervical loop the cells show few randomly distributed mitotic figures.

(2) Stellate Reticulum:

Although the intercellular spaces are wider, and the cells have become now star shaped many mitotic figures are still observed in this layer (Fig. 19).

(3) Stratum Intermedium:

They are more differentiated cuboidal cells, showing mitotic figures. The stratum intermedium cells overlying the inner enamel epithelium in the enamel-free area of cusp 2 and those over the future enamel-free area of cusp 1 and cusp 3 remain flat and do not show any mitoses.

(4) Inner Enamel Epithelium:

The observation is now confined to the first molars, since the second molars were included in the previous stage. This is because, as is known, the first molars start development earlier than the second.

As cusp formation advances, the inner enamel
epithelium (pre-ameloblasts) continue to divide in the regions shown by the wax models (Figs. 20, 21). The main growth occurs in the cervical loop region, where the cells are actively dividing, giving rise to new cells which are added to the outer enamel epithelium, stratum intermedium as well as the inner enamel epithelium. This region can be considered as a "growth centre". Other growth centres are in the bands of intense mitoses across the tooth, which can be followed in later stages to become the main transverse valleys.

In the case of the upper molar, with the growth at the anterior region of the crown, cusp 1 is developed, while growth at the back end of the crown leads to the establishment of cusp 3 (Fig. 20). Cusp 2 is the first to appear and can be easily distinguished at 20 to 21 days in utero (Fig. 17), i.e., the triserial division of the crown is established. In the case of the lower molar, deepening of the valleys leads to the establishment of the paired cusps L1 B1 and L3 B3 (Fig. 21). Cusps L2 B2 are the first to arise as can be seen in Fig. (18).

It is observed that the frequency of mitoses in the inner enamel epithelium of the first molars at this age (Figs. 20b, 21b) is greater than the previous stage (Figs. 17b, 18b), and the succeeding stage (Figs. 23b, 24b).

In short, the general distribution pattern over the crown becomes more complex, and the valleys separating
the cusps and ridges become deeper.

The inner enamel epithelium over the tips of the cusps show an increase in height with ovoid nuclei, lying more distally, i.e., furthest from the basement membrane. From this stage onward, (2 – 3 – 5 days) they show gradual increase in height with more distally placed nuclei.

However, the cells overlying the enamel-free areas do not show this feature. They remain cuboidal (low columnar). In other words, they are never of the same height as the rest of the inner enamel epithelium.

5) Odontoblasts:

Differentiation of the odontoblasts has begun. The cells become elongated. The membrana praeformativa has increased in thickness and a thin layer of dentine is seen over the tips of the cusps where the odontoblasts are fully differentiated. In this region the membrana praeformativa becomes the dentine-enamel membrane, which is the first to calcify and is then termed the dentine enamel junction. No mitoses were found in the odontoblasts upon which dentine matrix has been formed.

Vascularity of the pulp has also increased.

6) Tooth Follicle:

The follicular cells show few mitotic figures.

The follicular wall and bony encapsulation has enveloped the tooth germ up to the neck of the enamel organ.
It has been observed that starting from 17 to 18 days in utero, the position of the root bifurcation had commenced to form, and the position of the roots roughly indicated before dentine had commenced to be laid down in the tooth germ of both upper and lower first molars.

This can be clearly seen on the twentieth to twentyfirst day in utero (Figs. 17a, 18a), and even more clearly one day after birth (Figs. 20a, 21a).

As to the roots on the second molars, they can be recognized one day after birth (Figs. 15a, 16a) and they become more apparent two days after birth (Fig. 22).

It should be remembered that the upper molars have three roots and the lower have two.

Sections and wax model reconstructions show that embryos of the same litter killed at the same time show some variation in the degree of development.

VI. Two days after Birth:

(1) Outer Enamel Epithelium:

Two days, three days, and five days after birth, this layer is almost the same, composed of flat cells interrupted by the invasion of blood vessels.

The frequency of mitoses among these cells is diminished and it becomes even less in the subsequent stages examined.

(2) Stellate Reticulum:

It is a region now rich in blood vessels, composed
of star-shaped cells, some of which possess large nuclei. Mitotic figures are still frequent, and in some sections one can find up to three or four cells at different stages of mitosis in the stellate reticulum.

(3) **Stratum Intermedium:**

This layer varies in thickness from one to three layers. Some of the cells are flat, others are large cuboidal. Over the tips of the cusps where the ameloblasts have ceased to divide, the stratum intermedium cells still go on dividing.

(4) **Inner Enamel Epithelium:**

(A) **The second molars**:

They show a higher frequency of mitoses, i.e., the number of dividing cells in this layer is very much increased compared with the previous stage. This stage might be considered equivalent to the first molars one day after birth, which also shows an increase in the number of mitotic figures.

**Upper second molar** (M2):

The tips of the cusps and the enamel-free areas are the only parts devoid of mitoses. Owing to this high frequency, the valleys become deeper.

**Lower second molar** (M2):

Again the tips of the paired cusps L2 B2, L3 B3 are the only parts lacking mitotic figures (Fig. 22b). The anterior transverse valley separating L2 B2 from L3 B3
is deeper and covers a larger area of the crown.

The posterior transverse valley separating \( L_3 \) \( B_3 \) from the future median cusp 4 (unpaired) is not as deep as the anterior one.

By comparing Fig. (16b) with Fig. (22b), one can see that the latter figure shows a higher mitotic frequency.

In both upper and lower second molars, the cervical loop region shows a high frequency of mitotic figures.

(B) The first molars:

The high frequency of mitoses seen after one day of birth in the first molars is followed by a decrease in the number of mitoses on the sloped of the cusps, accompanied by a further increase in the number of mitoses in the valleys. The valleys correspond to the bands of intense mitoses in the earlier stages, as revealed by comparison of wax models.

Upper first molar (\( M^1 \)):

The ameloblasts over the tip of cusp 2 have now reached their full maturity. They do not divide any more, and the nuclei are all in the same level. The cells start their functional activity of enamel matrix production.

As to the general distribution pattern, the tips of the central cusps 1, 2, 3 and the associated enamel-free areas are completely devoid of mitoses (Fig. 23b). Also
cusps L₂ and L₃ (Figs. 23c and e) and B₂ and B₃ (Figs. 23d and f).

Although cusp 3 is the last central one to appear, yet the cusp and its slopes together with its lingual and buccal cusps L₂ and B₂ are devoid of mitoses. While cusp 2, which is the first to develop, shows some mitotic figures on the lower parts of its slopes, so do its lingual and buccal cusps L₂ and B₂ which show mitoses on the lower parts of the slopes. Cusp 1 which is the second to develop shows an enormous number of mitotic figures on its slopes. This might account for the fact that it is of the same height as cusp 2 at this stage. The same with its lingual and buccal cusps.

Thus, as the figures (23b, c, d, e, f) show, mitoses is still frequent in the valleys and along the cusp slopes, where dentine and enamel has not yet been laid down.

In other words, whilst dentine and enamel matrix were being laid down over the highest part of the cusps, differentiation of the basal cellular part of the cusps to the cervical loop continued.

Mitotic activity of the inner enamel epithelium in the cervical loop region can be observed in Fig. (23a). Also the position of the three roots are marked.

Lower first molar (M₁):

The main paired cusps L₁ B₁, L₂ B₂, L₃ B₃ as well
as the slopes are devoid of mitoses. However, the three valleys and the slopes of median cusp 4 which is the last to develop, show numerous mitotic figures (Figs. 24b, c, d, e, f). In the lower parts of the crown, especially those lying in the active cervical loop region, mitoses are frequent (Fig. 24a).

Although the first lower molar develop a short time after the first upper, yet it seems that it grows faster and this might explain why the occlusal view of the first lower molar (M₁) (Fig. 24b) have fewer mitotic figures than that on the first upper molar (M₁) (Fig. 23b).

VII. Three days After Birth:

(1) Outer Enamel Epithelium:

It is a discontinuous layer of flat cells. Mitotic figures are rare among these cells.

(2) Stellate Reticulum:

In this layer, mitotic figures are still very frequent, sometimes reaching 4 or 5 cells in different stages of division (Fig. 25a).

The cells are of different size and shape, mainly star-shaped. The nucleus is large, surrounded by a narrow cytoplasm drawn into processes, joining the cells together, thus giving a network appearance.

(3) Stratum Intermedium:

It is a layer varying in thickness from one to three cells, which are either flat or cuboidal. In most
cases the large cuboidal stratum intermedium cells lie over the inner enamel epithelium which has not commenced enamel matrix production, i.e., in the valleys and the lower parts of the crown, and they show many mitotic figures. While over the cusp tips they tend to be flat with few mitoses among them (Figs. 25a, b, and 26a, b).

(4) Inner Enamel Epithelium:

The cells lying over the cusps are fully differentiated and are now called ameloblasts since they are in their secretory function of enamel matrix production. In these regions, where a considerable amount of enamel matrix is secreted, the ameloblast cells are usually closely applied to one another, with hardly any intercellular spaces. The intercellular membrane cannot be observed easily; however, the cell border run a fairly straight course. The formative end of the ameloblasts have a clear cytoplasm which shows a vacuolated structure (Figs. 25a and b). The nuclei are oval and all arranged in the same level. Over the enamel-free areas of the crown (central cusps of upper molars and paired cusps of lower molars) the ameloblasts are not of the same height as the other cells, with oval centrally placed nuclei, and no clear vacuolar structure is seen at their formative ends.

On the other hand, the inner enamel epithelium lying in the valleys and the lower parts of the crown are
not yet fully differentiated and might be called pre-ameloblasts since they have not started their secretory function. In these regions, the nuclei are not in the same level (Figs. 26a, b). The membrana praeformativa is observed to be paralleled by another thin limiting membrane which separated the pre-odontoblasts from the epithelial cells (Figs. 26a, b). Together, these two membranes formed a double membrane enclosing a thin even space.

In some instances, this double limiting membrane is not demonstrable and the inner enamel epithelial cells are only separated from the underlying pre-odontoblasts by the membrana praeformativa.

The distribution of mitoses in the inner enamel epithelial layer is as follows:-

(A) Second Molars:

They still show a higher frequency of mitoses as can be seen by comparing this three days old stage (Figs. 27, 28) with the previous two days old stage (Fig. 22).

Upper second molar (M2):

No mitoses are found over the tips of the cusps and in the enamel-free areas, but the number of dividing cells in the valleys and along the slopes of the cusps is very much increased (Figs. 27b, c, d). As a result, the valleys are getting deeper and the cusps elevated. The cervical loop region is still an area of high mitotic
activity (Fig. 27a). This figure also shows the position of the roots.

**Lower second Molar (M₂):**

The anterior transverse band of mitoses separating L₂ B₂ from L₃ B₃ is getting denser and, consequently, the valley is deeper. In the same way, the posterior band separating the paired cusps L₃ B₃ from the median cusp 4 is loaded with mitotic figures.

The tips of the cusps L₂ B₂, L₃ B₃ and 4 are not covered by any mitotic figures (Fig. 28b), also the number of mitoses along the slopes is less. However, the lingual cusp slopes show less mitotic figures than the buccal ones (Figs. 28b, c, d).

The cervical loop region is still actively dividing as can be observed from the distribution of mitoses in this part of the crown (Fig. 28a), which also shows the position of the roots.

(B) **First Molars:**

There is generally a decrease in the mitotic distribution over the crown (Figs. 29, 30) as compared with the previous stage (Figs. 23, 24).

**Upper First Molar (M¹):**

There are fewer mitotic figures in the valleys, and along the lower parts of the cusp slopes (Figs. 29b - g). As mentioned before, although cusp 2 appears before cusp 3, the anterior most part of the former still shows quite a
number of mitotic figures while the latter is devoid of them.

The cervical loop region till this stage is active as can be observed from the distribution of mitoses (Fig. 29a).

**Lower First Molar (M̄):**

Mitoses are confined here to the lower parts of the crown (Figs. 30c, d, e, f). The occlusal view (Fig. 30b) shows that the cusps as well as the valleys are devoid of mitoses as compared with the previous stage (Fig. 24b). However, the cervical loop region is the only part showing numerous mitotic figures (Fig. 30a).

As previously mentioned, comparison of the occlusal view of M̄ (Fig. 29b) with that of M̄ (Fig. 30b), one can observe that in the former, mitotic figures are still present in the valleys, while in the latter mitosis has almost completely ceased, persisting only in the low parts of the crown.

(5) **Odontoblasts:**

Where a thick layer of dentine is produced, the cells are very tall, while in places where there is only a thin layer of dentine the cells are less tall; mitoses are not observed except in the deep lying mesenchyme cells of the dental papilla.

**VIII. Five Days After Birth:**

(1) **Outer Enamel Epithelium:**
It is an incomplete layer of flat cells devoid of mitotic figures. This layer is indistinguishable from the cells of the tooth follicle. The bone encapsulation surrounds the whole tooth germ except at the oral attachment.

(2) **Stellate Reticulum:**

There are few mitotic figures in the second molars and even fewer in the first molars. Nevertheless, numerous large blood vessels are present.

(3) **Stratum Intermedium:**

This layer is one to two layers of flat (on the cusps) to cuboidal (in the valleys) cells. No mitoses are observed in this layer in the first molars, but very few are seen in the second molars.

(4) **Inner Enamel Epithelium:**

The cells of this layer, being all fully differentiated by this time, are termed ameloblasts. The cells have reached their maximum height, with the nuclei at one end of the cell away from the enamel, which is pale pink in colour (i.e., Feulgen-positive). The "non-formative" ameloblasts, overlying the enamel-free areas, never attain the height of the formative cells.

By the fifth day after birth all mitoses on the crown of the first and second molars have ceased entirely (Figs. 29b – g; Figs. 30b – g; Figs. 31b – f; and Figs. 32b – f). However, the mitoses persist in Hertwig's sheath which is associated with the development of the
roots (Figs. 29a, 30a, 31a, 32a). The number of dividing cells on the second molars (Fig. 31a, 32a) exceed that on the first molars (Fig. 29a). This might be explained on the basis that the first molars appear before the second molars.

(5) **The Odontoblasts:**

They are well arranged in one layer of highly columnar cells. No mitoses at all were observed except in the second molars near the cervical loop. A fairly thick layer of dentine is present. There is a clear zone below the odontoblasts.

The odontoblasts underlying the enamel-free areas (apical pits) do not differ from the rest, i.e., they all pass through the usual developmental stages. In other words, whereas the roof of the apical pit is enamel-free, covered by short non-functional ameloblasts, its base is lined with a thick layer of dentine secreted by very tall columnar fully differentiated odontoblasts.

The following are some interesting additional observations made during this investigation.

(1) It was observed occasionally that one daughter cell of a dividing stratum intermedium cell "seemed" to lie among the inner enamel epithelium (Fig. 35a). Also, many dividing cells of the inner enamel epithelium showed one daughter cell or part of it among the stratum inter-
medium cells (Figs. 35b, c, d). In many of these cases, the basement membrane next to the dividing cell is intact but only slightly elevated (Fig. 35d).

(2) In all the stages examined, starting from 13 days in utero to five days after birth, the nuclei revealed by the Feulgen method were all more or less alike, and stained with the same intensity. In other words, I could not differentiate two different types of cells among the inner enamel epithelium in the early stages or among the ameloblasts in later stages, i.e., no narrow cells with pyknotic nuclei among larger cells. (This will be discussed when the "Kionoblasts" are mentioned).

(3) It has also been observed that the position and distance between the cusps remain constant during development once dentine and enamel are deposited, as can be shown by comparing the wax models at the age of two, three, and five days after birth, where the length of the tooth remains approximately constant as revealed by the scale below the model.

(4) When the crown of the molars at one day, two days, and three days after birth were examined in a basal view, it was observed that the posterior end of $M^1$ (Figs. 20a, 23a, 29a) showed mitotic figures. This region is actually a continuous layer of the inner enamel epithelium of the ameloblasts, the enamel organ formed by the union of the cervical loop on each side. $M^2$ shows this feature anteriorly and posteriorly
(Fig. 27a). This region is in fact the cervical loop at the end. Both $M_1$ and $M_2$ (Figs. 21a, 22a, 24a, 28a, 30a) show the reverse of $M_1$, that is, mitotic figures are observed in a basal view in the anterior region.

Table showing the relation between the length of the tooth germ at 120 magnification and the age.

<table>
<thead>
<tr>
<th>Tooth</th>
<th>Length of tooth germ in centimetres</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days in utero</td>
</tr>
<tr>
<td></td>
<td>15-16</td>
</tr>
<tr>
<td>$M_1$</td>
<td>6</td>
</tr>
<tr>
<td>$M_1$</td>
<td>4</td>
</tr>
<tr>
<td>$M_2$</td>
<td>3</td>
</tr>
<tr>
<td>$M_2$</td>
<td>2.5</td>
</tr>
</tbody>
</table>

From this table one can see the following:

1. The length of the first molars (upper and lower $M_1$ and $M_1$) remains the same from two days after birth onwards.
2. At 5 days after birth, the second upper molar ($M_2$) is of the same length as the second lower ($M_2$), while the first upper ($M_1$) exceeds the first lower ($M_1$) in length.
3. The second upper molar ($M_2$) is about $2/3$ the length of the first upper ($M_1$) at 5 days.
A relationship seems to exist between increase in length and appearance of the bands of intense mitoses: increase in length from 6 to 10 cms. \((M^1)\) and from 4 to 9.5 cms. \((M_2)\) is accompanied by the appearance of the first band of mitoses. The appearance of the second band of mitoses accounts for the increase from 11 to 15 cms. \((M^1)\) and 10 to 12 cms. \((M_1)\). Similarly, the occurrence of the third band of mitoses is followed by an increase from 15 to 18 cms. \((M^1)\) and 12 to 15 cms. \((M_1)\). On the second molars, however, there are only two bands of mitoses which can be explained in the same way.

In \(M^2\), the increase from 3 to 5 cms. is due to the appearance of the first band, and from 6 to 10 cms. for the second band. Similarly, \(M_2\), from 2.5 to 5 cms. is due to the first band, while from 6 to 10 cms. is due to the appearance of the second band. This can be followed easily on the models.
Fig. (1): a, b & c are three successive sections of the bud stage of \( M^2 \), 15-16 days in utero, each showing mitotic figures in the neck region and central cells and none in the basal peripheral layer (X 440).
Fig.(2): Occlusal views of wax model reconstructions of the bud stage of: (a) $M^2$, (b) $M_2$, 15-16 days in utero. Mitoses at the periphery while the central part is free from mitoses.
Fig. (5): Occlusal view of wax model reconstruction of the cat third milk molar, bud stage. Central area free of mitotic figures with a peripheral zone of mitoses.
Fig. (4): (a) Section of the early cap stage of M₁ (15-16 days in utero) showing mitotic figures in stellate reticulum, cervical loop and outer enamel epithelium (X 340)

(b) Higher magnification of the central part of the same section to show that the central basal layer is devoid of mitoses (X 760).
Fig. (5): Wax model reconstructions of the early cap stage, 15-16 days in utero.
(a) - M₁ and (b) - M₂.
First mitoses-free area - dots represent mitotic figures at the cervical loop.
Fig. (6): Wax model reconstruction of the early cap stage of $M_2$, 17-18 days in utero. The same distribution pattern as Fig. (5)
Fig. (7): Wax model reconstruction of the cat third milk molar, early cap stage. Same distribution pattern as in the mouse (Figs. 5a, b, and 6).
Fig. (3): Wax model reconstruction of the third molar of the bat (*Hipposideros beatus*), early cap stage. Same distribution pattern as in the cat and the mouse.
Fig. (9): Wax model reconstruction of the third molar of the bat (*Hipposideros beatus*), late cap stage. This shows the beginning of another mitoses-free area, separated from the first by a band of intense mitoses.
Fig. (10): Wax model reconstruction of the late cap stage of $M_2$, 20-21 days in utero. Two mitoses-free areas separated by a band of intense mitoses across the tooth.
Fig. (11): Wax model reconstructions of the late cap stage, 17-18 days in utero.
(a) - $M^1$, and (b) - $M_1$
Fig. (12a): Section in $M_1$, 17-18 days in utero, mitoses in pre-odontoblasts, inner enamel epithelium, stratum intermedium, outer enamel epithelium and cervical loop (X 760).

Fig. (12b): Section in $M_1$, 17-18 days in utero, mitoses in the different layers. Notice the intense Feulgen-positive granules on the faintly Feulgen-positive chromonemata (X 760).
Fig. (12c): Section in M₁, 17-18 days in utero. Two mitotic figures in the pre-odontoblasts in the vicinity of two mitotic figures in the inner enamel epithelium (same notice as Fig. 12b). (X 760).

Fig. (13): Section in M₁, 17-18 days in utero. Mitosis of a follicle cell (layer adjacent to outer enamel epithelium). Notice also mitotic figures in stellate reticulum, stratum intermedium and inner enamel epithelial layers (X 760).
Fig. (14): Wax model reconstruction of the third molar of the bat (*Hipposideros beatua*), early bell stage, showing more distinctly mitoses-free area, separated by bands of mitotic figures.
Fig. (15): Wax model reconstruction of the early bell stage, of M\(^2\), one day after birth.

(a) - basal view, showing mitoses in the cervical loop region.

(b) - occlusal view, showing more clearly the band of mitoses separating two mitoses-free areas.
Fig. (15): (c) - $M^2$, lingual side,
(d) - $M^2$, buccal side (upper surface is the top of the crown).
These views show that the crown surface is more or less flat, with no signs of cusp elevations.
Fig. (16): Wax model reconstruction of the early bell stage of $M_2$, one day after birth.
(a) - basal view (anterior to the left).
(b) - occlusal view (anterior to the right).
Fig. (16): (c) - M₂, lingual side (anterior to the right). (d) - M₂, buccal side (anterior to the left).
Both views show that the crown (upper surface) is flat.
Fig. (17): Wax model reconstruction of the late bell stage of $M^1$, 20-21 days in utero.

(a) - basal view, anterior to the left.
(b) - occlusal view, anterior to the left.
Fig. (18): Wax model reconstruction of the late bell stage of $M_1$, 20–21 days in utero.
(a) - basal view, anterior to the left.
(b) - occlusal view, anterior to the right.
Fig. (18): $M_1$, (c) lingual side, anterior to the right.  
(d) buccal side, anterior to the left.  
The cusps on the crown are clearly seen.
Fig. (19): Section in $M^1$ showing mitotic figures in all the layers: outer enamel epithelium, stellate reticulum, stratum intermedium, inner enamel epithelium and in the cervical loop (X 320).
Fig. (20): Wax model reconstruction of $M^1$, one day after birth.
(a) – basal view, anterior to the right.
(b) – occlusal view, anterior to the left.
(c) – lingual view, anterior to the left.
(d) – buccal view, anterior to the right.
Fig. (21): Wax model reconstruction of $M_1$, one day after birth.
(a) - basal view, anterior to the left.
(b) - occlusal view, anterior to the right.
Fig. (21): (c) lingual side, anterior to the right.
(d) buccal side, anterior to the left.
Fig. (22): Wax model reconstruction of $M_2$, 2 days after birth.

(a) - basal view, anterior to the right.
(b) - occlusal view, anterior to the left.
Fig. (22): (c) lingual side, anterior to the right.
(d) buccal side, anterior to the left.
Fig. (23): Wax model reconstruction of M, two days after birth. 
(a) - basal view, anterior to the right. 
(b) - occlusal view, anterior to the left.
Fig. (23): (c) - lingual side, anterior to the left.
(d) - buccal side, anterior to the right.
Fig. (23): (e) - lingual side, anterior to the left. (f) - buccal side, anterior to the right.
Fig. (24): Wax model reconstruction of $M_1$, two days after birth.
(a) – basal view, anterior to the right.
(b) – occlusal view, anterior to the left.
Fig. (24): (c) - lingual side, anterior to the right.
(d) - buccal side, anterior to the left.
Fig. (24): (e) - lingual side, anterior to the right. 
(f) - buccal side, anterior to the left.
Fig. (25a): Section in M, 3 days after birth. Ameloblasts vacuolated, 4 mitotic figures in stellate reticulum (X 440)

Fig. (25b): Section in M, 3 days after birth. Ameloblasts vacuolated. Notice Feulgen-positive granules some of which are surrounded by a Feulgen-positive shell (X 760).
Fig. (26): (a) - Section in $M_1$, (b) - Section in $M_2$, 3 days after birth, showing that the membrana praeforativa is paralleled by another thin membrane ($X$ 760).
Fig. (27): Wax model reconstruction of $M^2$, 3 days after birth.

(a) - basal view, anterior to the right.

(b) - occlusal view, anterior to the left.
Fig. (27): (c) - lingual side, anterior to the left.
(d) - buccal side, anterior to the right.
(Fig. (28)): Wax model reconstruction of $M_2$, 3 days after birth.

(a) — basal view, anterior to the left.
(b) — occlusal view, anterior to the left.
Fig. (28): (c) - lingual side, anterior to the right.
(d) - buccal side, anterior to the left.
Fig.(29): Wax model reconstruction of $M^1$, 3 days after birth. 
(a) - basal, (b) - occlusal, (c) - lingual; 
(d), (e), (f), and (g) buccal. Arrows point to the anterior end.
Fig. (30): Wax model reconstruction of $M_1$, 3 days after birth.
(a) - basal view, (b) - occlusal view, (c) and (d) - lingual side, (e) and (f) - buccal side. Arrows point to the anterior end.
Fig. (31): Wax model reconstruction of $M^2$, 5 days after birth.
(a) - basal view, anterior to the right.
(b) - occlusal view, anterior to the right.
Fig. (31): (c) - lingual side, anterior to the left.
(d) - buccal side, anterior to the right.
Fig. (31): (e) - postero-lingual view.
(f) - postero-buccal view.
Fig. (31): (g) - posterior view
Fig. (32): Wax model reconstruction of $M_1$, 5 days after birth.

(a) – basal view, anterior to the left.
(b) – occlusal view, anterior to the left.
Fig. (32): (c) - lingual side, anterior to the right.
(d) - buccal side, anterior to the left.
Fig. (32): (e) - lingual side, anterior to the right.
(f) - buccal side, anterior to the left.
Fig. (32): (g) - anterior view.
Fig. (33): Wax model reconstruction of \( M^1 \), 5 days after birth.
(a) - basal view, anterior to the left.
(b) - occlusal view, anterior to the right.
Fig. (33): (c) - lingual side, anterior to the left.
(d) - buccal side, anterior to the right.
Fig. (33): (e) – lingual side, anterior to the left.
(f) – buccal side, anterior to the right.
Fig. (34): Wax model reconstruction of $M_1$, 5 days after birth.

(a) - basal view, anterior to the left.
(b) - occlusal view, anterior to the left.
Fig. (34): (c) - lingual side, anterior to the right.
(d) - buccal side, anterior to the left.
Fig. (34): (e) - lingual side, anterior to the right.
(f) - buccal side, anterior to the left.
Fig. (35): (a) – Dividing stratum intermedium cell, one of the daughter cells seems to lie among the inner enamel epithelium. (X 760)

(b) – Dividing inner enamel epithelial cells showing part of the daughter cells among the stratum intermedium (X 440).
Fig. (35): (c) - Dividing cell in the inner enamel epithelial layer, one of the daughter cells seems to lie among the stratum intermedium (X 440).

Fig. (35): (d) - The basement membrane next to the dividing cell is elevated (X 760).
Crown view drawing of left m\(_1\) of 33 days mouse. The lingual border is lowermost. - b) Buccal view drawing of left m\(_1\) of 33 days mouse. - c) Lingual view drawing of left m\(_1\) of 33 days mouse.

Crown view drawing of left m\(_2\) of 33 days mouse. Buccal border is lowermost. - b) Buccal view drawing of left m\(_2\) of 33 days mouse. - c) Lingual view drawing of left m\(_2\) of 33 days mouse.

Fig. (36): (a) and (b) - after Gaunt (1955).
C - DISCUSSION AND CONCLUSIONS.

The process of mitotic cell division has been studied for many years and from many angles. The literature on this subject has been reviewed by Bullough (1952).

Mitosis takes place in tissues in process of normal development, repair and regeneration and in tumours. The question why cell division happens is related to the larger problem of why it sometimes ceases to occur; if it were understood why cell division largely ceases in maturity, it might become clearer how cells are released from this control in tumours.

During the present investigation, it has been observed that the size of the interphase nuclei in any one of the layers examined is not constant. Generally, the size of the nucleus is related to that of the whole cell, and most of the literature of this subject is concerned with this proportionality, to which R. Hertwig (1903) gave the well known name of "karyoplasmic ratio". Berrill (1943) concluded that: "cell division must, therefore, occur when the cell size exceeds a certain critical value". To state this, however, raises questions about how the cell increases
in size, and whether mere increase in size would really be a sufficient stimulus to mitosis. Nevertheless, increase in size is usually associated with mitosis. During this investigation, it has been observed that before the cell enters the mitotic phase the nucleus enlarges. This is in agreement with Conklin's observations (1912), in the embryo of the mollusc *Crepidula*. Another example, from a vertebrate tissue, in which the nuclear size is doubled, has been described by Beams and King (1942) in their work on the regenerating liver of the rat. According to Cohen & Berrill (1936), the cells of the columnar epithelium of the gut wall of the lower vertebrates lose their shape during mitosis and become rounded. This has been noticed in the present study in the case of the inner enamel epithelial cells.

Examination of the Feulgen preparations in which mitoses are frequent suggests that a nucleus in division contains very much more Feulgen-positive material than one in interphase. Quantitative measurements by Leuchtenberger (1950) and Swift (1950), however, do not confirm this. The chromosomes of both the interphase and early prophase nucleus show two components: the intense Feulgen-positive hetero-chromatic granules which are segments of the chromosomes, and the faintly Feulgen-positive threads of the chromonemata (Fig. 12a, b, c). Animal nuclei in which the latter can be demonstrated are regarded by Serra (1947) as characteristic
of secretory cells, and of tissues in which frequent cell division is occurring. The cells of the tooth germ exhibit both features, i.e., in the early stages, they are actively dividing, and later they start secreting the hard tissues of dentine and enamel.

1 - OUTER ENAMEL EPITHELIUM.

(a) Mitoses:

In the bud stage, mitoses among the cells of the peripheral layer are observed only in the neck region, while in the early cap stage, mitoses are sporadically distributed in the outer enamel epithelium though those on the lingual side predominate. In the late cap and early bell stages, mitoses are still frequent in this layer. During subsequent stages of development, the cells of the outer enamel epithelium show a steady reduction in the frequency of mitoses, which becomes rarer as the 5 days stage is reached. Numerous mitotic figures have been observed in the vicinity of the cervical loop in all the stages.

Very few investigators made observations of the frequency of mitoses in the outer enamel epithelium, and these were limited.

Canalis (1886), working on the development of teeth of rabbit, stated that many mitotic figures were observed in the bud stage. He believed that the cap stage
and the successive stages were the result of mitotic proliferation of the cells of both the enamel organ and dental papilla. He also stated that while the inner enamel epithelium and stratum intermedium layers showed numerous mitoses, the outer enamel epithelium showed only few.

Schaper & Cohn (1905), using pig embryos and young dogs in a study of the growth of the enamel organ, stated that, being epithelial in character at the beginning, the outer enamel epithelium became more and more flat as development proceeded. They added that in the early stages, mitotic figures were found everywhere in this layer, but with the growth of the enamel organ, cell division disappeared gradually from the outer enamel epithelium.

It is evident that Canalis as well as Schaper & Cohn admitted the presence of mitoses in this layer, but they did not give a detailed description of the mitotic distribution in serial sections of successive developmental stages.

There are, however, some investigators who deny the existence of mitoses in this layer. Reichenbach (1926) stated that no mitoses occurred in the outer enamel epithelium of the bell stage, and that this layer showed signs of being stretched.

Lefkowitz et al. (1955) reported that greater growth was evident lingually, although they did not state whether this growth was due to mere mitotic figures on that
side. Nevertheless, the present observations agree with this statement.

(b) **Morphology:**

Considering now the morphology of these cells, the present observations have shown that the cells of the peripheral layer of the bud stage, continuous with the Malpighian layer, are of low columnar form. They maintain this shape till they are invaded by blood vessels. The observed gradation in shape from low columnar to cubical to flat is only due to the plane of sectioning, as it has been found that sometimes the outer enamel epithelium cells are all of the columnar form on one side, while those on the other side of the same section are all cubical. During subsequent stages of development, i.e., after the enamel organ is invaded by blood capillaries, the cells of the outer enamel epithelium show a steady reduction in height, becoming cuboidal and finally flat.

Santone (1935) stated that the structure of the outer enamel epithelium varied in the various stages of enamel organ development. In the bud stage, the basal peripheral epithelium is made up of several layers of columnar cells. In the cap stage, when the internal epithelium is distinguished from the external one, the latter is composed of one layer of cells; later they become cuboidal then flattened. In the bell stage, the cells in the neck region
are flat and of one layer only; towards the base they are cubical, and in the cervical loop, the cubical cells grow in height and form transition stages towards the columnar cells of the inner enamel epithelium. That the cells of the outer enamel epithelium of the bell stage in the neck region are flat is contrary to the present description in which they were observed as low columnar cells.

Lefkowitz et al. (1953) stated that the outer enamel epithelium layer is a band of columnar cells which remain unchanged, showing no apparent morphologic change until they are invaded by the blood capillaries, then they become squamous. The present results are in agreement with the findings of Lefkowitz et al. A similar description of the morphology of the outer enamel epithelium during the developmental stages as that obtained during the present investigation was given by Gaunt (1955) and Turner (1961), i.e., being at first columnar, then cuboidal, then flat.

Prior to 20 days in utero, the enamel organ is avascular, blood capillaries appear only outside the outer enamel epithelium. Capillary invasion from the surrounding tissues, namely the follicle, commences at 20 to 21 days in utero, thus breaking the continuity of this layer. These blood capillaries penetrate the outer enamel epithelium and invade the stellate reticulum. This is in agreement with previous findings (Addison & Appleton, 1922; Lefkowitz et al.,

From the above, it is concluded that there is a general agreement that in the early stages of development, the outer enamel epithelium is cubico-columnar, and that after the invasion of blood vessels, they change to cubical and finally they become flat in the advanced stages.

2 - STELLATE RETICULUM.

(a) Mitoses:

Mitotic figures have been observed in the stellate reticulum over a considerable period of time starting from the bud stage at 14 days in utero up to 5 days after birth. Their frequency in the early stages exceeds that in the later stages.

The distribution of mitoses in the stellate reticulum, or even the occurrence of mitoses in this layer, was studied only by very few workers.

Canalis (1886), stated correctly that before a proper stellate reticulum could be recognized, mitoses took place uniformly throughout the enamel organ and not only in the central part as denied by others, but later, very few occurred in this layer, when the cells began to undergo modification characteristic of the mucous zone.

Conversely, Brugger (1949) stated that the interior of the enamel organ was mainly occupied by a highly vaculated
tissue known as the stellate or the enamel pulp, whose cells contained large pyknotic nuclei, which did not divide. Similarly, Santone (1935) stated that vacuolization in the enamel pulp cells occurred in the bud stage, while Lefkowitz et al. (1953) believed that the stellate reticulum cells undergo "senescence" by vacuolization of the cytoplasm and the nuclei being pyknotic at 17 days in utero.

I think that these writers have been misled through using routine histological methods. By using special cytological methods as those applied in the present investigation, the preparations showed this layer to be composed of large cells with nuclei uniformly stained with the same intensity, which remained active and showed many mitotic figures up to 5 days after birth.

Recently, Paynter & Hunt (1961) followed the mitotic activity in the molar tooth germs during development, by injecting young rats intraperitoneally with tritium labelled thymidine. They found a considerable number of dividing cells in the stellate reticulum of the enamel organ, even after the formation of the crown was well advanced. This observation support the present findings and disproves Santone's statement (1935) that the stellate reticulum does not persist throughout the entire period of enamel formation. I have found that this layer persisted as active cells showing mitotic figures up to 5 days after birth.
It is likely that numerous mesenchymatous cells would invade the stellate reticulum (enamel pulp) with the penetration of the blood capillaries. It is very difficult to distinguish these latter cells from the cells of the stellate reticulum; and so it is impossible to say whether the dividing cells are originally stellate reticulum cells or those invading the enamel organ. Nevertheless, though mitotic figures have been observed in this layer frequently in the early stages, they are rare in the 5 days stage.

(b) **Morphology:**

Orban (1957) described the central cells of the enamel organ as an epithelial structure containing a large quantity of fluid and consisting of stellate epithelial cells joined together by long intercellular bridges. Schour (1953) described them as a cellular network which resembled embryonic connective tissue or mesenchyme of epithelial origin. In contrast to these descriptions, sections of tooth germs in the bud and cap stages showed that the central cells were large and irregularly shaped and had narrow intercellular areas normal to an epithelial tissue. Later, transitional cells occurred between the outer enamel epithelium and stratum intermedium accompanied by spacing between the stellate cells, mainly over the future cusp tips.

Nearly the same description has been given by
Frisbie (1952). From his studies on dogs, cats and pigs, he found that the stellate reticulum cells, early in development, were large, ranging in size up to 50 μm by 75 μm, and that owing to this extreme size, only the nuclei were seen. He also observed that the large stellate reticulum cells undergo reduction in size, and this took place adjacent to the cuspal tips, and thus these cells in later stages, were blended with the outer enamel epithelium and stratum intermedium layers into a single layer of uniformly small compact cells, and that during this process the cells of the outer enamel epithelium were brought closer to the surface of the ameloblastic layer. He suggested that, on this account, the three layers: stellate reticulum, stratum intermedium and outer enamel epithelium, were probably one functional unit.

Observations obtained during the present study showed that the central cells of the bud and early cap stages were rounded with large nuclei nearly filling the cells. Heaping up of these cells in the latter stage was observed giving a resemblance to the so-called "enamel knot". In the late cap stage, it was observed that the stellate reticulum cells became more spaced, and that such spacing between these cells in the case of the lower molars was most obvious on the lingual and buccal sides, i.e., above the future lingual and buccal cusps, thus leaving a
compact mass of cells between the two areas. While in the case of the upper molars, this spacing occurs in the middle over the future primary central cusp. In certain regions (anterior and posterior) of the enamel organ, the spacing might occur very late or not at all.

This observation leads to the suggestion that transformation of the central cells of the enamel organ of the early stages to acquire the stellate form in advanced stages follows a certain pattern, or occurs in certain regions.

One of the current opinions about the mode of origin of the stellate reticulum was expressed by G. Tomes (1898) as follows: "The cells on the periphery of the enamel organ remain prismatic, but those in the centre become transformed into a stellate network. This conversion of cells into a stellate reticulum is most marked quite in the centre of the enamel organ. The transformation of the central cells occupying the centre and constituting the bulk of the enamel organ into a stellate reticulum goes on progressing from the centre outwards but it stops short of reaching the layer of the columnar cells which constitute the surface of the enamel organ, next to the dentine papilla. A layer of unaltered cells remains between the stellate cells and the columnar cells and is known as the stratum intermedium".

According to this quotation, there should be in
the enamel organ but a single point where the transformation into the stellate reticulum begins, and from which the process would progress. This point is situated in the centre of the enamel organ. This description agrees only in part with the present observations, i.e., in the case of the upper molars.

However, Bolk (1913-1922) disagrees with Tomes' opinion. Bolk believes that, as a rule, there is not a single centre, but there are two centres in the enamel organ, a lingual and a buccal one, from which the transformation into stellate cells radiate. Ahrens (1913) agrees with Bolk that the pulp formation does not begin in the centre of the organ.

From the above discussion one can say that the present observations still agree with Bolk's interpretation, but only in the case of the lower molars. It would appear that Tomes and Bolk were describing different teeth and, therefore, both interpretations might be considered correct.

Bolk gave the name "enamel septum" to the compact layer of cells between the two centres which were recognizable by a lesser density of nuclei, and he called this condition the "double pulp centre". This enamel septum was adduced as evidence that the tooth germ was a double structure and this was the basis of his "dimer theory". Renaut (1897), had given the first short
description of the enamel septum and called it the "directing enamel core" ("cone adamentin directeur"). The three types of "enamel septa" (Enamel cord, cleavage septum and epithelial septum) within the enamel organ have been discussed by Butler (1956).

It is clear that the "enamel septum" of Bolk (1913) is in general the same as the "enamel cord" of Ahrens (1913) as Bolk himself admitted. However, there is a point of disagreement between Ahrens and Bolk. The first believed that even the cells of the enamel cord became vacuolized and were then no longer distinguishable from the rest of the stellate reticulum. The latter stated that the elements of the septum during the further development of the organ were not transformed into stellate cells, but kept their indifferent character.

In conclusion, the present observations showed that transformation into stellate cells occurred in the centre (in agreement with Tomes) in the case of the upper molars, and in two places, lingual and buccal (in agreement with Bolk) in the case of the lower molars. In the latter case, the cells in between the two centres retain their shape for quite a long period, although, as Bolk stated, they sometimes stretch out parallel to the axis of the organ and, therefore, appear to be formed by much elongated and flattened cells. Also, in agreement with Bolk, there is a
relationship between these cells and the stratum intermedium.

(c) **The Enamel Knot:**

The enamel knot was first described by Ahrens (1913, 1914) in the cap stage, as a mass of cells which develops in the centre of the inner enamel epithelium by a localized rapid multiplication of the cells.

Bolk (1921) stated that in an advanced enamel organ, when the level of the section ran obliquely with regard to the direction of the septum, the latter appeared as a rounded heaping up of undifferentiated cells, wholly enclosed by reticular cells.

From the above, it would appear that both authors were describing one and the same structure, although in different stages.

The most detailed study of the enamel knot is that of Santone (1935a) who found it in guinea pig, rabbit, rat, cat, sheep and calf. He presented some photographs showing the enamel knot as a mass of cells characterized by the "concentric" arrangement of its cells. The presence of the enamel knot was confirmed in most mammals by different investigators (Gaunt, 1955 in the mouse). Its presence in the rat has been disputed by Lefkowitz et al. (1953), who interpreted this appearance as an artifact due to oblique sectioning (as Bolk explained oblique sectioning of the enamel septum). Butler (1956) reviewed the literature on
the presence, position and significance of the enamel knot and cord.

From the present observations, "heaping up" of the cells superficial to the basal columnar inner enamel epithelium was noted, but not in a "concentric" manner as described by Santone and his followers. The presence of this concentration of cells took different positions. In the early cap stage, it is observed to occupy the central basal depression in both upper and lower molars. In the late cap stage and early bell stage of the lower molars it occupies the valley between the lingual and buccal cusps, while in the upper molars, concentration of the cells is observed over the slopes of the primary median cusp, i.e., the valley between the median cusp and its lingual and buccal cusps. This might add support to the previous observations that rarefaction of the enamel pulp cells occurs over the cusp tips.

(d) Functions of the Stellate Reticulum:

Many functions have been attributed to the stellate reticulum and the literature has been reviewed by Reichenbach (1928), Lehner and Plenk (1936) and Green (1938). These functions were summarized by Butler (1956). They fall into two classes: (a) Nutrition of the cells of the inner enamel epithelium (Williams, 1884; Sudduth, 1884). This is argued by Santone (1935a), Hamp (1940) and Lefkowitz et al. (1944
and 1947). (b) Mechanical functions: (1) Protecting the inner enamel epithelium from mechanical disturbance (Todd & Bowman, 1859; Poulton, 1888; Santone, 1935a), or (2) forming a space into which cusps can grow (Waldeyer, 1869; Jewel, 1886; Hoffmann, 1894; von Korff, 1933; Avery, 1951), or (3) acting as an elastic cushion which maintains the shape of the enamel organ (Reichenbach, 1928; Grulat, 1936; Rizzoli, 1951). However, Butler (1956) suggested that polysaccharides were used to absorb water and cause swelling and that the "stellate reticulum may thus be regarded as a tissue specialized for the imbibition of water and, therefore, exerting pressure on its surroundings".

My opinion is that the stellate reticulum has both functions, i.e., nutrition as well as mechanical. In the early stages, when the enamel organ is avascular, nutritional material needed by the inner enamel epithelium is supplied from that stored in the stellate reticulum as proved from the high concentration of the polysaccharides in this tissue (the presence of polysaccharides in this tissue was observed in the present study and will be discussed later). In later stages, when the enamel organ is invaded by blood capillaries from the follicle, the stellate reticulum still acts as a pathway for the transference of material needed for the formation and maturation of the enamel. This might explain its persistence during
tooth development.

As to its mechanical function, the role of the stellate reticulum is mainly that of a space maintainer for the developing crown. As mentioned before, spacing of the enamel pulp cells and their subsequent transformation to the stellate reticulum in the bell stage was observed to take place over the future cusps, thus giving space for the proliferating inner enamel epithelium. With the developmental process, the cusps displace the stellate reticulum so that in the advanced stages the stratum intermedium comes in contact with the outer enamel epithelium and the stellate reticulum is pushed in the valleys between the cusps.

3 - STRATUM INTERMEDIUM.

(a) Mitoses and Morphology:

In the early cap stage, the stratum intermedium layer is not clearly demarcated from the stellate reticulum. However, those cells adjacent to the inner enamel epithelium are crowded and more differentiated with mitotic figures among them. These cells will eventually form the stratum intermedium.

In the late cap stage, this layer consists of large cells arranged 1 - 3 cells thick, and some of the cells are actively dividing.
In the bell stage, it is composed of 1 to 3 layers of flat to cuboidal cells. In later stages, they retain this thickness, form and continue to divide, even over the tips of the cusps where the ameloblasts have ceased to divide. However, the mitotic frequency gradually decreases. In most cases, the cuboid stratum intermedium cells lie over the inner enamel epithelium which has not commenced enamel matrix production, i.e., in the valleys and the lower parts of the crown, and these parts show many mitotic figures; while over the cusp tips they tend to be flat with few mitoses among them. Finally, mitosis gradually disappears from this layer with the establishment of the crown pattern.

Once the enamel-free areas can be distinguished, the stratum intermedium cells overlying the inner enamel epithelium in these areas are flat and do not show mitoses.

Most previous investigators did not give an accurate description of the mitotic frequency during the successive developmental stages. They concentrated their observations on the morphology and the role of this layer in the process of enamel formation, and made only casual observations on mitoses based on the examination of single sections and not the whole series through a tooth germ.

Santone (1935) stated that the thickness of the stratum intermedium was much greater near the tips of the
cusps than on the side walls of the crown and in the valleys. In this latter region, it was often reduced to a single series of cells and sometimes it was lacking in them, contrary to the present findings. He added that the stratum intermedium layer, unlike the stellate reticulum cells, persisted at the margins of the tooth throughout the entire period of enamel formation. I found that both layers were present in all the stages examined.

Santone stated that mitosis was relatively frequent in the stratum intermedium in stages which are not too much advanced.

Lefkowitz et al (1953) stated that, in the rat, the stratum intermedium at 17 days in utero appeared as round epithelium 4 to 5 cells thick, at 18 days it became squamous, finally at 20 - 21 days and after birth, it became again cubical.

Gaunt (1956) confirmed their finding in the teeth of the mouse, i.e., the stratum intermedium cells change from cuboidal to flat and then to cuboidal again. He stated that in the early cap stage these cells are cuboidal, by the sixteenth day they become squamous and by the 20 - 21 days stage, these cells once again become cuboidal; and that this change in form from cuboidal to squamous and then to cuboidal again was first observed at the highest parts of the crown and in time it extends towards the cervical region.
My observations do not confirm these changes of form. Apparent differences seen in different parts of a single section are due to the plane of sectioning. In later stages all the stratum intermedium cells become flattened.

Gaunt (1956) stated that the stratum intermedium cells overlying the future enamel-free areas retained their squamous form and did not show this secondary cuboidal form. This is, in a way, in agreement with the present results. To be more precise, the stratum intermedium cells in the early stages of development, i.e., cap stage, when the enamel-free areas are difficult to distinguish, are all alike; and cuboidal. Later, when these areas are apparent, i.e., early bell stage, the cells remain cuboidal except those in these areas, which change and become flat, and remain so throughout the stages.

Hunt (1959) stated that the stratum intermedium cells first appear as a single flattened layer of cells between the inner enamel epithelium and the stellate reticulum. They divide especially at the cervical loop and form several layers of cuboidal cells. It is understood that this layer starts with flat cells one cell thick, which due to mitoses in the cervical loop later becomes formed of many layers of cuboidal cells. This is contrary to the findings of all other workers.

Paynter and Hunt (1961), after intraperitoneal
injection of young rats with tritium labelled thymidine, found numerous dividing cells in the stratum intermedium layer, even after the formation of the crown was well advanced.

These results agree with the present observations. However, they did not give any further information about the parts in which these mitoses are more frequent.

(b) Insinuation:

It was observed occasionally that one daughter cell "seemed" to lie among the inner enamel epithelial cells. Also, many dividing cells of the inner enamel epithelium showed one daughter cell or part of it among the stratum intermedium cells.

Prenant (1921) and Santone (1935) believed that the stratum intermedium layer probably provided for the multiplication of the ameloblasts throughout the entire period of growth. This does not give any indication as to how these cells are added to the ameloblasts.

Ten Cate (1959), working on human teeth, reported (in abstract) the phenomenon of "recruitment" and "insinuation" of the stratum intermedium cells between and among the inner enamel epithelium. In 1961, Ten Cate amplified his previous statement. He observed that some cells were in an intermediate position between the stratum intermedium and the inner enamel epithelium. He described
these cells as having "pear-shaped" nuclei and gave them the term "insinuating". He found that some of the insinuating cells were in mitosis, and presented a figure of a cell in the telophase stage of division, showing one daughter nucleus situated at the level of the inner enamel epithelial nuclei, and the other approximately to the level of the stratum intermedium nuclei. He also stated that no insinuating cells were found in the lower parts which has not been fully differentiated. Ten Cate, following suggestions made by a number of earlier authors (Waldeyer, 1871; Carter, 1918; Lehner & Plenk, 1936; Widdowson, 1947), believed that these insinuating cells were passing from the stratum intermedium into the inner enamel epithelium.

Turner (1961), also using human teeth, agrees with Ten Cate's findings.

I believe this picture of the dividing cells seems to be a misleading one. I think that neither the cells of the stratum intermedium recruit among the ameloblasts, nor the reverse. Both of the two daughter cells will eventually lie in their original layer, for in many cases the basement membrane can be seen to remain intact next to the dividing nuclei, but only slightly elevated, as has been shown in Fig. 35d. As to the explanation of the "pear-shaped" nucleus, it is probable that the inner enamel epithelial cells after division, the two rearranged
daughter cells, coming to lie beside each other in one layer, undergo a "sliding" movement, and as the space is sometimes not sufficient for the two resultant cells, the nuclei appear compressed, thus giving this "pear-shaped" appearance.

I should like to mention two other points which might have some bearing on insinuation. The first is the fact that in the stages examined, the columnar inner enamel epithelial cells were more or less alike in size, and the cuboidal stratum intermedium cells are smaller in size compared with them. So in coming to lie among the more columnar cells of the inner enamel epithelium, they should attain the same dimensions and, therefore, one might expect to find transitional stages, but this has not been observed. Secondly, according to the laws of division of labour, each layer has its own function, though these layers might be dependent on each other. The stratum intermedium cells, if assumed to change position will consequently change function; it would take some time to acquire the new function, and one might expect to see some cells lagging behind, thus showing difference in staining reactions, but such a case has not been met with in this investigation. On general grounds, it seems improbable that cells would change function in the manner postulated. Specialization is a character of the different types of tissues, especially in higher vertebrates.
More recently, Hunt and Paynter (1962) followed the migration of cells from the stratum intermedium layer. This was done by injecting guinea pigs with tritium labelled thymidine. They confirmed previous interpretations which showed that the stratum intermedium cells form the epithelial attachment in erupting teeth, and that cells from this layer form the preameloblasts, the latter statement being in agreement with Ten Cate's observations.

Hunt and Paynter found that the stratum intermedium cells are the precursors of the stellate reticulum. Hence, they made the following statement: "These studies suggest that the stratum intermedium cells are relatively undifferentiated cells which are capable of forming preameloblasts, stellate reticulum cells or stratified squamous epithelium, depending upon the existing environmental conditions".

To begin with, as is known, both the stellate reticulum and the stratum intermedium arise from the central cells of the enamel organ. The only difference is that those adjacent to the inner enamel epithelium will remain more differentiated and will form the stratum intermedium. So it is not easy to find out which layer is the precursor of the other since both of them are derived from one and the same mass of cells. Secondly, I cannot accept the fact that "the stratum intermedium cells are capable of forming preameloblasts", especially if the results were
obtained by the above mentioned method. The incorporation of the thymidine in the nuclei of two cells, say one of them in the stratum intermedium and the other in the inner enamel epithelium, does not give evidence that one cell comes from the other, especially if both cells were found apart from each other. Finally, it is concluded that the thymidine method only shows which cells have divided and does not give any indication of the direction of movement of any cell.

4. INNER ENAMEL EPITHELIUM

As mentioned before, the object of this investigation is to study the relation between the distribution of mitoses in the inner enamel epithelium and the development of the cusps.

When the enamel organ assumes the early cap stage, mitotic figures are to be seen in the inner enamel epithelium only in the vicinity of the cervical loop. In the following stages of development, mitotic activity is accelerated and consequently these figures become most pronounced in certain areas which lie on the slopes of the cusps and between them in the valleys and not at the highest parts of the crown.

By comparing the models of successive developmental stages, it is concluded that the areas distinguished
by the absence of mitoses in the cap stage become the most elevated parts of the final crown. In fact, they also include the enamel-free areas of the crown. The interest of this observation is that the inner enamel epithelium seems to be mapped out even before it has folded to form the crown topography.

Continued mitoses in the intervening positions cause the enamel epithelium to fold downwards. This downward growth undercuts the cusps and causes them to tilt. The pattern is, therefore, established in principle at a stage prior to the development of the cusps.

With the differentiation of the inner enamel epithelial cells into ameloblasts and the formation of a thin layer of enamel matrix on the cusp tips, mitotic activity gradually ceases on the slopes of the cusps. However, mitotic figures are to be observed in the inner enamel epithelium at the lowest parts of the crown and in the valleys, these persisting until again the enamel matrix is laid down in these areas. Therefore, the areas of most active mitoses are those associated primarily with the future ridges and valleys of the crown.

(a) Cervical loop region:

The term "cervical loop" used in the present description represents that part where the outer enamel epithelium merges into the inner enamel epithelium.
The term has also been used by Schour (1953), Orban (1957), Scott & Symons (1958), and Turner (1961), while Lefkowitz et al. (1953) used the term "marginal proliferative zone.

In all the developmental stages studied, starting from the early cap stage, when an outer enamel epithelium could be distinguished from an inner enamel epithelium until the crown is fully formed, numerous mitotic figures have been observed in the cervical loop region. Growth of the cervical loop region of the enamel organ appears to be concerned with two functions: during the early stages, it is related to cusp development, and during later stages, it is related to the basal apertures where the roots will eventually form. But root apertures are found in principle before cusps have all developed.

The exclusive occurrence of mitoses in the cervical loop region is not a new discovery, as can be seen from numerous articles on the development of the enamel organ: Canalis (1886), Rose (1891), Sachse (1895), von Ebner (1902), Kolliker (1902) and it was also emphasized by Schaper & Cohen (1905). Recently, it has been confirmed by many dental research workers as Santone (1935), Sicher (1942a, b), Lefkowitz et al. (1953) and Baume et al. (1954).

Canalis (1886) stated that numerous mitoses occurred in the inner enamel epithelial cells uniformly in the early stages, but when a dentine cap was formed, mitoses became
rarer in the inner enamel epithelium above it, while they were still numerous lower down in the epithelium where no dentine was formed.

A similar interpretation was made by Schaper & Cohen (1905), who stated that in the cap stage all the cells of the inner enamel epithelium were capable of proliferation and that mitotic figures were scattered diffusely especially in the parts near the edges. In the succeeding stage, they found a similar mitotic distribution, although the cervical loop was very well developed and the inner enamel epithelium already showed a more developed differentiation. Later, they underwent histologic differentiation and changed to a simple layer of high epithelial cells. Further differentiation took place and the cells gave up their proliferating function, and at the same time, took over a special function or prepared for such functions as a consequence of the early disappearance of karyokinesis in the inner enamel epithelium on top of the papilla; mitoses occurred only in the lowest part of the enamel epithelium and were especially numerous in the cervical loop region. In the next stage, formation of the enamel started at the top of the papilla, then proceeded at the lower parts. The authors concluded that once the inner enamel epithelial cells were differentiated into the characteristic columnar ameloblasts, and have taken over their specific functions, all karyokinesis disappeared.
without exception on the crown. However, it continued in
the lower part of the tooth (root), i.e., in that part where
there was no trace of enamel substance and where the enamel
cells more or less preserved the indifferent form. Schaper
& Cohen emphasized that from this time onwards proliferation
occurred only where the outer enamel epithelium turned into
the inner enamel epithelium (i.e., cervical loop), where
the enamel organ grows by apposition in a basal direction.
The embryonic nature of this region of growth could be
recognized by the primitive epithelial character of these
cells and it is for this reason that they called this part
of the enamel organ "epithelscheide" (= place of point of
separation, or the zone of indifference in the cell
structure of the enamel organ).

Taking into consideration that this work was not
done in successive stages of the same animal (pig), and that
they had to fill this gap by examining corresponding stages
in other animals (young dogs), and that their interpretations
were not obtained from serial sections, the results reached
by Schaper & Cohen on tooth development are very accurate.
However, there is one point which I would like to make clear.
They stated that, in the cap stage, mitotic figures were
scattered in the inner enamel epithelium, and they presented
a figure of a section showing this distribution. This
statement is correct only if we consider this section as
passing in the area of intense mitoses, mentioned in the present work. On the other hand, if the section was in a mitoses-free area, we should get a different picture in which mitotic figures in the inner enamel epithelium are restricted to the cervical loop region.

These findings remained unchallenged up to the present time, and those who followed Schaper & Cohen did not add new information to what had been said before.

Marsland (1951), Gaunt (1955), Ten Gate (1959) and others, all agree that development of the tooth occurs by proliferation in the early stages and then once differentiation starts mitoses cease.

Lefkowitz et al. (1953) stated that, in rat, from the sixteenth to the nineteenth day in utero the preamelo-blasts show no mitoses. This stage corresponds to the early cap stage described in this work, although the timing is different. They added that from the nineteenth day they start dividing and that "mitotic activity is greatest in the dentine grooves" (valleys).

Although they did not show how these mitoses are distributed, the writer agrees with them that the valleys are regions of mitotic activity, and this was also confirmed by Glasstone (1938).

Poole (1957) stated that the tooth germ increases in size by a downward extension of the enamel organ; new
ameloblasts are continuously produced by division at the base where the inner and outer enamel epithelia can be distinguished until later stages. In other words, the cells of the cervical loop region are capable of dividing till later stages.

Recently, Paynter & Hunt (1961), by injecting tritium labelled thymidine in rats, were able to detect many dividing cells in the inner enamel epithelium at the base of the fissures in the molar teeth, after deposition of dentine and enamel was well advanced down the sides of the cusps. This agrees with the present findings.

Although it is generally accepted that the inner enamel epithelial cells do proliferate at a certain time of their life history, only Baume et al (1954) are against this view. Using the rat as the experimental animal, they believe that such cells are too specialized to proliferate. However, they admit that the epithelial tissues are in a highly proliferative state as far as the oral epithelium and the enamel epithelium at the cervical loop ("proliferative zone") are concerned, and that the inner enamel epithelium originates from the centre of proliferation—"proliferative zone". In other words, they believe that from the cervical loop, the inner enamel epithelial cells travel around the loop to the inner side, while the new outer enamel epithelial cells move upwards on the outer side.
of the enamel organ. Consequently, they made this statement: "In accordance with the rule that highly specialized cells such as the inner enamel epithelium have lost the potency to proliferate, present observations give uniform evidence that the epithelial growth takes place at the outer basal side of the loop".

(b) Presence of mitoses on cusp tips:

The presence of mitoses at the cusp tips was described by many workers.

Von Korff (1932) believed that cell division was particularly active at the tips of the cusps.

Glasstone (1938) also found mitoses on the tips of the cusps after they have been formed. But she also observed that at the time when the cusps were growing in height most rapidly, mitoses were confined to their vertical sides and the valleys between them. Similarly, Lefkowitz et al. (1953) stated that, in the rat, mitotic activity was greatest at the tips of the cusps and in the valleys.

The existence of proliferative cells at the tips of the cusps appears to contradict with the observations of Blechschmidt (1953), of the lack of mitoses at the tip of the primary cusp, and with the present observations which have shown that cessation of mitoses in the cusp areas is evident, even at the earliest stages of development. In fact, the cuspal areas were devoid of mitoses from the very
early stages and consequently when the cusp elevations were apparent, no mitoses were, likewise, found on their tips.

It is generally known that a secondary cusp develops on the slope of a primary cusp. In other words, ridges are frequently the site of origin of new cusps (Butler, 1956). Ridges are defined as folds passing down the slopes of the cusps. They frequently cross the valleys so as to joint two cusps. The term "cingulum" is applied to a ridge passing round the base of the crown and bounded internally by a valley which roughly parallels the basal outline of the tooth. The cingulum has long been recognized as an important source of new cusps (Huxley, 1871; Allen, 1874; Cope, 1883; Bolk, 1914, 1922). The origin of a secondary cusp may be imagined as an early maturation of a group of cells in a zone which becomes divided into a basal zone which continues to add to the height of the crown and a zone which deepens the valley separating it from the primary cusp. In the very early stages of ontogeny, when the crown relief is low, it is exceedingly difficult to determine from the sections the absolute anatomical tip of the cusps. However, comparison of the wax models have shown that these cusp tips lie in the mitoses-free areas, and that mitotic figures are present on the cusp slopes though absent from their tips, and this might well give the appearance
of mitoses at the absolute cusp tip. The presence of mitoses at cusp tips, as thought by some authors, might also be due to the fact that the mitoses-free area is too small to be detected, even by the present method.

Finally, it is concluded that the cusp tip is the first region to cease dividing and the first to produce dentine and enamel, and as Butler (1956) has suggested, "the cusp should not be regarded as a centre of growth but rather as a centre of precocious maturity, and that increase in the crown relief occurs by continuous growth in the valleys between the cusps".

(c) Enamel-free areas:

According to the literature, the occlusal surface of the cusps of molar teeth of murids is always devoid of enamel. This was first shown by Mahn (1890).

Addison & Appleton (1921) have shown that the crowns of the molar teeth in the albino rat have enamel-free areas on the cusps. Similarly, Gaunt (1956) has shown that, in the mouse, there are areas where the ameloblasts fail to develop normally and deposit no enamel at all, i.e., enamel-free areas. However, recently, Johannessen (1961) concluded that prior to occlusal wear, the summits of the cusps of the rat molar teeth are not always devoid of enamel. In other words, the ameloblasts deposit only a very thin layer of enamel which is worn out as soon as the
tooth is erupted.

As a result of this study, these enamel-free areas can be recognized very early in the development of the tooth germ, when the crown is almost flat and there are no cusp elevations, as shown in Fig. 15c, d, and Fig. 16c, d, from which it can be seen that certain areas are already recognizable by their lack of mitoses. These areas will come to lie on the distal or mesial sides of the cusps, as demonstrated by the models of the successive stages. In other words, a mitoses-free area represents a cusp with its enamel-free area.

Enamel-free areas are specialization of the mouse family, and one would not expect to find such a striking differentiation among the ameloblasts in mammalian teeth generally. This raises the question: Does the mitoses-free area represent the position of the enamel-free area or the position of the cusp? If the first alternative is correct one would not find such a distribution pattern in teeth which do not show enamel-free areas. For this reason comparison of the distribution pattern in teeth of different groups of animals was needed. Wax models of the cat and the bat have shown the same distribution pattern as the mouse, i.e., there are certain areas devoid of mitoses separated by bands of intense mitoses. This leads to the belief that the picture obtained in the case of the mouse is not wholly
due to the enamel-free areas, but is partly due to them as they lie at the tips of the cusps.

Over the enamel-free areas of the crown, the inner enamel epithelium does not show mitotic figures and the ameloblasts are found to be much less columnar and never attain the height of the fully differentiated formative ameloblasts. This confirms the observations of Gaunt (1956); Lefkowitz et al. (1953); Addison & Appleton (1921) and Mahn (1890).

(d) **Cusp formation:**

According to Butler (1956), the ontogeny of the crown pattern is fundamentally a process of folding of the membrana praeformativa during the development of the tooth germ.

The development of the bell stage from the more or less flattened cap stage of the enamel organ might be expected to shed some light on the process of cusp formation. In other words, what is the stimulating element which causes the inner enamel epithelium to fold and form the cusps?

Earlier work on this subject has been reviewed by Hoffmann (1925), Reichenback (1928) and Lehner & Flenk (1936). The main point of controversy was whether the base of the enamel organ was pushed upwards by growth of adjacent mesenchyme, or whether the bell stage was reached by unequal growth of the epithelium itself.
In fact, cusp outline requires cell division to accommodate the increased area of the membrana praeformativa. From the present observations, it has been found that folding of the inner enamel epithelium is a result of mitoses in certain definite regions of this layer in the early stages, long before the mesenchyme cells organize to form a definite layer of odontoblasts. So it was concluded that cusp outline is caused by unequal growth due to the occurrence of mitotic figures in localized regions of the crown. In other words, the inner enamel epithelium is responsible for determining the crown pattern.

Von Korff (1932) attributed the folding of the surface of the developing tooth to localized growth at definite places in the inner enamel epithelium, comparing the process with other folding epithelia such as embryonic nervous system, glands and villi of the gut.

Butler (1956) put forward the hypothesis that the unequal growth of the inner enamel epithelium played a major part in moulding the crown pattern. The present observations confirm this hypothesis. The anterior faces of the median upper cusps have many mitoses while the posterior faces are occupied by the enamel-free areas (mitosis-free). Hence, backward tilting of the cusps takes place. Similarly, the lower cusps tilt forward because the posterior faces of the cusps have many mitoses while the
anterior faces are enamel-free (mitosis-free). This supports the view that cusp shape is controlled by epithelia rather than mesenchyme.

Finally, though I cannot claim to explain all factors involved in the folding of the inner enamel epithelium, I think it is fair to say that unequal growth within the epithelium itself is an important factor, and as Butler (1956) stated, "the folding of the epithelium is controlled by its intrinsic growth pattern".

Moreover, I believe that the inner enamel epithelium being itself a definite layer, might have an effect on the underlying mesenchyme cells causing them to form a row of cells which later differentiated into the odontoblast layer. This view is supported by many workers, i.e., most investigators subscribe to the theory that the inner enamel epithelium initiates the activity in the mesenchyme.

According to Glasstone (1936, 1938), Sicher (1942a) and Shapiro et al (1944), the inner enamel epithelium is thought to be responsible for the organization of the odontoblast layer. Similarly, Huggins, McCarroll & Dahlberg (1934) believe that the enamel organ induces the formation of an odontoblast layer from specialized pulp cells. This view is also supported by Orban (1957) and James (1957) who believe that the inner enamel epithelium seems to exert
an influence upon adjacent connective tissue cells causing them to differentiate into odontoblasts. Similarly, Nylen & Scott (1960) state that the early alignment of the inner enamel epithelium fixes the location of the dentine enamel junction even before the odontoblasts begin to differentiate. More recently, Noble et al (1962), in an electron microscope study, state that the odontoblasts differentiate from the surface cells of the dental papilla under the organization of the inner enamel epithelium.

There is another view that considers the odontoblasts as the stimulating element which causes the inner enamel epithelium to fold (Turner, 1961).

Earlier, Canalis (1886) believed that the mesenchyme pushed the adjacent enamel organ and took part in the invagination. Likewise, Schaper & Cohen (1905) stated that the papilla grows upwards facing the enamel organ to be pushed downwards enclosing the papilla. This statement might in a way, lead to the belief that the mesenchyme induces the inner enamel epithelium to fold.

I cannot accept this view of Turner and the others that cusps are forced by the localized growth in the papilla. But, while the inner enamel epithelium exerts a dominating influence over the adjacent connective tissue, the condensation of the latter should not be considered a positive reaction to the crowding by the proliferative epithelium. The peripheral layer of the papilla does indeed
grow along with the inner enamel epithelium by showing mitotic figures, and likewise, ceases growth first where cusp tips will form.

Finally, a third view exists which holds that the folding is due neither to the inner enamel epithelium itself nor to the dental papilla. This was discussed by Butler (1956). Ahrens (1913) attributed to the "enamel knot" an important role in the formation of the crown pattern. He suggested that the enamel knot pushes the basement membrane into the surface of the papilla, to form what Ahrens believed to be the precursor of the central basin of the crown, and that slight occlusal convexities of the basement membrane at the buccal and lingual borders of the knot ("enamel grooves") were regarded as the first signs of the buccal and lingual series of the cusps. This is argued by Reichenbach (1928), Norberg (1929), and Santone (1955) who showed that the enamel grooves have no effect on the final shape of the crown, since they flatten out as the tooth germ develops. A similar effect was seen by Rose & Barthels (1896) in the calf and was interpreted as evidence of the "Concrescence Theory". The presented observations showed that Ahren's interpretation appeared only in the early cap stage, but on examining the whole series of sections in later stages, besides observing this concentration of cells of the enamel organ (enamel knot of Ahrens) in the middle
over the basin-like depression, it was found at the tips of
the future primary cusp in some sections and then over the
slope of the cusp in the succeeding sections. This agrees
with Reichenbach (1928), Santone (1935a), Fernandez-Rama
(1951) and Blechschmidt (1953).

(e) Distance between cusps and position of roots:

By comparing the wax models, it was observed that
once established, the position and the distance between the
cusps remain more or less constant during the developmental
stages, although the valleys deepen considerably. These
results were also reported by Gaunt (1955). Previously,
Kronfeld (1935) (in man) and von Beust (1928) (in the pig)
had shown that no further separation of the cusps took place
once calcification started. Similarly, Gantz (1922) and
Meyer (1954) found that the main features of the pattern of
human milk molars were present long before the teeth reached
their full diameter. Rhomberg (1932) found that this is
also true for the pig.

On the other hand, Turner (1961) stated that
"cusps continued to be moved apart from each other as the
tooth germ increased in volume". In fact, Turner is right,
and this might be explained in two ways:

(1) Schour & Massler (1940) stated that in man cusps are
regions of maximal thickness of enamel. Accordingly, the
crown pattern of the completed tooth might deviate from that
of the surface of the dentine, owing to the uneven thickness of the enamel. Thus, Kraus (1952) had shown that, in man, the relative distances between the cusps and the proportions of the crown as a whole are modified in this way. (2) Growth in the valleys not only deepens them, but might widen them, thus increasing the distance between the cusps (Butler, 1956). This had been shown to a marked degree in the horse (Friant, 1933) and the ox (Kupfer, 1935).

However, the present observations in which the dentine-enamel-junction is considered, show that the main features of the crown, and many of its minor details, are already present in the membrana praeformativa before the hard tissues have developed.

During this investigation it has also been observed that the position of the roots were roughly indicated at an early stage of development, before dentine had commenced to be laid down in the tooth germ of both upper and lower molars. A similar report was made by Orban & Mueller (1929) in the rat molars and by Turner (1961) in human teeth.

(f) Klonoblasts:

In all the stages examined, the nuclei revealed by the Feulgen method were all more or less alike and stained with the same intensity. In other words, I could not differentiate between two types of cells among the inner
enamel epithelium in the early stages, or among the ameloblasts in later stages.

The presence of a cell, the "kionoblast", different from, but lying amongst the ameloblasts of developing teeth was first pointed out by Saunders et al (1942), who considered it as a supporting cell. Compared with the ameloblasts, it was described as "a more slender cell, its nucleus is narrow and compact, and stains more deeply than the ameloblasts". It has also been studied by Westin (1952) who suggested, however, that the cell is a tubular structure concerned with carrying blood to the region of enamel formation. Its presence has been confirmed by Symons (1955a, 1956, 1957) and Ten Cate (1961).

Symons (1955a) stated that with the haematoxylin and eosin the difference in staining was noted, while by using the Feulgen method he showed that, in the early stages, there is only a slight difference between the kionoblast and the inner enamel epithelial nuclei, and in later stages all the nuclei of the inner enamel epithelial layer were alike. In other words, he could not see a marked staining difference with Feulgen although he saw it by using different staining methods. The present findings are in agreement with this.

I think that what has been described as the kionoblasts are nothing but the intercellular spaces of the
inner enamel epithelium and ameloblasts filled with some sort of secretion, contributing in the formation of the enamel matrix. This might explain the reason why it appeared more slender and more intensely stained. (The "kionoblasts" will be discussed in more detail in the next chapter).

(g) **Double basement membrane:**

In this study, the membrana praeformativa was observed to be paralleled by another thin limiting membrane which separated the pre-odontoblasts from the epithelial cells. Together, these two membranes, formed a double membrane enclosing a thin even space. This has been shown in Fig. (26a, b). In fact, this is the same structure, which with other technical methods, appears as a thickening of the separating membrane.

Orban (1957) stated that, prior to the formation of dentine, the connective tissue of the dental papilla is separated from the inner enamel epithelium by the basement membrane. When a thin layer of dentine is laid, the inner enamel epithelial cells begin their amelogenetic activity by forming a continuous thin membrane on the enamel side of the basement membrane, and this has been termed the dentine-enamel membrane, which later calcifies and is thus called the dentine-enamel-junction.

Quigley (1959), in his electron microscope study,
stated that the first indication of the dentine enamel junction appeared as a double membrane before any differentiation of the inner enamel epithelium and connective tissue (odontoblasts), and that subsequent thickening of the membrane and differentiation of the odontoblasts followed. Similarly, this has been shown by the electron microscope by Fearnehagh (1960a, 1961). He showed that, at the beginning of dentinogenesis, the pre-ameloblast layer was separated from the newly differentiated odontoblasts by a basement membrane. He stated that in regions where dentine was found, but the enamel matrix had not yet formed, there was a space between the cell membrane of the ameloblasts at its formative end and the newly formed dentine. This space widened and the extracellular region became packed with small granules round the formative end.

More recently, the above observations were confirmed by Noble et al. (1962) in an electron microscope study. They suggested that this picture represented the earliest stage in dentine matrix formation, classically described as a thickening of the basement membrane. A double membrane has also been observed in other epithelia. Gibbins (1962) stated that in common with other epithelia (Selby, 1955), the oral epithelium is usually separated from the underlying connective tissue by a limiting membrane which varies in thickness (range 300 - 600 Å) and is usually
separated from the plasma membrane of the basal cell by a space of 200 Å approximately, and that in some instances the limiting membrane is not demonstrable and the basal cell is then in contact with the connective tissue. Similarly, in this work the double membrane was not always distinguished and then only one membrane could be seen.

(h) Proliferation and differentiation:

Ameloblasts on different parts of the tooth reach maturity and begin to form enamel matrix at different times. As is well known, this process starts at the tips of the cusps and progresses downwards, so that the tooth may have dentine and enamel at its tip while at the base of the crown the inner enamel epithelium is still growing. Wax models of the cheek teeth of the mouse show that growth, expressed in terms of mitotic figures, continues in the valleys and on the lower parts of the crown after it has ceased on the highest parts of the cusps. This fact led to the conclusions (1) that the tip of the cusp is the first part to stop growing (no mitoses) and the first part to begin apposition; (2) that cusps do not grow up from the crown, it is the valleys which grow down; and (3) that maturation of the ameloblasts, recognized by the absence of mitoses, spreads down the slopes until eventually even the cells next to the base become mature, the proliferative zone, i.e., the cervical loop is no longer active and growth ceases.
The results of these observations gave a clear picture of the relation between proliferation and differentiation during the development of the enamel organ. With the beginning of formation of the enamel at the cusp tips, mitoses are gradually pushed back in the direction of the cervical loop, so that finally the cell proliferating growth of the enamel organ only occurs by means of the embryonic cells of this region which show an extraordinary independence in its life functions. The seeming antagonism or incompatibility between proliferation and functional differentiation has been recognized at least since 1905 (Schaper & Cohen), and has frequently been restated by Strangeways (1924), Lorain Smith (1932), Reinmann (1940), Needham (1942), Nicholson (1950), Marsland (1951, 1952) and Gaunt (1955). It is discussed by Dawson (1940), especially in the light of the views of Bloom (1937) and Weiss (1939).

Bloom (1937) stated that: "cellular multiplication and cellular differentiation are distinct processes each of which usually takes place only in the absence of the other". While Weiss (1939) stated: "In general it can be said that the more specialized a cell is in its structure and function, the less apt it is to divide". He also stated that "..... proliferation does not interfere with differentiation, but it does impede the elaboration of certain manifest products of differentiation".
Dawson (1940), while agreeing with Weiss that "a differentiating cell ...... gradually loses its capacity to divide and factors promoting differentiation automatically reduce proliferation", points out the converse supposition, found in the literature, that the apparent inability of certain cells to divide, cannot be accepted as primary evidence of their high degree of specialization.

Fischer (1946) stated that, whereas in the early stages of embryonic development all the cells of an organism are able to divide, as differentiation proceeds, this ability may be reduced or lost as a result of a kind of "physiological division of labour".

Hughes (1952) expressed his view as follows: "that cell multiplication and cell differentiation appear to be mutually exclusive, so far from indicating as Bloom (1937) thought - that they are "distinct processes", may show that they form two aspects of a single kind of cellular activity, differently conditioned by the environment". An example of this is provided by the cells of the inner enamel epithelium in the enamel-free areas, which lose their capacity of dividing at a very early stage, unlike the rest of the highly specialized ameloblasts.

5 - PAPILLA (ODONTOBLASTS)

As soon as the lamina begins to show the localized
swellings or tooth buds which mark the sites of the future teeth, the mesenchyme becomes concentrated round each bud, each mass of mesenchyme being covered with a capillary network. This has been shown very clearly by Gaunt (1959) in the cat, by Adams (1962) in three species of rodents, namely, rat, hamster and mouse, and by Boyer & Neptune (1962) who described the patterns of blood supply to teeth and adjacent tissues in rats.

Mitotic figures in the pre-odontoblasts were found to show a similar cycle and distribution to those of the inner enamel epithelium. As the enamel organ increases by division in the inner enamel epithelium, in certain areas, the peripheral pulp cells facing the inner enamel epithelial layer and the deep lying cells of the pulp also increase by division. In the early stages, the pre-odontoblasts are concerned entirely with increase in size of the papilla (growth). With the formation of dentine matrix mitosis ceases. As, however, this matrix commences to be laid down before that of enamel, mitotic activity in the pre-odontoblasts ceases earlier than in the inner enamel epithelium. The same results were previously obtained by Canalis (1886), who stated that while the enamel organ grew by active proliferation of its epithelial elements, the mesenchyme cells of the papilla also showed many mitoses. He also showed that the odontoblasts ceased to divide under dentine,
but continued to do so, near the base. Similarly, numerous mitoses occurred in the connective tissue in the centre of the papilla, but later became rarer. The odontoblasts in the enamel-free areas, unlike the ameloblasts, did not differ from those elsewhere on the crown and passed through the usual developmental cycle. This is in agreement with Gaunt's findings (1956).

Paynter and Hunt (1961) noticed that, in young rats, many of the labelled cells were related to the development and growth of the blood vessels of the pulp, and that most of the early pulpal mitoses were confined to the area adjacent to the root sheath, with little activity elsewhere. The present findings agree with this result, so far as the later stages (3 to 5 days after birth) are concerned. These investigators, however, did not extend their investigation to these earlier stages.

With the Feulgen method used in this investigation, all the nuclei of the pulp tissue, especially those forming the peripheral layer of the dental papilla, i.e., the odontoblasts, were similar in shape and intensity of stain, both in the early stages and the advanced ones. In other words, the "radial cells" first described by Jasswoin (1924) among the odontoblasts, and confirmed by Symons (1955a, 1956, 1957) could not be recognized. The "radial cells" and the "hionoblasts" will be discussed in more detail in the next chapter.
In conclusion, I think that in general, the shape of the tooth is due partly to the enamel organ and partly to the mesenchyme of the dental papilla. The former is responsible for the cusps and valleys on the crown, while the latter is responsible for the size of the tooth, the shape of its basal outline, and the position of its roots.

CONCLUSION

Mitoses in the Inner Enamel Epithelium:

The findings recorded for the first molars and second molars were compared, and it was concluded that the mode of growth in both cases were the same. When judged from the point of view of the mitotic frequency in the inner enamel epithelium, both showed five distinct stages: (1) The first is characterized by a mitoses-free area. This includes the bud and early cap stages. (2) The second stage is marked by the appearance of bands of intense mitoses (future valleys) separating mitoses-free areas (future cusps and enamel-free areas). (3) In the third stage, there is an increase in the frequency of mitoses till the tooth size is attained. (4) In the fourth stage, few mitoses were recorded on the slopes of the cusps with increasing age (except for the enamel-free areas and cusp tips where no mitoses were found in an early stage) and an increase in the valleys. (5) The fifth stage, is
characterized by the disappearance of all mitotic figures from the crown. The last final change occurred with the onset of amelogenesis. It is probably true to say that this follows the natural laws of the relation between proliferation and differentiation.

As a final conclusion, it can be stated that proliferative growth is a function of the cervical loop. In all the stages, more mitoses were recorded for the cervical loop, a region which is always actively providing new cells to the outer enamel epithelium, stratum intermedium, inner enamel epithelium to make up for the continuous increase in size of the enamel organ. During the early stages of development, mitoses in the cervical loop are related to cusp formation, i.e., the crown is elevated as a whole by growth at the base, and during later stages, it is related to the basal apertures where the roots would eventually join the crown of the tooth.

The areas free of mitoses within right and left teeth of the same head were approximately identical. The parallelism noted proves that the whole process is genetically controlled.

It is hoped that a similar attempt to plot the distribution of mitoses in the odontoblasts and stratum intermedium layers will be carried out in the future, so that by comparing the distributions in the different layers
a more accurate explanation may be obtained of the growth of the tooth germs with regard to cusp formation.
The chi-square test, which provides a suitable significance test, has been used to determine to what extent the differences in density of mitoses in the inner enamel epithelium are significant.

The Poisson test has been employed as a check to randomness.

Further confirmation was obtained by applying the chi-square test to the results obtained from the Poisson test (Simpson and Roe, 1960).

These tests were applied to the first upper and lower molars as well as the second upper and lower molars, in the cap stage (Figs. 11a, 11b; 15b; 16b). It must be remembered that the wax models of the tooth germs at this stage show a flat surface, i.e., with no cusp elevations. Mitotic counts in these experiments were made by counting the number of dividing cells in each section over the whole surface of the tooth.

(1) In the first case, i.e., the chi-square test, the value
of the chi-square is used as a measure of the extent to which the observed numbers of dividing cells in each section depart from the values one would expect if the number of the dividing cells in all the sections was identical. The number of dividing cells in each section is counted and tabulated. This is the observed number (O). Then the number of dividing cells to be expected in each section (E) is calculated from the following formula:

\[ E = \frac{\sum O}{\text{number of sections}} \]

The value of chi-square (\( \chi^2 \)) is calculated from the equation:

\[ \chi^2 = \sum \frac{(O - E)^2}{E} \]

The probability that the observed results are due to chance or otherwise is obtained from the table of the chi-square, using \((N - 1)\) degrees of freedom, where \(N\) = the total number of sections. If the calculated chi-square exceeds that of the table, therefore, it provides very strong evidence that it is not a matter of chance, especially if it falls under a probability less than 0.001.

Because the tooth germ sometimes consists of a large number of sections (especially in the first molars), grouping was necessary for easy calculations. The total number of sections in the tooth germ was divided into groups or classes. Each class consists of five successive sections. The observed number of dividing cells in the five successive sections is added, and this is (O). The expected
number (E) in this case is given by dividing the total number of mitoses in all the sections by the number of classes.

Both experiments, i.e., the chi-square calculated by considering each section separately, and the chi-square calculated by dividing the total number of sections into classes (each of five sections) gave the same results. In other words, the calculated chi-square always exceeded the chi-square table under a probability of less than 0.001. (Table Ia, IIa, IIIa, IVa).

(2) In the second case, i.e., the Poisson test, the observed frequency was tabulated in six classes (1) number of sections containing no mitoses, (2) sections containing 1 mitosis, (3) sections containing 2 mitoses, (4) sections containing 3 mitoses, (5) sections containing 4 mitoses, (6) sections containing 5 or more mitoses. Then the Poisson probability (P) is calculated in each of the above six cases by the following equations (Simpson and Roe, 1960, p.129):  

\[
\begin{align*}
X &= 0, \text{ probability (P)} = \frac{1}{e^X} \\
X &= 1, \quad " \quad  " = \frac{X}{1} \frac{1}{e^X} \\
X &= 2, \quad " \quad  " = \frac{X^2}{2!} \frac{1}{e^X} \\
X &= 3, \quad " \quad  " = \frac{X^3}{3!} \frac{1}{e^X} \\
X &= 4, \quad " \quad  " = \frac{X^4}{4!} \frac{1}{e^X} \\
X &= 5, \quad " \quad  " = 1 - \text{sum of the above probabilities } = .00^+
\end{align*}
\]
Multiplying the Poisson probability by the total number of sections (N) gives the expected frequency. Direct comparison of the observed frequency and the expected or calculated frequency gives a clue to the type of distribution. If both observed and calculated frequencies are almost identical, this means that it is a Poisson or random distribution.

However, in the present experiments (Table Ib, IIb, IIIb, IVb), the observed frequencies always deviated and never fitted the Poisson frequencies.

If an observed distribution fits a Poisson distribution, the observed variance of the distribution should be equal to the theoretical variance of the distribution to which it is being fitted (Simpson and Roe, 1960, p.129). The observed variance and the theoretical variance are calculated by using the equations:

**Observed variance:** \( S^2 = \frac{\sum fx^2}{N-1} - \frac{NX^2}{N} \)

**Expected (Poisson) variance:** \( \bar X = \frac{\sum fx}{N} \)

Accordingly, the observed variance in the present experiments always exceeded the theoretical one.

(3) In the third case, the chi-square test is done against a Poisson distribution (Simpson and Roe, 1960, p.310). In this case, the chi-square is calculated from the data of observed frequencies and expected or calculated frequencies of the Poisson distribution (Table Ic, IIc, IIIc, IVc).
The results obtained from these experiments are presented in the following table:

<table>
<thead>
<tr>
<th>Tooth</th>
<th>$X^2$ Test</th>
<th>$X^2$ on Poisson</th>
<th>$\frac{s^2}{\bar{X}}$</th>
<th>Calculated variance</th>
<th>Probability</th>
<th>Calculated Table \</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>N^2</td>
<td>35.76</td>
<td>26.125</td>
<td>5.305</td>
<td>22.45</td>
<td>&lt;0.001</td>
<td>16.266</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N^1</td>
<td>53.6</td>
<td>26.125</td>
<td>9.405</td>
<td>22.508</td>
<td>0.001</td>
<td>18.47</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N^1</td>
<td>59.814</td>
<td>34.528</td>
<td>4.53</td>
<td>20.544</td>
<td>0.001</td>
<td>13.815</td>
<td>18.89</td>
</tr>
<tr>
<td>N^1</td>
<td>35.760</td>
<td>34.528</td>
<td>4.2</td>
<td>3.014</td>
<td>&lt;0.001</td>
<td>16.27</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
From this table it is evident that in all the cases in which the chi-square is applied, there is less than one probability in 1000 that the observed distribution of mitoses is due to chance, or that this is an even distribution.

Similarly, from the Poisson test it is shown that the observed variance is always greater than the expected or theoretical variance and never fits it.

These results give support to the assumption that the observed distribution of mitoses in the inner enamel epithelium, reflected in the presence of mitoses-free areas and areas of intense mitoses, must have a significant meaning, since it is not an even or random distribution.

In interpreting the observations on mitoses, there is a striking resemblance between right and left teeth (Figs. 5a, b; 6a, b; 10, 11a, b). In other words, it is assumed that the variances of the right and left teeth are identical. However, there are two cases which seem to be somewhat misleading (Figs. 10 and 11b). A statistical test for the equality of the variances is, therefore, needed in these two cases, i.e., Figs. (10 and 11b). This is done by the so-called "variance ratio" (Bailey, 1959, p.50).

The method used consists of dividing the surface of the tooth (Figs. 10 and 11b) into squares, and counting
the mitoses in the right and left comparable squares. Mitotic counts in these squares are tabulated and then the standard deviation in each case (right and left) is calculated from the equation:

\[
\text{Standard deviation} = \sqrt{\frac{\sum x^2 - \frac{1}{N}(\sum x)^2}{N-1}}
\]

Finally, the variance ratio is calculated from the equation:

\[
\text{The variance ratio} = \frac{\text{square of standard deviation of right tooth}}{\text{square of standard deviation of left tooth}}
\]

i.e., \[ F = \frac{\text{St. d}^2 \text{ right}}{\text{St. d}^2 \text{ left}} \]

The results obtained are as follows:

For Fig. (10):

Calculated variance ratio (8 degrees of freedom) = 2.234

Variance ratio from the (F) table (8 degrees of freedom) at 0.05 probability level = 3.44 and at 0.01 " " = 6.03

For Fig. (11b):

Calculated variance ratio (16 degrees of freedom = 1.311

Variance ratio from (F) table (16 degrees of freedom) at 0.05 probability level lies between 2.35 and 2.28, and at 0.01 " " " " 3.41 and 3.26

In both cases the calculated results are less than the value in the (F) table at both probabilities. This means that there is no significant difference in the distribution of mitoses between right and left teeth.
This statistical method shows that the distribution of mitoses in the right and left teeth is identical, within the limits of experimental error.
TABLE Ia.

Second Upper Molar

\( \chi^2 \) Test

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>E</td>
<td>0 - E</td>
<td>(0 - E)^2</td>
<td>(0 - E)^2 / E</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>-------</td>
<td>-----------</td>
<td>--------------</td>
</tr>
<tr>
<td>20</td>
<td>9.44</td>
<td>10.56</td>
<td>111.5136</td>
<td>11.75</td>
</tr>
<tr>
<td>9</td>
<td>9.44</td>
<td>-0.44</td>
<td>.1936</td>
<td>.02</td>
</tr>
<tr>
<td>4</td>
<td>9.44</td>
<td>-5.44</td>
<td>25.5936</td>
<td>3.13</td>
</tr>
<tr>
<td>3</td>
<td>9.44</td>
<td>-6.44</td>
<td>41.6736</td>
<td>4.41</td>
</tr>
<tr>
<td>17</td>
<td>9.44</td>
<td>7.56</td>
<td>57.1536</td>
<td>6.05</td>
</tr>
<tr>
<td>16</td>
<td>9.44</td>
<td>6.56</td>
<td>43.336</td>
<td>4.59</td>
</tr>
<tr>
<td>10</td>
<td>9.44</td>
<td>.56</td>
<td>.3136</td>
<td>.33</td>
</tr>
<tr>
<td>5</td>
<td>9.44</td>
<td>-4.44</td>
<td>19.7136</td>
<td>2.09</td>
</tr>
<tr>
<td>3</td>
<td>9.44</td>
<td>-6.44</td>
<td>41.4736</td>
<td>4.39</td>
</tr>
</tbody>
</table>

\( O = \) observed number of mitoses over five successive sections

\( E = \) expected number of mitoses over five sections

\[ \chi^2 = \sum \frac{(O - E)^2}{E} = 36.76 \]

\( \chi^2 \) from table, under 8 degrees of freedom and probability .001 is 26.125.

In other words, the calculated \( \chi^2 \) (36.76) corresponds to a probability of less than 0.001.
TABLE Ib.

Second Upper Molar

Poisson Test

<table>
<thead>
<tr>
<th>No. of mitoses</th>
<th>Observed frequency</th>
<th>Probability</th>
<th>No. of sections</th>
<th>Expected frequency</th>
<th>No. of sections</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>f</td>
<td>p</td>
<td>N = f</td>
<td>p x N</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>18</td>
<td>.211</td>
<td></td>
<td>9.495</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>.328</td>
<td></td>
<td>14.76</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>.255</td>
<td></td>
<td>11.475</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>.132</td>
<td></td>
<td>5.94</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>.051</td>
<td></td>
<td>2.295</td>
<td></td>
</tr>
<tr>
<td>5+</td>
<td>4</td>
<td>.023</td>
<td>45</td>
<td>1.035</td>
<td>44.99</td>
</tr>
</tbody>
</table>

Expected (Poisson) variances: \( \bar{x} = \frac{\sum f X}{N} = 1.555 \)

Observed variance: \( s^2 = \frac{\sum f X^2 - N \bar{x}^2}{N - 1} = 5.363 \)

\[ \frac{s^2}{\bar{x}} = 3.448 \]

The observed variance is 3.448 times what would be if the observation fits the Poisson distribution exactly.
TABLE Ic.

Second Upper Molar

$x^2$ on Poisson

<table>
<thead>
<tr>
<th>O</th>
<th>E</th>
<th>O - E</th>
<th>(O - E)^2</th>
<th>$(O - E)^2 / E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>9.495</td>
<td>8.505</td>
<td>72.335</td>
<td>7.61</td>
</tr>
<tr>
<td>9</td>
<td>14.76</td>
<td>-5.76</td>
<td>33.177</td>
<td>2.24</td>
</tr>
<tr>
<td>4</td>
<td>11.475</td>
<td>-7.475</td>
<td>55.876</td>
<td>4.8</td>
</tr>
<tr>
<td>7</td>
<td>5.94</td>
<td>1.06</td>
<td>1.1236</td>
<td>.19</td>
</tr>
<tr>
<td>3</td>
<td>2.295</td>
<td>.705</td>
<td>0.497</td>
<td>.21</td>
</tr>
<tr>
<td>4</td>
<td>1.035</td>
<td>2.965</td>
<td>8.791</td>
<td>8.4</td>
</tr>
</tbody>
</table>

$x^2 = \sum \frac{(O - E)^2}{E} = 23.45$ (calculated value of $x^2$)

$x^2$ from table under 3 degrees of freedom and probability 0.001 is 16.266. i.e., there is less than one chance in 1000 that this is an even distribution.

(Lumping the degrees of freedom, we have 5-2 constants which are N and $\bar{X}$ in the Poisson).

Under 3 degrees of freedom the calculated $x^2$ (23.45) exceeds that under a probability of less than 1 in 1000.
TABLE IIa.  
Second Lower Molar  

test  

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>E</td>
<td>(O - E)</td>
<td>(O - E)²</td>
<td>(O - E)²/E</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---------</td>
<td>----------</td>
<td>-----------</td>
<td>---</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>- 4</td>
<td>16</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>10</td>
<td>4</td>
<td>16</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>10</td>
<td>14</td>
<td>196</td>
<td>19.6</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>10</td>
<td>100</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>10</td>
<td>3</td>
<td>9</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>- 5</td>
<td>25</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>- 5</td>
<td>25</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>- 7</td>
<td>49</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>-10</td>
<td>100</td>
<td>10.0</td>
<td></td>
</tr>
</tbody>
</table>

(O = observed number of mitoses over five sections)  
(E = expected number of mitoses over five sections)  

\[ x^2 = \sum \frac{(O - E)^2}{E} = 53.6 \]  

\[ x^2 \] from table under 8 degrees of freedom and 0.001 probability = 26.125. Therefore, the calculated \( x^2 \), which is 53.6, corresponds to a probability of less than .001, i.e., there is less than one chance in 1000 that the observed distribution of mitoses would be obtained when an even distribution is expected.
TABLE IIb.
Second Lower Molar

Poisson Test

<table>
<thead>
<tr>
<th>No. of mitoses</th>
<th>Observed frequency</th>
<th>Probability</th>
<th>No. of sections</th>
<th>Expected frequency</th>
<th>No. of sections</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>f</td>
<td>p</td>
<td>N = f</td>
<td>p X N</td>
<td>(p X N)</td>
</tr>
<tr>
<td>0</td>
<td>12</td>
<td>.125</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>.259</td>
<td>10.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>.267</td>
<td>10.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>.185</td>
<td>7.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>.097</td>
<td>3.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5+</td>
<td>7</td>
<td>.067</td>
<td>40</td>
<td>2.68</td>
<td>40</td>
</tr>
</tbody>
</table>

Expected variance: \[ \hat{\lambda} = \frac{\sum f X}{N} = 2.075 \]

Observed variance: \[ s^2 = \frac{\sum f X^2 - N \bar{X}^2}{N - 1} = 9.405 \]

\[ \frac{s^2}{\hat{\lambda}} = 4.53 \]

The observed variance is 4.53 times what would be if the observation fits the Poisson distribution exactly.
### TABLE IIc.

**Second Lower Molar**

**$X^2$ on Poisson**

<table>
<thead>
<tr>
<th></th>
<th>$E$</th>
<th>$O - E$</th>
<th>$(O - E)^2$</th>
<th>$\frac{(O - E)^2}{E}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>5</td>
<td>7</td>
<td>49</td>
<td>9.80</td>
</tr>
<tr>
<td>6</td>
<td>10.36</td>
<td>-4.36</td>
<td>19.009</td>
<td>1.83</td>
</tr>
<tr>
<td>5</td>
<td>10.68</td>
<td>5.68</td>
<td>32.2624</td>
<td>3.02</td>
</tr>
<tr>
<td>8</td>
<td>7.40</td>
<td>0.60</td>
<td>0.36</td>
<td>0.048</td>
</tr>
<tr>
<td>2</td>
<td>3.88</td>
<td>-1.88</td>
<td>3.5344</td>
<td>0.91</td>
</tr>
<tr>
<td>7</td>
<td>2.68</td>
<td>4.32</td>
<td>18.6624</td>
<td>6.9</td>
</tr>
</tbody>
</table>

$$X^2 = \sum \left( \frac{O - E}{E} \right)^2 = 22.508$$

$X^2$ from table under 4 degrees of freedom and probability $0.001 = 18.47$, i.e., since the calculated $X^2$ (22.508) exceeds the $X^2$ table (18.47) under a probability 0.001, this means that there is less than one chance in 1000 that this is an even distribution.
<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>12.4</td>
<td>1.6</td>
<td>2.56</td>
</tr>
<tr>
<td>15</td>
<td>12.4</td>
<td>2.6</td>
<td>6.76</td>
</tr>
<tr>
<td>21</td>
<td>12.4</td>
<td>8.6</td>
<td>73.96</td>
</tr>
<tr>
<td>13</td>
<td>12.4</td>
<td>0.6</td>
<td>0.36</td>
</tr>
<tr>
<td>8</td>
<td>12.4</td>
<td>-4.4</td>
<td>20.36</td>
</tr>
<tr>
<td>3</td>
<td>12.4</td>
<td>-9.4</td>
<td>88.36</td>
</tr>
<tr>
<td>11</td>
<td>12.4</td>
<td>-1.4</td>
<td>1.96</td>
</tr>
<tr>
<td>19</td>
<td>12.4</td>
<td>6.6</td>
<td>43.56</td>
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<tr>
<td>13</td>
<td>12.4</td>
<td>0.6</td>
<td>0.36</td>
</tr>
<tr>
<td>29</td>
<td>12.4</td>
<td>16.6</td>
<td>275.56</td>
</tr>
<tr>
<td>16</td>
<td>12.4</td>
<td>3.6</td>
<td>12.96</td>
</tr>
<tr>
<td>6</td>
<td>12.4</td>
<td>-6.4</td>
<td>40.96</td>
</tr>
<tr>
<td>3</td>
<td>12.4</td>
<td>-9.4</td>
<td>88.36</td>
</tr>
</tbody>
</table>

\(X^2 = \sum \frac{(0 - E)^2}{E} = 59.814\) (calculated \(X^2\))

\(X^2\) from table under 13 degrees of freedom and 0.001 probability is 34.528, i.e., there is less than one chance in 1000 that the observed number would be obtained when an even distribution is expected.
### TABLE IIIb

**First Upper Molar**

**Poisson Test**

<table>
<thead>
<tr>
<th>No. of mitoses</th>
<th>Observed frequency</th>
<th>Probability</th>
<th>No. of sections</th>
<th>Expected frequency</th>
<th>No. of sections</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>.087</td>
<td></td>
<td>5.916</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>.212</td>
<td></td>
<td>14.416</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>.258</td>
<td></td>
<td>17.544</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>.209</td>
<td></td>
<td>14.212</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>.127</td>
<td></td>
<td>8.636</td>
<td></td>
</tr>
<tr>
<td>5+</td>
<td>15</td>
<td>.107</td>
<td>68</td>
<td>7.276</td>
<td>68</td>
</tr>
</tbody>
</table>

Expected or Poisson variance: \( \bar{X} = \frac{\sum f X}{N} = 2.441 \)

Observed variance: \( S^2 = \frac{\sum f X^2 - N \bar{X}^2}{N - 1} = 27.328 \)

Observed variance is \( \frac{S^2}{\bar{X}} \) times the theoretical one, i.e., \( \frac{27.328}{2.441} = 11.15 \) times what it would be if the observation fits the Poisson distribution exactly.
TABLE IIIc.
First Upper Molar

\[ \chi^2 \sim \text{Poisson} \]

<table>
<thead>
<tr>
<th>O</th>
<th>E</th>
<th>O - E</th>
<th>((O - E)^2)</th>
<th>(\frac{(O - E)^2}{E})</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>5.916</td>
<td>6.084</td>
<td>37.015</td>
<td>6.257</td>
</tr>
<tr>
<td>15</td>
<td>14.416</td>
<td>.584</td>
<td>0.341</td>
<td>.023</td>
</tr>
<tr>
<td>10</td>
<td>17.544</td>
<td>-7.544</td>
<td>56.911</td>
<td>3.243</td>
</tr>
<tr>
<td>8</td>
<td>14.212</td>
<td>-6.212</td>
<td>39.588</td>
<td>2.785</td>
</tr>
<tr>
<td>8</td>
<td>8.636</td>
<td>-0.636</td>
<td>0.404</td>
<td>0.046</td>
</tr>
<tr>
<td>15</td>
<td>7.278</td>
<td>7.722</td>
<td>59.629</td>
<td>8.190</td>
</tr>
</tbody>
</table>

\[ \chi^2 = \sum \frac{(O - E)^2}{E} = 20.544 \]

\(\chi^2\) from table under 2 degrees of freedom (after lumping) and probability 0.001 = 13.815.

Therefore, there is less than one chance in 1000 that this is an even distribution.
### TABLE IVa

**First Lower Molar**

**$X^2$ Test**

<table>
<thead>
<tr>
<th></th>
<th>$E$</th>
<th>$O - E$</th>
<th>$(O - E)^2$</th>
<th>$(O - E)^2/E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>17</td>
<td>-1</td>
<td>1</td>
<td>.059</td>
</tr>
<tr>
<td>12</td>
<td>17</td>
<td>-5</td>
<td>25</td>
<td>1.529</td>
</tr>
<tr>
<td>10</td>
<td>17</td>
<td>-7</td>
<td>49</td>
<td>2.882</td>
</tr>
<tr>
<td>18</td>
<td>17</td>
<td>1</td>
<td>1</td>
<td>.059</td>
</tr>
<tr>
<td>22</td>
<td>17</td>
<td>5</td>
<td>25</td>
<td>1.529</td>
</tr>
<tr>
<td>21</td>
<td>17</td>
<td>4</td>
<td>16</td>
<td>.941</td>
</tr>
<tr>
<td>29</td>
<td>17</td>
<td>12</td>
<td>144</td>
<td>8.470</td>
</tr>
<tr>
<td>23</td>
<td>17</td>
<td>6</td>
<td>36</td>
<td>1.760</td>
</tr>
<tr>
<td>21</td>
<td>17</td>
<td>4</td>
<td>16</td>
<td>.941</td>
</tr>
<tr>
<td>18</td>
<td>17</td>
<td>1</td>
<td>1</td>
<td>.059</td>
</tr>
<tr>
<td>9</td>
<td>17</td>
<td>-8</td>
<td>64</td>
<td>3.765</td>
</tr>
<tr>
<td>8</td>
<td>17</td>
<td>-9</td>
<td>81</td>
<td>4.764</td>
</tr>
<tr>
<td>7</td>
<td>17</td>
<td>-10</td>
<td>100</td>
<td>5.88</td>
</tr>
<tr>
<td>9</td>
<td>17</td>
<td>-8</td>
<td>64</td>
<td>3.765</td>
</tr>
</tbody>
</table>

($O = $ observed number of mitoses over five sections)
($E = $ expected number of mitoses over five sections)

\[
X^2 = \sum \frac{(O - E)^2}{E} = 35.760
\]

$X^2$ from table under 13 degrees of freedom and 0.001 probability = 34.528. That means that there is less than one chance in 1000 that this is an even distribution.
## TABLE IVb

**First Lower Molar**

**Poisson Test.**

<table>
<thead>
<tr>
<th>No. of mitoses $X$</th>
<th>Observed frequency $f$</th>
<th>Probability $p$</th>
<th>No. of sections $N = f$</th>
<th>Expected frequency $p \times N$</th>
<th>No. of sections $p \times N$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>.049</td>
<td></td>
<td>3.332</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>.147</td>
<td></td>
<td>9.996</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>.220</td>
<td></td>
<td>14.96</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>.228</td>
<td></td>
<td>15.504</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>.164</td>
<td></td>
<td>11.152</td>
<td></td>
</tr>
<tr>
<td>5⁺</td>
<td>12</td>
<td>.192</td>
<td>68</td>
<td>13.056</td>
<td>68</td>
</tr>
</tbody>
</table>

**Expected variance:** $\overline{X} = \sum \frac{f \times X}{N} = 3.014$

**Observed variance:** $s^2 = \frac{\sum f \times X^2 - N \times \overline{X}^2}{N - 1} = \frac{903 - 617.44}{67} = \frac{285.56}{67} = 4.2$

$$s^2 = \frac{\sum f \times X^2}{\overline{X}} = 1.39$$

i.e., the observed variance is 1.39 times what would be if the observation fits the Poisson distribution exactly.
IV. CYTOLOGY OF THE AMELOBLASTS AND ODONTOBLASTS.

A. Methods


Material should be fixed as soon as possible. There are many reasons to believe that the Golgi apparatus is an exceedingly sensitive barometer to cellular conditions. It is certain that the Golgi apparatus is subject to definite post-mortem changes (Cajal, 1914).

(a) Silver Methods:

I. Aoyama's Method (block staining):

This method is essentially a modification of Cajal's, which is itself derived from Golgi's. Golgi first studied his "apparatus" by fixing nerve cells in a fixative similar to Altmann's, and then treating them with silver nitrate. The Golgi bodies slowly reduced silver nitrate to silver, and thus became blackened. Then Cajal published his "photographic" method which involved fixation in silver nitrate and reduction in a photographic reducer, hydroquinone. Later, he improved this by fixation in formaldehyde, followed by silver impregnation, and then reduction. He then discovered that the addition of certain nitrates to the fixative was favourable to the subsequent impregnation of the Golgi apparatus with silver. He found uranium
nitrate the best.

Afterwards, Da Fano reinvestigated the matter and recommended cobalt nitrate as giving more constant results.

Aoyama discarded the uranium salt because it penetrates badly, and cobalt because the salt is very hygroscopic. He chose cadmium chloride not only because it is very stable and diffusible, but also because it has a greater affinity for lipoids than Cajal’s or Da Fano’s salts. There is not, however, a great deal of difference between Cajal’s, Da Fano’s and Aoyama’s fluids. Therefore, the Aoyama’s technique was preferred to other silver impregnation methods (Ramon y Cajal and Da Fano) because it gives excellent results, showing a clear picture of the Golgi apparatus. Because of its ease of application and clarity of impregnation it was used as a basic method for comparative study. Aoyama’s technique consists of the following steps:

1. Fix in Aoyama’s fluid for four hours. Aoyama’s fluid consists of 1 gm. cadmium chloride, 15 c.c. neutral formalin, and 85 c.c. distilled water.

Because I used four large pieces of tissue (whole heads), I increased the time of fixation from four hours to eight hours and then to twentyfour hours. Apparently, there was no difference, i.e., impregnation was not improved by the longer time of fixation. This proved that this fixative
penetrates fairly quickly (the four hours fixation was on small pieces of nervous tissue, Gatenby, 1950).

(2) Wash well in several changes of distilled water for five minutes.

(3) Transfer to newly made 1.5% silver nitrate solution and leave for two days in the dark, changing the fluid every day.

(4) Wash well in distilled water for three to five minutes.

(5) Transfer to the reducing fluid for one day. The reducing fluid consists of: 1.5 gm. hydroquinone, 15 c.c. neutral formalin, 85 c.c. distilled water, and about 0.3 gm. sodium sulphite, sufficient to produce a yellow tinge.

(6) Wash in distilled water for fifteen minutes.

(7) Dehydrate in alcohol, clear in xylol or better in terpeneol, embed, cut sections, mount on slides and leave to dry in oven.

It should be remembered that the sections are now already stained with silver, so remove the wax and mount in canada balsam for examination. In this method, the silver is taken up by the Golgi apparatus and its inclusions (secretory granules), but the rest of the cell remains unstained, i.e., the position of the nucleus is pale yellow, with no nuclear details or nuclear membrane stained.

If the material needs decalcification, it is important to choose a suitable decalcifying fluid, which
will not interfere with the reaction. It is also necessary to decide whether to decalcify before or after impregnation. As mentioned before (page 8), the results obtained from this investigation led to the following conclusion:

(1) Decalcification must be carried out after impregnation, i.e., after step 6 in the procedure.

(2) Nitric alum must not be used, as it removes the brown colour; the iron replaces the silver.

(3) Although trifluoroacetic acid gave good results the 3% nitric acid in 70% alcohol was preferred to avoid the acetic acid.

Over-impregnated sections were toned with yellow gold chloride. The preparations were brought down to distilled water, and then left for about fifteen minutes in a 0.1% solution of gold chloride. Sections may be placed in a 1% oxalic acid solution to complete the reduction of gold chloride, but this is not necessary. Metallic gold will now have replaced silver, and the cytoplasm will be very pale transparent bluish grey. Rinse in distilled water, and leave for five minutes in a 5% solution of sodium thiosulphate (hypo) to remove any silver salt that might be still in the sections. Then wash, dehydrate, clear, and mount in the usual way.

Some of the Aoyama silver nitrate sections were counterstained with haematoxylin-eosin, to show the mito-
chondria in the same cell. In this case, the alum bath is short, because silver is extracted with the long method.

II. Power's method (1952) (on the slide staining):

This method is a modification of Romanes' silver chloride method (1950), which was originally devised to trace the nerve fibres in teeth. The modification was to mordant the sections before staining by Romanes' method in a 0.5% cupric nitrate overnight in a 37°C oven in order to diminish the background staining and to intensify the staining of the nerve fibres.

This method is as follows:

1. Remove paraffin from sections with xylol and hydrate through graded ethyl alcohols to distilled water.
2. Place in 0.5% aqueous cupric nitrate solution over night in 37°C oven.
3. Rinse through several changes of distilled water.

From here on follow Romanes' silver chloride method (1950) which is as follows:

4. Place overnight in the following solution in 56°C oven

\[
\begin{align*}
\text{Distilled water} & \quad \ldots \quad \ldots \quad \ldots \quad 50 \text{ c.c.} \\
0.1\% \text{ silver nitrate} & \quad \ldots \quad \ldots \quad 2.9 \text{ c.c.} \\
0.1\% \text{ sodium chloride} & \quad \ldots \quad \ldots \quad 1.0 \text{ c.c.} \\
1\% \text{ ammonia} & \quad \ldots \quad \ldots \quad 16 \text{ drops (0.7 c.c.}}
\end{align*}
\]

5. After incubation, the sections now light brown in colour, are rinsed in distilled water and developed for five minutes
Hydroquinone ... ... ... 1.0 gm.
Sodium sulphite, anhydrous ... ... 5.0 gm.
Distilled water ... ... ... 100 c.c.

(6) Wash in distilled water for two minutes.
(7) Place in 2% oxalic acid, not more than three minutes.
(8) Wash and place in 5% sodium thiosulphate for three to five minutes.
(9) Wash, dehydrate, clear and cover.

It must be stressed that, although the Aoyama method is widely used to demonstrate the Golgi apparatus in all tissues, it has been used for the first time in the present work to show this organelle in the tooth germ layers. Similarly, the Golgi apparatus has been demonstrated, also for the first time, by the "on the slide" method (Power 1952), which is a method for tracing the innervation of dentine.

In all silver impregnation methods care must be taken of the following points in order to ensure good impregnation of the Golgi apparatus: (1) Fixation and subsequent treatment are to be carried out in the dark, in glass stoppered bottles. (2) As with all silver routine, all glassware should be chemically clean to avoid precipitation. Impurities in tap water have an important role in the silver methods, for example, if the glassware is contaminated with tap water, this might produce a
precipitate of silver chloride, in which case the solution must be discarded. Therefore, it is preferable to use double distilled water to ensure complete cleanliness.

(b) Osmic methods:

I. Osmic acid method:
(1) Fix in 2% osmic acid for a week in the dark at 37° C.
(2) Wash in distilled water and leave overnight.
(3) Decalcify if necessary.
(4) Dehydrate, clear and embed.

II. The Nassonov's modification of the Kolatchev's technique:

For simplicity it is called Kolatchev-Nassonov technique or modified Champy.

(1) Fix in modified Champy for 24 hours. Modified Champy consists of: equal volumes of 6% potassium dichromate, 1% chromic acid and 2% osmic acid.
(2) Wash in running water overnight.
(3) Transfer to 1 to 2% osmic acid solution at 35° C for seven days.
(4) Transfer to distilled water for one day.
   (Decalcification is usually carried out after this step).
(5) Upgrade through alcohol, clear in xylol, and embed in paraffin.

In both the above osmic methods, I and II, over-osmicated sections can be cleached in 0.25% potassium permanganate, followed by 1% oxalic acid to remove the brown
precipitate.

Osmium tetroxide solutions fix and blacken the Golgi apparatus and fat. Tissues fixed in osmium tetroxide should be washed overnight in running water, for any osmium tetroxide left in the tissues would be reduced by alcohol and leave a precipitate. However, it is a magnificent preservative of the living structure of cells. If used on very thin slices of tissues, it gives very life-like preservation, and this is the reason for its use in electron microscope studies.

Although the Kolatchev-Nassonov technique usually gives good results, especially with nervous tissue, and although the Kolatchev technique was used by Beams and King (1933) and Jasswijn (1924) to demonstrate the Golgi apparatus in developing teeth, I was not successful in showing this organoid as clearly as with the Aoyama silver method. I think this failure was mainly due to the fact that the teeth were not dissected out. This resulted in an uneven fixation at different depths. It seemed that the osmic acid has a low penetration power, because the peripheral tissue was over-osmicated while the inner parts had only a pale grey colour.

Sections obtained from this method were bleached as mentioned above, and used for demonstrating other structures after staining in Heidenhain's iron haematoxylin.
2. Cytological method for demonstrating the Mitochondria:

Regaud's Champy's and Helly's fluids were used as fixatives for the study of the mitochondria. The sections were stained in Heidenhains iron haematoxylin. The first fixative gave the best results.

(a) **Fixation with Regaud's fluid:**

1. Fix for 24 hours in Regaud's fluid (20 c.c. neutral formalin, 80 c.c. potassium dichromate).

2. Transfer to 4 to 5% potassium dichromate solution for one week, changing the fluid every day.

3. Wash in running water for 24 hours. (Decalcify if needed after this step).

4. Dehydrate in alcohol, clear in xylol, and embed in paraffin wax.

(b) **Fixation in Champy's fluid:**

1. Fix for 24 hours in Champy's fluid (7 c.c. of 3% potassium dichromate, 7 c.c. of 1% chromic acid, and 4 c.c. of 2% osmic acid).

2. Wash in running water for 24 hours. (Decalcify if needed after this step).

3. Dehydrate, clear, and embed.

(c) **Fixation in Helly's fluid (Zenker formol):**

1. Fix for 24 hours in Zenker formol fluid (100 c.c. distilled water, 5 gm. mercuric chloride, 2.5 gm. potassium dichromate, 1 gm. sodium sulphate). To each 100 c.c. of
this solution add 5 c.c. neutral formalin (40% formaldehyde) immediately before use. (The pH of this fluid is 4.7).

(2) Transfer to 4 to 5% potassium dichromate solution for a week, changing the fluid every day.

(3) Wash in running water for 24 hours. (Decalcification after this step).

(4) Dehydrate, clear, and embed.

In this method 0.5% iodine in 70% alcohol is used to remove the mercury precipitate. This step is done during dehydration in 70% alcohol, or by treating the sections with iodine solution.

Potassium dichromate ($K_2Cr_2O_7$) is one of the oldest and commonest fixing reagents. The usual solution used is 3% in distilled water. A saturated solution contains about 12%. Potassium dichromate gives a homogeneous fixation of the cytoplasm without precipitation. It preserves lipines and hence is used for the fixation of mitochondria. However, it requires thorough removal from the tissues with running water. Solutions of potassium dichromate have pH values of: 1% : 4.10; 2.5% : 4.05; 5% : 3.85. The degree of acidity of this salt appears to condition the type of fixation (Zirkle, 1930).

When the reagent is less acid than a variable critical pH 4.2 to 5.2, fixation is good. But when acidified (e.g. by chromic acid) beyond the critical point, then both
chromation and cytoplasm are precipitated as meshworks, i.e., chromosomes are well fixed, and mitochondria destroyed. There are two characteristic fixation pictures depending upon the pH of the reagent: (1) with chromic acid and more acidic dichromates (barium, calcium, mercuric or silver) there is destruction of mitochondria and disorganization of the cytoplasm and nuclear contents resisted only by the nucleolus, (2) with potassium dichromate and other less acidic dichromates (ammonium, lithium, or sodium), the mitochondria, cytoplasm and nucleus are well fixed. Thus, it can be said that the morphological features of the fixed cells are determined by the fixing reagent. Postchroming (i.e., the subsequent treatment of tissues which have been previously fixed in fluids of Regaud or Helly with a solution of 2½ to 3% potassium dichromate for three to seven days) can influence staining properties. It is especially used to mordant mitochondria. Also they oxidize unsaturated lipids to substances which are insoluble in the usual fat solvents. This is the basis of Ciaccio's (1909) method for preserving lipids for demonstration in paraffin sections. Staining using the Heidenhain's Iron Haematoxylin method: (1) Bring sections, fixed in Regaud, Champy or Helly to water. (2) Mordant in 2.5% solution of ferric alum (not less than three hours (short method), it might reach 24 hours (long
method). There was no difference in the results between the short and long methods.

(3) Wash in distilled water.

(4) Stain in "ripened" haematoxylin solution for the same length of time as in the mordant.

(5) Rinse in distilled water.

(6) Differentiate in the mordant solution (2.5% ferric alum), which washes out the stain. The process of differentiation ought to be controlled under the microscope. The sections are removed from time to time from the alum solution, and put into tap water whilst they are examined. This is favourable to the stain. As soon as a satisfactory differentiation is obtained, the preparations are washed in running water for 10 minutes to remove the excess of the mordant.

(7) Dehydrate in alcohol, clear in xylol, mount in canada balsam. Eosin can be used as a counterstain.

Haematoxylin stain was prepared by dissolving 0.5 gm. haematoxylin in 100 c.c. distilled water. The solution was left for a month to "ripen", i.e., for the haematoxylin to undergo partial oxidation into haematin.

It was also prepared by dissolving 0.5 gm. haematoxylin in 10 c.c. of 95% alcohol, then 90 c.c. of distilled water is added. This solution takes only a few days to "ripen".

This method, introduced by M. Heidenhain (1892)
remains the standard cytological stain. After Regaud and similar fixatives it demonstrates the mitochondria, various secretory products and fibrils.

Sections of material fixed in Regaud's fluid were also stained by Held's molybdic acid hematoxylin (Lee, 1937, page 374), and Gomerl's method (Gray, 1954, page 592), for reticulin fibres (von Korff's fibres).

Wax embedded serial sections on the same slide sometimes reacted differently, although they had all been treated identically. This may have been due to the partial removal of some lipid component from the section or due to over-washing the fixative. This explanation is not wholly satisfactory. It is probable that these anomalies are due to the fact that it is practically impossible to control exactly the conditions of wax embedding.

3. Cytological Method for demonstrating Fats:

Sudan black technique. Paraffin sections:

Fixation:

(a) Aoyama's fluid (without silvering):

(1) Fix for four hours in Aoyama's fluid.

(2) Wash in running water for twentyfour hours.

(3) Dehydrate in alcohol, clear in xylol, and embed in paraffin wax.

(b) Regaud's fluid

(1) Fix in Regaud's fluid for twentyfour hours.
(2) Transfer to 4% potassium dichromate for a week at 37° C, changing the fluid every day.
(3) Wash under running tap water for twentyfour hours.
(4) Dehydrate, clear and embed in the usual way.

**Staining: Sudan Black:**

(1) Bring sections down to 70% alcohol.
(2) Stain in a saturated solution of Sudan Black B in 70% alcohol, for 5 - 10 minutes, or better, in a dilute solution of Sudan Black B, made by mixing equal volumes of the saturated solution and 70% alcohol, for 2 - 3 hours.
(3) Differentiate in 50% alcohol for 30 - 60 seconds.
(4) Stop differentiation in distilled water.
(5) Mount in Apathy gum (25 gm. gum Arabic, 25 gm. sugar, 25 c.c. distilled water) or glycerine jelly, both of which are useful in mounting after water.

4. **Histochemical method for polysaccharides.**

Slides obtained by the Feulgen technique described in the previous chapter (page 50) were examined for the presence of polysaccharides.

Sections were photographed by ordinary light.

The optical equipment was a Watson Microscope. A camera was fitted on its eyepiece, and photomicrographs were taken at the following magnifications: 80, 340, 440, 760. All photomicrographs in this chapter were produced by the writer.
B. OBSERVATIONS.

I. Golgi Apparatus

A. Aoyama's Method:

Since the different developmental stages of the molar teeth of the mouse have been described in the previous chapter, it will be sufficient to point out that the cells of the inner enamel epithelium differentiate first at the cusp tips and then downward towards the cervical loop, so that a marked but gradual transition between the short undifferentiated ameloblasts and the taller differentiated ameloblasts can be observed in the same section. It will also be recalled that, during the transition from undifferentiated to differentiated ameloblasts, the nuclei migrate from their position at the base of the inner enamel epithelium to the opposite end of the cell, i.e., the end directed towards the enamel pulp. In this way, the original basal or mesenchymal end of the inner enamel epithelium has become the free or discharging end of the differentiated functional ameloblasts, as indicated by the laying down of the enamel. Differentiation starts usually in the late bell stage. I should mention that, in the silver preparations,
no nuclear details are demonstrable, except in the case of dividing nuclei, especially in the late anaphase stage when the two daughter chromosome sets can be easily distinguished (Fig. 37b). In other words, the position of the nucleus is represented by a colourless space to which is attached the classical Golgi apparatus.

(a) **Inner Enamel Epithelium**:

In the undifferentiated inner enamel epithelial cells, the Golgi apparatus has a compact net-like form and is located above the nucleus, i.e., at the enamel pulp end of the cell (Fig. 37a, b, c, and Fig. 38). This position of the Golgi apparatus is similar to that of the cells of the Malpighian layer of the oral epithelium (Fig. 39), from which the inner enamel epithelial cells have been derived.

In the inner enamel epithelium, at the beginning of differentiation, i.e., when the nuclei start to move away from the basement membrane, a striking migration of the Golgi apparatus to the opposite pole of the nucleus is observed.

It has been noticed that the Golgi apparatus migrates in the form of lengthened cords or strands some of which are broken into short rods, along the sides of the nuclei, to take up a position in the differentiated ameloblasts adjacent to the nucleus at the discharging end of the cell which was originally the basal end of the undifferentiated ameloblasts (Fig. 40a, b, c, d). Thus the terminology
used to describe the ameloblasts in this investigation is in line with that applied to other secretory cells of the body. It takes into consideration the repolarization which the cells have undergone. Thus, the part of the cell adjacent to the stratum intermedium is referred to as the "basal end" and the part facing the enamel is referred to as the "distal end" (secretory or formative or discharging end of other investigators). The Golgi apparatus in the ameloblasts immediately following the reversal of polarity again assumes a rather compact net-like structure, which gradually becomes less compact as the cells rapidly grow in height. In the tall ameloblasts which have usually produced a well developed layer of enamel, the network of the Golgi apparatus becomes drawn out into long strands, which in some cells extend throughout the greater part of the distal cytoplasm. The Golgi apparatus assumes different forms of neighbouring cells. In some cells, it forms a number of very short rods or granules arranged in a line distal to the nucleus, in others it is a normal net, while in other instances it is diffused or dispersed (Fig. 40c and Fig. 45) or even not represented at all (Fig. 40b). As mentioned before, when decalcification is carried out before silver impregnation, the staining reaction is changed and thus the nuclei are demonstrable while the Golgi apparatus is not represented by this method (Fig. 41).
Golgi apparatus remains constantly polarized on the side of the nucleus next to the enamel, i.e., the distal end (Figs. 42 and 43), even after the cell becomes short, in a general position comparable to the so-called secretory zone of many gland cells. There is no marked difference between the form, position and staining reactions of the Golgi apparatus in the decalcified and the undecalcified material.

Less attention has been paid to the Golgi apparatus of the other layers of the enamel organ, while that of the ameloblasts was studied in more detail during the developmental stages, due to the fact that the ameloblasts are considered to play the most important part in enamel formation.

(b) **Stellate Reticulum:**

The cells possess a compact localized Golgi apparatus. However, not all are polarized in the same direction.

(c) **Stratum Intermedium:**

The Golgi apparatus of the stratum intermedium cells, similar to that of the stellate reticulum, is localized in the form of a compact network usually at one side of the nucleus, but not polarized in the same direction in all the cells (Fig. 40a). However, it seems that those adjacent to the ameloblasts which have started enamel matrix
production have also changed polarity and the Golgi apparatus is observed next to the ameloblast end (Fig. 42). Thus the migration of the reticular apparatus in the cells of the stratum intermedium does occur, but can be observed only in those cells of this layer which are adjacent to the ameloblasts.

(d) Odontoblasts:

In the very early stages of development, the Golgi apparatus of the dental pulp cells is in the form of a compact network at one side of the nucleus, resembling in a general way what has been described for the stellate reticulum cells. In other words, these cells, while probably individually polarized, are not so in any one particular direction. However, it is interesting to note that when these cells differentiate and become arranged in one layer to form the odontoblasts the Golgi apparatus becomes polarized in one direction, towards the dentine enamel junction (Fig. 40a, b, c, d). The odontoblasts possess a well formed net-like Golgi apparatus which is very easily demonstrated by this silver method, in a localized position between the nucleus and the discharging end of the cell (dentine end), similar to other secretory cells. The Golgi apparatus of the odontoblasts is very well developed even before that of the inner enamel epithelium changes polarity. This is due to the fact that dentine is deposited
before enamel. However, the Golgi apparatus of the odontoblasts like that of the ameloblasts, differs in form from cell to cell according to its physiological state, i.e., sometimes anastomosing strands of the Golgi filaments form a typical network, in some cases the Golgi apparatus appears hypertrophied, in others diffused, and still in others an atrophied type of Golgi apparatus is present.

Other Structures shown by the Aoyama Method:

(a) Ameloblast layer and Enamel:

(1) In both the undifferentiated and differentiated inner enamel epithelial cells, some compact granules which give the appearance of a globular body are often observed in the basal end (stratum intermedium end). They appear dark brown after this method (Fig. 40a, b, d, and Fig. 43).

(2) In the distal cytoplasm of the actively secreting ameloblasts, there are often found some granules of different sizes which are probably connected with the formation and calcification of enamel, and topographically related to the Golgi apparatus (Figs. 40d, 42 and 43).

(3) The preameloblasts are observed to be surrounded throughout their whole length by an apparently homogenous substance, "cementing substance". This substance appears, sometimes, to be discontinuous due to the fact that the nuclei at this stage are at different levels, or due to the plane of sectioning (Fig. 40c). This cementing substance
can be seen to be continuous with the interprisms of the enamel. In most instances, this interprismatic substance appears as a row of projections extending between the cells from the last formed matrix (Fig. 44). In some preparations, it appears to form clumps at the basal end (Fig. 40a, b, d). In the later stages of enamel formation, the intensely staining "cementing substance" is not observed between the ameloblasts.

(4) The presence of a double basement membrane separating the inner enamel epithelium and the odontoblast layers is demonstrated by this Aoyama silver nitrate method as shown in Fig. (45).

(5) As is known, the outer surface of the enamel organ, which has not completed its growth, is very similar to a "honeycomb" which has a hexagonal shape. This observation is demonstrated in Fig. (46).

(6) The prisms appear to be dotted with the minutest black grains of reduced silver on the lateral surfaces of the prisms cut longitudinally (Fig. 47a, b), these grains acquire the appearance of fine lines outlining both sides of each prism.

(7) In young enamel, the prisms show the "ladder-like" appearance separated by the interprisms whose fibres run longitudinally (Fig. 48a, c, d).

(b) Odontoblast layer and dentine:
(1) A great abundance of fibres in the dental pulp are stained dark brown by this method, especially the so-called "Korff's fibres" between the odontoblasts (Fig. 40a, c, d). The fibres are observed to originate from the pulp cells as thin fibres, thickening at the periphery of the pulp to form relatively thick bundles which pass between the odontoblasts.

(2) In a high magnification, the silver is seen to precipitate in the dentinal tubules. The precipitate can also be observed in the side branches of the tubules (Fig. 47a, b, c).

(3) The nerve fibres are observed passing parallel to the odontoblastic layer, i.e., transversely to the tubules (Fig. 47c) and they seem to extend along the sides of the dentinal tubules.

An interesting structure was observed and is shown in Fig. (49) which appears as two fibrils passing in the enamel which is more intensely stained in this vicinity. Another intensely stained region at the periphery of the dentinal tubules is observed. This feature might be explained as an abnormality in the formation of the hard tissues due to either hyper- or hypo- calcification.

B. Power's Method:

(1) Golgi Apparatus:

Power's method was mainly used for the demonstration
of the nerve fibres. However, it also showed the Golgi apparatus, especially that of the odontoblasts which was observed in the form of a classical network (Fig. 50 a, b, d) in the same position seen in the Aoyama silver nitrate preparations (Fig. 40b, c, d), but less compact. The Golgi apparatus in the ameloblasts was also observed (Fig. 50a) in the same position, i.e., distal end and the same form as in the previous method, but the intensity of the stain was less. The slight variation is attributed to the difference in the technical methods, i.e., the Aoyama method is stained in "bulk", while Power's method is an "on the slide" silver method.

(2) Nerve Fibres:

In both decalcified and undecalcified material, Power's method has shown that the pulp contains numerous nerve fibres, which can be followed accompanying the pulpal blood vessels and into the subodontoblastic (zone of Weil) and odontoblastic layers (Figs. 50a, b, c and 51a, b). The nerve fibres stained black in contrast to the much lighter staining of the surrounding non-nervous structures. By Power's method and with serial sections, the continuity of the nerve fibres was traced in the pulp as shown in Fig. (50a, b) and Fig. (51a, b). The nerve fibres maintain a close relationship with blood vessels, either as single fibres intimately associated with vessel walls, or as small
nerve bundles which accompany the vessels, from which very thin branches could be easily traced passing between the odontoblasts (Fig. 50a, b, c).

Gomeri's Method for Korff's Fibres:

Korff's fibres revealed by Gomeri's method were best observed at the base of the tooth arising from the subodontoblastic layer as simple fibres, corkscrewing on their way between the odontoblasts (Fig. 52a, b).

2 - Mitochondria.

(a) Ameloblasts:

I was able to detect the mitochondria in the ameloblasts only when enamel matrix production had started. This is due to the fact that in the very early stages, the nucleus nearly fills the cell and later, the inner enamel epithelium nuclei are at different levels which makes it difficult to see the mitochondria. They are in the form of granules which are fairly numerous and distributed throughout the greater part of the cytoplasm, at both ends of the nucleus, i.e., basal and distal. However, the mitochondria at the distal end show less distinctly, while at the basal end they are closely packed (Fig. 53a, c, and Fig. 54 a, b).

The number of the mitochondria and the intensity of staining vary in the cells of different zones of the tooth, as well as in the same zone, during the different
physiological states (Fig. 55a, b, c, d).

(b) **Odontoblasts:**

The mitochondria are observed in the form of fine granules which are fairly evenly distributed throughout the cytoplasm of the odontoblasts (Fig. 54a). In this figure, the lighter appearing regions in the cells are those occupied by the Golgi apparatus (see Fig. 40a).

However, the mitochondria in the odontoblasts do not show any striking characteristics and seem to show little or no change in form and distribution.

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3. **Fats.**

(a) **Ameloblasts:**

In both calcified and decalcified Sudan Black preparations, a number of fine lipoidal granules which varied in amount from cell to cell were observed in the ameloblasts at the basal end as well as the distal end. However, some cells showed a homogeneous sudanophilia at the distal end, Golgi zone, (Fig. 56a, b, c, d).

(b) **Enamel Matrix:**

The newest formed enamel, in both calcified and decalcified sections stained strongly with Sudan Black (Fig. 43b). This figure shows clearly the arrangement of the prisms and the interprisms, and the "ladder-like" character of the rodent enamel is well demonstrated.
(c) Odontoblasts:

Lipid granules or a general sudanophilia are observed in the cytoplasm of the odontoblasts (Fig. 43b and Fig. 56b). The number of granules and intensity of stain vary from cell to cell according to the physiological state.

(d) Dentine:

The dentine is stained faintly with Sudan Black (Fig. 43b), in both calcified and decalcified preparations. However, the dentine predentine line is sudanophile.

4. Polysaccharides

The Feulgen technique, or the periodic acid–leucofuchsin method, oxidises certain hydroxyl groups of carbohydrate portion (glycoprotein, glycogen, polysaccharides (acid, alkaline, neutral and nucleopolysaccharides) monosaccharides, free aldehydes, lipoprotein compounds, etc.) to aldehyde groups. These aldehyde groups react with colourless leucofuchsin to form a dye compound which stains pale pink to red violet. Since no specific histochemical test was made to differentiate these compounds, I shall refer to these Feulgen-positive red granules, observed during this study, as polysaccharides in general.

Polysaccharides, in the form of Feulgen-positive red granules of different sizes, were observed to be widely distributed in all components of the enamel organ and dental
pulp during the developmental stages up to five days after birth. Also the cytoplasm of the oral epithelium shows a positive reaction to Feulgen, in the form of a diffuse pink colour in the mucous layer and fine red granules in the Malpighian layer.

In the bud stage, red Feulgen-positive granules are present among the cells. During the early cap stage, numerous sparse Feulgen-positive granules of variable size are observed among the cells of the enamel organ, i.e., stellate reticulum and inner enamel epithelium (Fig. 4a, b). These red granules were observed in the different layers of the tooth germ during the successive developmental stages up to five days after birth. They are observed in extracellular regions such as in the ground substance of the dental pulp and of the stellate reticulum and stratum intermedium.

Feulgen-positive granules are present in the differentiating ameloblasts. As the cells elongate these granules increase, and are maintained throughout the period of enamel production. They are visible in both distal and basal portions of the cell. However, they are more numerous in the formative zone (Golgi apparatus region) (Fig. 57); some of these granules appear to be enclosed in a Feulgen-positive shell. Tomes processes and young enamel are also faintly stained, and some of these granules were observed
in the ground substance of enamel.

The differentiating odontoblasts and the predentine matrix contain numerous Feulgen-positive red granules. This is best seen in the cervical loop region (Fig. 35c) but towards the cusps, where the cells are more or less mature, the number of these granules and their intensity of stain decrease. The ground substance of dentine shows some of these granules. However, the pulp cells, fibres and ground substance are more Feulgen-positive.

It is of interest that the same red granules were observed in the developing bone of the jaws, while the cytoplasm of the cartilage cells of the nasal septum showed a diffuse pink colour.

*Some features observed in sections prepared by different techniques*

I. Ameloblast layer:

(1) A "cementing substance" is observed between the pre-ameloblasts and appears to extend from the stratum intermedium end and projects beyond the distal basement membrane. In some instances, it is discontinuous, due to the fact that the nuclei are at different levels. This feature seen clearly in Fig. (58a, b, d) is comparable to the silver preparation seen in Fig. (40c). The thickness of this darkly stained region's "cementing substance" when compared
with the width of the cells themselves makes one sure that it is not a different cell. This substance deposited on the lateral walls of the cell membrane initiates the formation of the interprism substance.

In the tall ameloblasts, this substance is observed only at the distal end, and appears as a row of projections bordering Tomes' processes (Fig. 59d); and attached to the last formed matrix (Fig. 44).

(2) A membrane is always observed between the stratum intermedium layer and the inner enamel epithelium (ameloblasts), (Fig. 58a, b). This membrane appears to be thickened at places (terminal bar apparatus), i.e., at places where the intercellular spaces are filled with the cementing substance, which gives the impression that this substance originates from the stratum intermedium layer. Another membrane is also present at the distal end, i.e., at the dentine enamel junction (Fig. 58a, b, d).

(3) In later stages, very little space exists between the tall ameloblasts. The cells appear closely applied to each other, giving the impression of a syncytial epithelium (Fig. 61a, b).

The secretory ameloblasts are rather tall, lined up in an orderly fashion, and hardly exceed the nucleus in width. The nuclei are oval in shape, basal in position (stratum intermedium end) and possess many small masses of
chromation (Fig. 61a, b). A small amount of cytoplasm is proximal to the nucleus while the largest amount is distally located. When the maximum length is reached, the ameloblasts exhibit an extremely complicated cytoplasmic organization characteristic of that observed in certain cells of known secretory function.

(4) The cytoplasm of the cells shows a wide variation in structure. It may be either finely granular (Fig. 59d), or have granules of coarse irregular type (Fig. 60b, c). The small granules fuse to form larger bodies. A wide variation also exists in the amount of granulation. This granular structure, in fact, is often entirely absent, and one finds in its place a vacuolation of the cytoplasm. These vacuoles are usually small near the nucleus, and increase in size as they approach the secretory border (Fig. 60a). These clear spaces are scattered through the cells in the vicinity of the Golgi apparatus. Each of these spaces, some of which contain secretory granules, sometimes appears to be enclosed by a membrane or shell (Fig. 60a, b, c) and (Fig. 57). These spaces possibly represent vacuoles the contents of which were dissolved out during the preparation of the tissue or were discharged in the enamel region.

(5) Figs. (59a, b, c) are three successive sections in the first upper molar, showing that in the floor of the apical
pit, there is dentine only, while the roof is lined by a thin layer of enamel, so that when the tooth erupts, this enamel layer is worn out by attrition and hence we get the enamel-free area characteristic of the mouse family.

II. Stratum Intermedium:

When enamel matrix production begins, the stratum intermedium exhibits an increase in cytoplasm. At this stage the cell borders of ameloblasts adjacent to the stratum intermedium are distinct (Figs. 54a, 58a, b, d, 60a, 61a, b).

During enamel matrix production, round bodies and granules appear in the stratum intermedium and these persist to the end of matrix production (Figs. 54c, 58a, b, d, 60a). During maturation, these cells appear cubical and globules and granules are seen in these cells as well as in the tissue spaces between these cells. Similar bodies are also seen within the cytoplasm of the ameloblasts (Fig. 55d). When enamel maturation is completed, the stratum intermedium becomes reduced to a single layer of flattened cells.

III. Odontoblasts:

Prior to initial predentine formation, the most peripheral cells of the dental papilla differentiate into a single layer of short columnar cells, the odontoblasts. As predentine formation commences, these cells increase in length and show a gradual rise in the quantity of cytoplasmic detail present. Some fibres, the so-called "Korff's fibres", originate from the subodontoblastic layer and pass between
the cells. As the cells continue to elongate and move away from the dentine enamel membrane, the fibres increase in thickness between the cells, forming bundles which spread fan-like toward the separating membrane to form the predentine (Figs. 61a, b, c, d, 62a, b, c, d). The fibrils are oriented approximately at right angles to the dentine enamel junction. As predentine reaches a certain thickness the fibrils become obscured by an amorphous investing substance and a solid layer, the dentine matrix is formed. In other words, these fibres, having reached the dentine matrix, disappear in the course of calcification of the matrix. It has been observed that near the ends of the odontoblasts the predentine contains a granular substance (Fig. 62c). In the more remote portion of predentine, which is formed earlier, no granular material is observed. These fibres occupy the same position as those demonstrated by Figs. (50a, b, c) and (52a, b), prepared by different techniques.

After good fixation, very little space exists between the odontoblasts except in regions where the intercellular fibrils are present (Figs. 54a, 57). The large bundles of fibres arising from the subodontoblastic layer in passing through the narrow intercellular spaces between the odontoblasts seem to overlap these cells, and are thus more heavily stained than the other cells, (Figs. 62a, b, d).
As to the presence of a fibril in the dentinal tubule — I am not sure. Sometimes the tubules appear to consist of an almost structureless transparent tissue (Fig. 54a); sometimes a short fibre which does not go further the predentine can be seen (Fig. 59d). However, this might be due to mere optical effect.
Fig. (37a): Bud stage. Aoyama silver nitrate method. Golgi apparatus of the peripheral layer polarized above the nucleus away from the basement membrane. Nerve fibres in the mesenchyme (X 80).
**Fig. (37b):** Bud stage. Aoyama silver nitrate method. Notice dividing cell, showing Golgi apparatus beside the daughter-chromosomes (X 440).

**Fig. (37c):** Bud stage. Aoyama silver nitrate method. Polarized compact network of Golgi apparatus (X 760).
Fig. (38): Cap stage. Aoyama silver nitrate method. Golgi apparatus polarized in the same position as the oral epithelium, away from the basement membrane (X 440).

Fig. (39): Aoyama silver nitrate method. Oral epithelium; Golgi apparatus in the Malpighian and mucous layers polarized above the nucleus (X 340).
Fig. (40a): $M_1$, one day after birth. Aoyama method. Golgi apparatus in the inner enamel epithelium streaming on the sides of the nuclei, in changing of polarity. That of the odontoblasts is compact and polarized. Korff's fibres arising from subodontoblastic layer (X 760).

Fig. (40b): Same section as above. Heavily stained scalloped membrana praeformativa. Golgi apparatus of inner enamel epithelium faintly demarcated. That of the odontoblasts shows different forms in neighbouring cells, and secretory granules in its vicinity (X 760).
Fig. (40c): Same series. Diffused Golgi apparatus in ameloblasts. Korff's fibres between two odontoblast cells distinguished from the Golgi apparatus (X 760).

Fig. (40d): Incisor, 3 days after birth. Aoyama method undecalcified. Different forms of Golgi apparatus in ameloblasts and odontoblasts, cork-screw Korff's fibres, secretory granules. (X 760).
Fig. (41): M^1, 5 days after birth, Aoyama technique, decalcified in trifluoroacetic acid before staining. Nuclei are shown, Golgi apparatus is not demonstrated, contrary to what is expected (X 440).
Fig. (42): M\(^1\), 10 days after birth, Aoyama technique, decalcified in nitric alcohol. Compact localized Golgi apparatus (X 760).

Fig. (43): M\(^1\), 14 days after birth, Aoyama technique, decalcified in nitric alcohol. Golgi apparatus still polarized, compact and closer to the nucleus in the short ameloblasts (X 760).
Fig. (44): M¹, Aoyama technique. Cementing substance continuous with interprisms which appear as a row of projections (X 760).

Fig. (45): Aoyama technique. Double basement membrane showing partitions (interprismatic substance). Hypertrophied diffused Golgi apparatus in both inner enamel epithelium and odontoblasts (X 760).
Fig. (46): Aoyama technique.
$M_1$, "honey-comb" (X 340).

Fig. (47a): $M_1$, 6 days after birth, Aoyama
technique, nitric alcohol
decalcification. Dentinal tubules
and enamel prisms (X 760).
Fig. (47b): $M_2$, 14 days after birth, Aoyama technique, decalcified in nitric alcohol. Enamel prisms and dentinal tubules ($X$ 760).

Fig. (47c): $M_1$, 14 days after birth, Aoyama technique, decalcified in nitric alcohol. Nerve fibres entering predentine. Silver is precipitated in the dentinal tubules and their side branches ($X$ 760).
Fig. (48a): $M_1$, 6 days after birth, Aoyama method, decalcified in nitric alcohol. Young enamel showing prisms and interprisms (X 760).

Fig. (48b): $M_1$, 7 days after birth, fixed in Regaud, undecalcified, Sudan Black preparation, showing the structure of enamel matrix. (X 760).
Fig. (48c): M1, 10 days after birth, Aoyama method, decalcified in nitric alcohol, showing the structure of enamel. Comparison of a, b & c shows that there is no difference between decalcified and undecalcified sections of young enamel (X 760).

Fig. (48d): M1, 14 days after birth, Aoyama method, decalcified in nitric alcohol. The greater part of the enamel disappeared, only in the cervical loop region the wedge-shaped portion of young enamel escaped destruction (X 760).
Fig. (49): $M^1$, 8 days after birth, Aoyama method, decalcified in nitric alcohol. This structure in enamel is considered an abnormality (X 440).
(Fig. 50a): M¹, 7 days after birth, fixed in Regaud, no decalcification, stained according to Power's method. Bundle of nerve fibres in the pulp. Individual nerve fibres between the odontoblasts. Golgi apparatus in both ameloblasts and odontoblasts can be seen (X 440).

(Fig. 50b): Same section as above (X 760).
Fig. (50c): Section in the same series as 50a. Golgi apparatus of odontoblasts and nerve fibres between the cells (X 440).
Fig. (51): M, 11 days after birth, fixed in chloral hydrate mixture, decalcified in sodium citrate-formic acid mixture, stained according to Power's method.

(a) Nerve fibre accompanying wall of blood vessel and part of the nerve bundle (X 760).

(b) The whole course of the nerve bundle in view (X 340). This preparation shows the surrounding pale non-nervous tissue and the darkly stained nerve bundle.
Fig. (52): M₁, 7 days after birth, fixed in Regaud, undecalcified, stained according to Gomeri's method to show Korff's fibres arising from the subodontoblastic layer.
(a) - (X 440), (b) - (X 760).
Fig. (53a): $M_1$, 2 days after birth, fixed in Regaud, undecalciﬁed, stained with Heidenhain haematoxylin and eosin. Heavily stained nuclei among oral epithelium, stratum intermedium, ameloblasts and odontoblasts. Mitochondria at the basal end of the ameloblasts ($X \ 80$).

Fig. (53b): Section in the same series as above. Heavily stained cells among the Malpighian layer of the oral epithelium ($X \ 340$).
Fig. (53c): Same section as Fig. (16b). Beginning of Tomes' processes (vacuoles at distal end of ameloblasts), basal granules (mitochondria), double basement membrane, appearances of the so-called "Kionoblasts" among the ameloblasts (X 440).
Fig. (54): $M_1$, 3 days after birth, fixed in Regaud, decalcified in trifluoroacetic acid, stained in Heidenhain haematoxylin. Mitochondria at the basal end of ameloblasts, those of the odontoblasts distal to the lighter region of the Golgi zone. All the cells of both layers are alike. A thin layer of dentine is deposited (a) - (X 760), (b) - (X 340).
Fig. (55a): M₁, 12 days after birth, fixed in Regaud, decalcified in nitric alcohol, stained by Held's method, showing the fibrous young enamel and the mitochondria at the basal and distal ends of the ameloblasts (X 340).

Fig. (55b): Part of the above section (X 760)
Fig. (55c): Same section of Fig. (55a). (X 760).

Fig. (55d): Same section of Fig. (55a) (X 440).

Figs. (55b, c and d) are higher magnifications of parts of Fig. (55a).
Fig. (56): $M^1$, 11 days after birth, fixed in chloral hydrate mixture, decalcified in sodium citrate-formic acid mixture, stained with Sudan Black. The preparation shows a sudanophilia in the basal and distal ends of the ameloblasts.

(a) - (X 340), (b) - (X 440).
Fig. (56c): $M^1$, 6 days after birth, fixed in Regaud, undecalcified (ester wax embedding). Sudan Black preparation showing sudanophilia in the basal and distal ends of ameloblasts (X 440).

Fig. (56d): Same series as above. It also shows sudanophilia in the odontoblasts (X 760).
Fig. (57): \( M^1 \), 3 days after birth, fixed in Regaud, decalcified in nitric alcohol, Feulgen method. Feulgen-positive granules at the distal end of the ameloblasts, some of which are enclosed in Feulgen-positive shell, also few red granules at the basal end, and in the stratum intermedium layer (X 760).
Fig. (58): $M^1$, 3 days after birth, osmic acid fixation, trifluoroacetic acid decalcification, Heidenhain haematoxylin and eosin staining. (a) - (X 340), (b) - part of (a) magnified (X 760). They show that the cementing substance (interprisms) extend from the stratum intermedium to the enamel surface. Also they show a membrane separating stratum intermedium and inner enamel epithelium, the nuclei of the latter are at different levels.
Fig. (58c): Section in M₁, same series as Fig. (58a). Ameloblasts cut transversely and longitudinally show no difference in staining reactions (X 760).

(Fig. (58d): Section in M₁, same series as Fig. (58a). Membrane between stratum intermedium and inner enamel epithelium and that at the distal end of the latter are clearly shown. Cementing substance between preameloblasts extends from stratum intermedium and dentine enamel junction, of the same thickness throughout their course (X 760).
Fig. (59a). See next page.

Fig. (59b). See next page.
Fig. (59c)

Figs. (59a, b & c) are 3 successive sections in $M_1$ 4 days after birth, fixed in Helly, decalcified in nitric alcohol, stained with Heidenhain haematoxylin and eosin, showing apical pit and enamel-free area ($X \times 340$).

Fig. (59d): Section in $M_1$ from the same series as the above. Cementing substance (interprisms) appear as a row of projections, bordering Tomes' processes ($X \times 340$).
Fig. (60a): $M_1$, 6 days after birth, fixed in Regaud, undecalcified, stained with Heidenhain haematoxylin. Granules in the stratum intermedium and in the vacuoles of the ameloblasts (X 760).
Both figures are from one section; (b) in $M_1$, (c) in $M_1$, 7 days after birth, fixed in Kolatchev-Nassonov, decalcified in nitric alcohol, stained in Heidenhain haematoxylin and eosin. They show the presence of droplets of secretory material at the distal end of the ameloblasts ($X \ 440$).
Two successive sections in $M_1$, 3 days after birth, fixed in osmic acid, decalcified in trifluoroacetic acid, stained in Heidenhain haematoxylin and eosin. Presence of vacuoles in the basal part of ameloblasts, where the basement membrane is interrupted, all the nuclei stain with the same intensity and very little space exists between the cells (X 760).
Fig. (61c): Lower incisor from the same section Fig. (61b); (X 440).

Fig. (61d): Part of the above section magnified (X 760)
Both Figs. (61c) and (61d) show the typical prism pattern of the mouse incisor. They are also showing fan-like Korff's fibres, and that the cells of the ameloblasts and odontoblasts are all alike.
Fig. (62): M, 3 days after birth, fixed in osmic acid, decalcified in trifluoroacetic acid, stained in Heidenhain haematoxylin and eosin. Bundles of Korff's fibres give the appearance of a different cell ("radial cell") among the odontoblasts. Both sections are in the same series and same magnification (X 760).
Fig. (62c): Section in the same series as Figs. (62a and b). Notice granule at odontoblast dentine end (X 760).

(Fig. (62d): M₁, 7 days after birth, fixed in Kolatchev-Nassonov, decalcified in nitric alcohol, stained in Heidenhain haematoxylin and eosin. Korff's fibres are clearly shown (X 440).
C - DISCUSSION AND CONCLUSIONS

1 - The Golgi Apparatus

Though the histological study of teeth has greatly advanced, their cytological study has, however, developed more slowly. Very few studies have been made with the light microscope of the Golgi apparatus, mitochondria and the inclusions in the different layers of the tooth germ. Recently, some cytological electron microscope observations have been made on the ameloblasts and odontoblasts. All these investigations were limited. Light microscope studies were made only in the young postnatal animals (just after birth), and with the electron microscope studies were made only on the fully differentiated ameloblasts and odontoblasts, concentrating mostly on the structure of enamel and dentine. In all these cases, the Golgi apparatus has been demonstrated by osmic methods. No attempt has been made hitherto to follow the organelles during the life cycle of the inner enamel epithelium till the cell becomes again reduced in size. So, much remains to be learnt regarding the inter-relationships within the cell between the Golgi apparatus and the inclusions (secretory granules) found in the cells.
In this investigation an attempt was made to study this organelle during the different developmental stages (from the bud stage till the tooth is ready to erupt), and to correlate the form and position of the Golgi apparatus with the cell inclusions. The Golgi apparatus was demonstrated here for the first time by one of the classical silver nitrate methods, namely, Aoyama, and also for the first time, by a method originally devised to show the nerve fibres in teeth, namely Power's method (1952). Aoyama's method is "block" staining while Power's method is "on the slide" staining. The Golgi apparatus has never previously been demonstrated by Power's method, either in teeth or any other type of tissue.

(a) **Inner Enamel Epithelium:**

Much attention has been paid to the inner enamel epithelium since it is a layer which shows the striking feature of changing polarity. The Golgi apparatus is net-like in form in the undifferentiated inner enamel epithelium and occupies a position in the cell between the nucleus and the enamel pulp, a position similar to that of the Malpighian layer of the oral epithelium. During mitosis, it is observed to be divided approximately equally between the two daughter cells; this is in agreement with previous generally accepted results, although Dalton (1951) found that the Golgi apparatus disappeared entirely in hepatoma
cells during mitosis. During change of polarity, i.e., when the nucleus is seen to move to the opposite pole of the cell at the beginning of differentiation, there is a striking migration of the Golgi apparatus to the opposite pole of the nucleus, i.e., distal to the nucleus. It passes along the sides of the nucleus in the form of lengthened strands. In 1934, Beams and King suggested that the movement of the Golgi apparatus, in the form of long streamers, in uterine gland cells was probably due to its fluid nature and to the necessity of passing through the small space between the nucleus and the cell membrane. It is usually well developed in the stage of cytomorphosis (secretion) appearing in different forms, and tends to decrease in size becoming more compact as the cell becomes older (i.e., when the ameloblast becomes reduced in size).

Massenti (1914) described the Golgi apparatus as a diffuse network in the ameloblasts. The observations of Jasswoin (1924) disagree with the findings of Massenti. Jasswoin described in the cat a polarized condition and in addition, he noted the change of polarity of the Golgi apparatus in the ameloblasts. It seems that Jasswoin (1924) was the first to describe such change of polarity in the inner enamel epithelial cells.

Schour (1932), in his chapter "The Teeth" in Cowdry's Special Cytology, suggested the necessity for a
re-examination of the polarity of the ameloblasts.

Beams and King (1933) using the rat as the experimental animal, confirmed Jasswain's findings and re-emphasized his interpretation of the change of polarity of the Golgi apparatus in the ameloblasts.

Morphology of the Golgi Apparatus:

As to the morphology of the Golgi apparatus, while it is true that it is a polarized compact network in the early stages, after changing polarity, it is not always in the compact form. As mentioned before in the present observations, the Golgi apparatus differs in form even in neighbouring cells. This difference in form has also been reported by Cowdry (1923) in the thyroid gland. Sometimes it appears to be diffused in the cytoplasm, sometimes it hypertrophies, sometimes the cell seems to be devoid of the Golgi apparatus, while other cells contain a disperse granular Golgi apparatus. Hypertrophy of the Golgi apparatus has been reported by Da Fano (1922) in mammary glands during pregnancy and lactation. The hypertrophied Golgi apparatus is a feature which Severinghaus (1937) associated with secretory activity. While Nelson (1934), whose cytological studies are in almost complete agreement with those of Severinghaus (1937), stated that "the enlarged Golgi might be taken as evidence of an increased secretory activity of the cells", he added, "we cannot always ascribe physiological
activity purely on the basis of a morphological picture". However, the latter statement has been quite firmly established in secretory cells in general. Moreover, the form of the Golgi apparatus appears to vary in accordance with the physiological states of the cell, as Bertelmez and Bensley (1932) and Beams and King (1934) have shown. The extreme variability in the form of the Golgi apparatus makes it likely that the apparatus in life is in a state of constant slow movement.

So I think that Massenti's description of a diffuse Golgi apparatus in the ameloblasts was in part correct. During this investigation, the Golgi apparatus is observed constantly in the distal end of the ameloblasts, though it becomes more compact and reduced in size. Apparently no observation has been recorded before of the Golgi apparatus when the functional life of the ameloblast terminates with its final reduction.

**Polarity of the Golgi Apparatus:**

Considering now the polarity of the Golgi apparatus, Beams and King (1933) agreed with Jasswoin (1924) that in the early stages of development, the reticular apparatus of the cells of the inner enamel epithelium is arranged above the pole of the nucleus, at the stratum intermedium end (basal end), similarly to what is observed in the Malpighian layer of the oral epithelium from which the cells of the
inner enamel epithelium have descended. In the later stages the reticular apparatus passed along one of the sides of the nucleus gradually moving to that pole which is directed inwards (distal end), i.e., towards the dentine enamel junction, which is referred to by other authors as the formative or secretory or discharging end. The present observations are in agreement with this interpretation of the localization and change of polarity. It must be noticed that both Jasswain and Beams and King demonstrated their findings by drawings and not by photomicrographs as those presented in this study; and apart from their work, no attempt has been made to follow the change of polarity of the Golgi apparatus in the inner enamel epithelium, till this investigation was made. Other investigators working on teeth with the light microscope (Nuckolls et al. 1943, 1947; Saunders et al. 1942; Marsland, 1951), mentioned such reversal of polarity by referring to previous work of Jasswain (1924) and Beams and King (1933).

This phenomenon of reversal of polarity as indicated by the position of the Golgi apparatus, was described for other cells by Golgi (1909); D'Agata (1910); Basile (1914); Cajal (1915); Cowdry (1922, 1923); Reiss (1922); Courrier and Reiss (1922); Ludford and Cramer (1923); Giroud (1928); Bertelmez and Bensley (1932), and Beams and King (1934). In many of the above mentioned cases,
the physiological condition of the cells in which the reversed position of the Golgi apparatus took place was not known. Accordingly, these results were open to question since it has been suggested that the reversed position of the Golgi apparatus in the cells may be due to a mechanical displacement (Golgi, 1909).

**Significance of change of Polarity of the Golgi Apparatus:**

The determination of polarity in the cells constitutes one of the many perplexing problems in biology. The position of the cytoplasmic components such as the nucleus, Golgi apparatus, secretion granules, etc., have been taken to indicate the structural expression of the physiological polarity of the cell. However, Lillie (1909) pointed out that polarity is not a result of the position of the nucleus or of any configuration of granules, but "is a property of the ground substance", i.e., is due to some heterogeneous physical chemical properties of the ground substance. On the other hand, the constant and regularly polarized condition of the Golgi apparatus in many types of epithelial and glandular cells led Golgi (1909) and Cajal (1915) to suggest the possibility of using this structure as an indicator of secretory polarity. Cowdry (1922, 1923) was one of the first to apply this suggestion in a study of the mechanism of secretion in the thyroid gland. In many other cases, the Golgi apparatus has been used to indicate
a reversal in direction of secretion and as an indicator of physiological activity.

The inner enamel epithelium of the enamel organ of the developing tooth might be an excellent material to test the hypothesis that the Golgi apparatus is an indicator of secretory polarity and its morphology is an indicator of different physiological states of the cell. In other words, the inner enamel epithelium of the enamel organ offers a more conclusive test than most of these cases in which reversal in polarity had been described. It differs in that we are dealing with a case where the known original basal end of the cell becomes the discharging end and remains such throughout the development of the tooth.

Jasswoin (1924) stated that although he observed that the secretory granules elaborated by the ameloblasts appeared in the same area occupied by the replaced reticular apparatus, he did not succeed in following the participation of the Golgi apparatus in the production of these secretory granules, as has been demonstrated convincingly by Nassonow (1923). Nevertheless, Jasswoin suggested that these granules participate in the transformation of Tomes' processes into enamel prisms.

Although Beams and King (1933) did not describe any secretion granules in the Golgi zone, they stated that "there is definitely an obvious morphological reversal of
polarity in the ameloblast corresponding to the change in functional polarity, and that these facts can be interpreted to favour the secretion theory of enamel formation". They added that "it is difficult to believe that the reversal in polarity of the Golgi apparatus in this particular case is not associated directly with a change in function of the cell. It is true no doubt, that as the cells beginning to secrete grow rapidly in height, the Golgi apparatus is stretched and extends proportionally, but this does not account for its migration to the other side of the nucleus".

The present observations support the view of Beams and King (1935) that the striking change in polarity and form of the Golgi apparatus represents an increased activity of the ameloblasts (secreting enamel), rather than the result of mechanical displacement caused by the extreme lengthening of the cells or by the accumulating secretion, since it precedes the appearance of the secretory granules.

Recently, the presence of the Golgi apparatus in the ameloblasts distal to the nucleus, has also been demonstrated in electron microscope studies by Kerebel and Grimbert (1958), Nylen and Scott (1960), Reith (1960) and Fearnhead (1960).

**Golgi Apparatus and Secretion:**

A large number of argentophil (probably secretory) granules, are observed in the Golgi zone. This observation
was also reported by Nylen and Scott (1960). In some cells, the cytoplasm of the ameloblasts in direct relation to the Golgi apparatus appeared condensed. This condensed cytoplasm is succeeded by a clear area of Tomes' process which contains very minute granules. Nuckolls et al. (1947) described the area above Tomes' process as being condensed.

Kirkman and Severinghaus (1938) stated that "the Golgi apparatus neither synthesizes secretory substances nor is transformed directly into them, but it acts as a condensation membrane for the concentration, into droplets or granules of products elaborated elsewhere and diffused in the cytoplasm".

Although the function of the Golgi apparatus in secretory cells is admitted, I am not sure whether the granules present in the Golgi zone were formed directly in association with the Golgi apparatus. This can only be determined by electron microscopy. Dalton and Felix (1954) and Gatenby, Dalton and Felix (1955) have shown by their electron micrographs that the small argentophile granules in intimate relationship with the membranes of the Golgi apparatus are formed "by budding or pinching off from the Golgi apparatus". Reith (1960), in his electron microscope study, stated that the Golgi apparatus exists just distal to the nucleus in the ameloblasts, and that gradations ranging from vesicles to flattened profiles of rods and filaments
can be seen in most sections. In addition, "the Golgi granules which vary in size are present with sufficient frequency to suggest that they are a part of this complex."

Fearnhead (1960) chapter 17, from his electron microscope study, stated that the part of the cell between the nucleus and the formative end, i.e., the Golgi zone, is packed with granules and numerous vesicles, some of which appear to be discharging their content into the extracellular region, and that convincing evidence that the ameloblasts are actively engaged in synthesis and secretion is provided by the character of the Golgi apparatus.

While more remains to be established regarding the inter-relationships within the ameloblast between the Golgi apparatus and the secretory granules, the general thesis that some connection exists between the function of this organelle and formation of these substances may meanwhile be accepted.

Although it is now generally accepted that enamel is the result of secretion by the ameloblasts, Quigley (1959) favours the view that enamel matrix arises by a transformation of, rather than a secretion by, the ameloblasts.

(b) Odontoblasts:

During this investigation, the Golgi apparatus of the peripheral cells, like the rest of the pulp cells, is observed as a compact polarized network. Polarization was
not in a particular direction, a character of the connective tissue cells. However, when the peripheral cells differentiate into odontoblasts and become arranged in a definite layer, the Golgi apparatus is seen to occupy the same position, i.e., the middle part in all the odontoblasts. In other words, the Golgi apparatus lies between the nucleus and the dentine enamel membrane, where it remains always in this position. There is a general agreement that the Golgi apparatus occupies this position in the odontoblasts (Nylen and Scott, 1958a, 1960; Reith, 1960).

As to the morphology, the Golgi apparatus shows different forms during dentine matrix production and calcification, varying from a compact net to a diffuse or hypertrophied apparatus. This variability was not as evident among the neighbouring odontoblast cells as that found among the ameloblasts.

Cajal (1914) first described the Golgi apparatus of the odontoblasts and dental pulp cells as a compact localized net. At the same time, Massenti (1914) described a diffuse network in the odontoblasts and dental pulp cells. Timofejev (1925) described a polarized condition of the Golgi apparatus in the odontoblasts without giving further information as to its morphology. Each of these authors was correct in his description since it is a known fact that the shape of the apparatus varies from cell to cell. As
mentioned before, the significance of this difference in
form of the Golgi apparatus is ascribed to the different
physiological states of the cells.

The role which this organelle plays during dentine
production might be explained on the same basis as that
during enamel formation.

(c) Stratum Intermedium:

The Golgi apparatus of the stratum intermedium
cells is localized in the form of a compact network at one
side of the nucleus but not polarized in the same direction
in all the cells. Whether the lack of uniform polarity of
these cells in any one direction is due to change in their
position during descent from the oral epithelium or to the
migration of the Golgi apparatus has not been determined,
and needs re-investigation. However, migration of the
reticular apparatus is observed only in those cells of this
layer which are adjacent to the ameloblasts, in which the
apparatus is localized in the stratum intermedium cell near
the ameloblastic end. Migration of the apparatus in those
cells adjacent to the ameloblasts was previously reported
only by Jasswoin (1924), with further comments. Such change
of polarity and uniformity of location in all the cells
adjacent to the ameloblast proves that this layer plays an
important part in enamel formation.
2 - Mitochondria

(a) Ameloblasts:

From the onset of differentiation through the entire period of matrix formation and maturation, the mitochondria were observed to be located in the basal end of the ameloblasts (stratum intermedium end). They were also observed in the distal end of the cell, but it is very difficult to differentiate them from the larger and finer secretory granules found at that end. It seems that mitochondria show little (if any) change in form, but its amount varied considerably.

Jasswain (1924) regarded Tomes' processes as extensions of the protoplasm which contained "condriosomes" (mitochondria). Through the years, the presence of granules in the basal end has been described (Shibata, 1927; Beams and King, 1933), but it has been denied rather emphatically that they were mitochondria by Saunders et al. (1942). According to Shibata and Beams and King, mitochondria in the ameloblasts do not show any striking characteristics, and are in the form of short rods and granules which are fairly numerous and distributed throughout the greater part of the cytoplasm and round the nucleus. However, in most secretory cells, the amount of mitochondria varies during the different physiological states of the cells. This feature has been pointed out by many investigators (Kater,
1931; Beams and King, 1934).

Recently, and with the electron microscope, these granules have definitely been identified as mitochondria (Watson and Avery, 1954; Nylen and Scott, 1958, 1960; Kerebel and Grimbert, 1958; Quigley, 1959; Reith, 1960; Fearnhead, 1960, 1961). In these recent electron microscope studies, only the form and position of the mitochondria in the ameloblasts have been reported. Nothing has been said of the possible functions, or the role that the mitochondria might play in the formation of enamel. According to Cowdry (1924), the mitochondria act as an interfacial film. Their surfaces absorb and concentrate such substances as fats or proteins, which gradually pass to the interior of the mitochondria, where they are deposited as such or undergo certain changes by the action of the enzymes. However, it has long been considered that the mitochondria play some special role in cell metabolism. The general evidence from work in this field is summarized by Bourne (1951).

From this brief discussion it is concluded that there is general agreement that the mitochondria is mostly located in the basal end of the ameloblasts, and that they might play a part in enamel production.

(b) Odontoblasts:

Very little attention has been paid to the mitochondria in the odontoblast layer. Shibata (1927) was the
first to make a study on the form and distribution of mitochondria in teeth of a number of mammals. His approach to the subject was mainly experimental, i.e., what happens to the mitochondria by applying heat, acids, freezing, etc. However, he stated that under normal conditions, they are in the form of granules, short rods or filaments, distributed in the odontoblast through the whole cytoplasm and round the nucleus. In recent electron microscope studies, they were observed in the distal end of the odontoblast (i.e., towards the dentine enamel junction) (Nylen and Scott, 1960).

In the present study, granular mitochondria were observed in this position. The order of arrangement is; the nucleus, the Golgi apparatus and then the mitochondria and finally the dentine enamel junction. They stain less intensely when compared with those of the ameloblasts. They do not show an apparent change in form or any striking characteristic. However, Stewart (1961) suggested that the mitochondria in the odontoblasts were associated with specific secretory activity.

3. Fats

(a) Ameloblasts:

In Sudan Black preparations, both basal and distal ends are sudanophil. In other words, lipid granules are found in the mitochondrial and Golgi zones. The amount and
size of granules varied considerably. A reciprocal relationship appears to exist between the amount of mitochondria and the amount of fat. De Robertis, Nowinski and Saez (1954) stated that, in general, where there are many drops of fat in a cell there are few mitochondria and vice versa.

Irving (1958a) observed that in Sudan Black preparations, deep blue or black granules were scattered throughout the cytoplasm of the ameloblasts in an almost haphazard way, but tended to be more on the enamel side of the nuclei (i.e., distally). In addition, some basal granules stained intensely with Sudan Black. Symons (1957) has reported findings with Sudan Black similar to those described by Irving. In 1960, Reith stated that the lipid bodies are present in the Golgi region. All these findings are in agreement and so it is concluded that sudanophil material or lipid bodies are found among the cytoplasmic inclusions of the ameloblasts, especially in the Golgi zone.

(b) Odontoblasts:

Lipid granules were observed in the odontoblasts, but less intensely stained compared with those of the ameloblasts. However, most of the cells showed a homogeneous sudanophilia, and vacuolation.

Symons (1957) and Irving (1958a) found that the sudanophil granules were distributed at random throughout the cytoplasm of the odontoblasts. In 1961, Stewart reported
the presence of vacuoles occurring in the odontoblasts. In an attempt to determine the contents of these vacuoles, he observed lipid inclusions in the cell body of the odontoblasts. Some investigators have considered the vacuoles to be indicative of degenerative changes (Gardner, 1959), or inadequate fixation (Kalnins, 1934), or have mentioned them without attaching apparent significance to them (Langeland, 1959). However, Stewart (1961) assumed them to be of physiological significance, a suggestion with which the writer agrees.

(c) Enamel and Dentine:

The present findings confirm those of Irving (1958b, 1960) that young enamel which started calcification and the dentine predentine junction stained with Sudan Black. In other words, the sudanophil material is found at the sites where calcification is being initiated, i.e., at the dentine predentine junction and in enamel which is becoming acid-soluble.

4. Polysaccharides

Red Feulgen-positive granules of different sizes were observed in all components of the tooth germ, i.e., ameloblasts, odontoblasts, stratum intermedium, stellate reticulum, as well as tissue spaces of the pulp tissue, enamel organ and tooth follicle. In addition, they were
observed in the oral epithelium. They were first detected in the early cap stage, and were seen during the onset of matrix production until the age of 5 days after birth, when they disappeared from both odontoblast and ameloblast layers. This indicates that an appreciable amount of polysaccharides is present.

The factual basis of classification of animal carbohydrates is too scanty to make a proper choice of a term. Each investigator used a certain term, for example, polysaccharides, acid polysaccharides, mucopolysaccharides, acid mucopolysaccharides, neutral polysaccharides, glycoproteins, glycogen, etc. All these terms are classified under the "polysaccharides", (Bourne and Danielli, 1957).

Previous description of glycogen distribution in teeth have been mainly confined to the early stages of development before calcification. Creighton (1896) described the presence of glycogen in the stellate reticulum of the developing incisor in guinea pig embryos. This observation was confirmed by Sundberg (1924) who reported the presence of glycogen in the oral epithelium, outer enamel epithelium, and stellate reticulum of teeth of rat embryos at the cap stage. Santone (1935) stated that glycogen is present in the stellate reticulum but is entirely lacking in the stratum intermedium. Horowitz (1942) confirmed Sundberg's findings and in addition, reported that
glycogen is present in the undifferentiated ameloblasts of the rat embryos. The same findings were reached by Glock (1940) in rat embryos and by Bevelander and Johnson (1946) in the developing pig's tooth. However, Bevelander and Johnson (1946) reported the disappearance of glycogen from the ameloblasts with the onset of amelogenesis. All the above investigators did not observe any in the pulp. However, Wislocki et al. (1948), and Wislocki and Sognnaes (1950) found that glycogen is present in the epithelial cells of the oral cavity, in the dental lamina and dental cord, in the outer enamel epithelium, stellate reticulum, inner enamel epithelium and dental pulp of the human tooth at the bell stage of development prior to the onset of dentinogenesis.

Engel (1948) indicated that glycoproteins were found in the cells of the stellate reticulum and stratum intermedium, and in the matrices of the enamel pulp and dental pulp; and that glycoproteins granules were also observed to appear in the cytoplasm of the odontoblasts and ameloblasts prior to the formation of enamel and dentine, and hence he suggested that the glycoproteins in these cells might be associated with the production of these matrices.

Glasstone (1958) reached the same results through her investigation on hamster, rat and mouse teeth. She correctly attributed the disappearance of glycogen from the
rodent tooth germ to the general metabolic changes occurring at birth. Pourtois (1961) stated that in the mouse the dental sac and the neck of the enamel organ are full of glycogen, and that mucopolysaccharides are abundant in the intercellular spaces while very few occur in the stellate reticulum. Ten Cate (1962) in his investigation of the distribution of glycogen in human teeth during dentinogenesis and amelogenesis, confirmed its presence into the stellate reticulum, stratum intermedium and inner enamel epithelium. He agreed with Kroon (1952) that the disappearance of glycogen from these layers coincides with the onset of enamel matrix production; and with Moog and Wenger (1952) that the role of glycogen in amelogenesis is as a source of hexosephosphate esters. These investigators have employed different staining methods and teeth of various animals of different ages. Among the methods employed were aqueous solution of iodine, Best's Carmine, Bauer-Feulgen method, Feulgen-method, and the PAS. The latter is used in recent years by most investigators. This might explain why there is some variation in the above interpretations and in the choice of the term. The PAS reaction (Schiff's reagent, 1866), which is used exclusively in the analysis of carbohydrates was introduced into histology by Feulgen and Ressenbeck (1924); Feulgen and Voit (1924a); McManus (1949); Lillie (1947a, b) and Hotchkiss (1948). All these
methods are based on the same principle, that certain chemical
groups in the tissues are oxidized by the periodic acid and
one of the reaction products is aldehyde. This aldehyde
can be identified by Schiff's solution (Leucobasicfuchsin),
a colourless solution, which gives a bright magenta red
colour when it is combined with the aldehyde.

Thus it might be concluded that there is a general
agreement that a polysaccharide is present in the tooth germ
layers and that it disappears from the ameloblasts and
odontoblasts during the onset of dentinogenesis and amelo-
genesis. It is suggested that this disappearance might be
explained by assuming that this substance is broken down
into simpler molecules which are used again for building
other substances used in the formation of the hard tissues.

SECRETION OF ENAMEL AND DENTINE

Structure of Enamel:

The morphological and histological investigation
of the formation of enamel has long been pursued by many
authorities. The enamel is no other substance that that
which is transformed from the material secreted from the
ameloblasts and from the substance intimately related to
these cells and probably contributed by other cells like the
stratum intermedium. According to the classical concepts
of amelogenesis, matrix formation begins with the thickening of the so-called dentine-enamel-membrane, following which a system of terminal bars and Tomes' processes are developed at the distal ends of the ameloblasts. It has also been generally agreed that the terminal bars give rise to the interprism substance and that Tomes' processes are the source of the prism substance proper (Orban et al. 1948, Noyes, 1948). There is some argument about the real identity of the structures at the distal ends of the ameloblasts which have been called "terminal bars". According to Maximow and Bloom (1949), terminal bars are commonly seen in tissues of epithelial origin and their supposed function is to close the intercellular spaces. They appear as smoothly outlined rods of a dense cementing substance located at the cellular interfaces. Two systems of "terminal bar apparatus" have been described in the ameloblasts, one at the stratum intermediate end (basal end) and the other at the formative end (distal end). The presence of a homogeneous substance between the pre-ameloblasts has already been described by von Ebner (1902) as the "cementing substance" (Kittsubstanz), and was confirmed by Smerecker (1905), Eklof (1915) and Jasswoin (1924) and in the present study, while its presence is denied by Studnicka (1917), who, however, interpreted the presence of clumps present between the ameloblasts as secretory granules protruding into the spaces between the
ameloblasts. These clumps were also reported by Jasswoin and the writer and are still considered as the cementing substance. Besides, Studnicka (1917), described another structure between the prisms and the cementing substance, which he named prism-sheath ("Prismenscheiden"). In agreement with Jasswoin (1924), the cementing substance disposed between the ameloblasts was observed in this study, to be directly continuous with the interprismatic substance of the enamel. Saunders et al. (1942) stated that the ameloblasts are united by a small amount of cementing or interprismatic substance, which arises in association with the "pre-enamel processes" and which extends through the membrane wall into the intercellular intervals, and that the interprismatic substance is essentially a similar material to that which is deposited as the "pre-enamel processes". By "pre-enamel processes" they mean the characteristic delicate processes extending peripherally on either side of the ameloblasts, which they demonstrated in a picture resembling that of Fig. (59d) in this work. From their figures and descriptions it is evident that their conclusions, Jasswoin's and the writer's are all in agreement. However, they made their statements confusing by denying the presence of the so-called terminal bar apparatus, which is actually (according to the previous definition) part of the interprismatic substance. The pre-enamel matrix or "honey-comb
layer" of earlier writers has been interpreted in various ways (Wellings, 1940). The evidence available from the study of rodent teeth, particularly rats and mice, suggest that it represents the organic constituent of enamel matrix. It is known to be devoid of calcium salts (Orban et al., 1943; Nuckolls et al., 1943 and 1947; Saunders et al., 1942); and corresponds to the cementing substance or primary enamel of von Ebner. The initial steps in enamel prism formation result from the secretory activity of the ameloblasts (Kitchin, 1933; Lams, 1921; Marsland, 1951; Nuckolls et al., 1943; Wassermann, 1944). Like other gland cells the ameloblasts maintain a definite relation to their secretory products (Beams and King, 1933).

Enamel matrix production can best be described as a two-step process. The first step is the formation intercellularly of the interprism matrix and appears as a dense material between the cells which completely surrounds the ends of the ameloblasts delineating Tomes' processes. These are the structures which ordinarily have been called the cementing substance or terminal bar system or pre-enamel matrix. The second step is the filling in the Tomes' process with material to form the enamel prisms. Enamel matrix production is then followed by a different process called maturation, which in its simple form, is the precipitation of a basic calcium phosphate from the calcium,
phosphate and other ions present in the tissue. According to Marsland (1952) the partial solubility of enamel in acid, i.e., when the prism structure can be seen as demonstrated in Figs. (42 and 47a, b), indicates that the second phase of amelogenesis, namely maturation, has started.

Cross striations in the enamel prisms were observed in this investigation along the prisms at the same regular intervals and were demonstrated in young enamel. (Fig. 48a, b, c, d). These have been previously and first described by Kitchin (1933) in the form of a "ladder". The problems encountered in defining these striations of the prisms and in ascertaining the presence or absence of the interprismatic substance in enamel have been described in previous publications (Scott, 1955; Helmcke, 1955; Helmcke et al., 1961; Hazossi et al., 1956). Many accounts of the ultrastructure of developing rodent enamel, which revealed stages in the development of the organic matrix, its calcification and the relationship between the organic and inorganic components, have been given (Nylen and Scott, Sogmaess, Frank and Sogmaess, Fearnhead, Reith, and many others). A review of the classical research into the fine structure of enamel is given by Schmidt and Keil (1958).

From decalcified and undecalcified stained preparations there is no doubt that around the periphery of each prism is a zone with a relatively high organic
content. Some consider this as the prism sheath while others regard it as the interprismatic substance. Even the electron microscope studies failed to solve the problem. Watson and Avery (1954); Lenz (1958); Quigley (1959) deny the existence of the interprismatic substance, while Frank (1959); Scott and Nylen (1960) and Avery and Visser (1960) confirm its existence. On the other hand, Frank and Sognnaes (1960) confirm it in human and deny it in rat.

Nylen and Scott (1960) observed that the "terminal bars" (of some workers) at the distal end (secretory) of the ameloblasts, formed as accumulations of dense material deposited between the cells, were present only during matrix production and are actually extracellular material identical with young enamel matrix. They concluded that these "terminal bars" at the distal ends are the source of the interprism material, and that Tomes' processes give rise to the prism substance proper, a conclusion which agrees with previous reports. However, they observed a second row of terminal bars at the opposite or basal ends of the ameloblasts, which were not structurally identical with the former ones (at the distal end), although these two terminal bar systems are optically the same structure. Although the interprismatic substance of the enamel is formed and organized before the substance of the enamel prism is formed, the enamel prism is calcified before the interprismatic
substance (Avery and Visser, 1960).

Kionoblasts:

Jasswoin (1924) described and figured "narrow and flattened cells" among the typical ameloblasts, which he, however, considered were immature forms. In his explanation he stated: "on account that some ameloblasts started the process of differentiation late, they can be observed to be staining deeply, and continuing their differentiation in the succeeding stages". In other words, he regarded an intensely stained cell as a less differentiated ameloblast. He thought that these cells, before their differentiation into fully mature ameloblasts might be responsible for the production of the "ectoplasmic substance", i.e., the "cementing substance" of von Ebner and others, now generally called the interprismatic substance.

Saunders et al., (1942) observed a darkly staining cell, morphologically different from, but lying amongst the lighter staining ameloblasts. They considered it as a supporting cell and called it the "kionoblast". It is found singly or in groups of four to five cells at irregular intervals between the ameloblasts. Compared with the ameloblasts, it was described as "a more slender cell, its nucleus is narrow and compact and its cytoplasm finely granular, but more uniform, and with routine stains more basophilic in reaction". They concluded that the inner enamel epithelium
differentiates into two types of cells, the ameloblasts and the "kionoblasts" and that the latter establishes the dentine enamel membrane, since it is derived from and continuous with the terminal extremity of the cell. They stated that "later, as the enamel is formed, the kionoblasts became incorporated in the matrix". At the same time they admitted that some material is deposited on the lateral walls of the ameloblasts, extending into the intercellular intervals as an interprismatic substance. The "kionoblast" has also been described by Westin (1952) who suggested, however, that the cell is a tubular structure concerned with carrying blood to the region of enamel formation. Symons (1955a), using different cytological methods, confirmed the presence of the kionoblast. He ascertained that the terminal extremity of this cell spreads out along the basement membrane so that at least it may play some part in thickening this membrane. Using the alkaline phosphatase technique, Symons (1955b) had shown the presence of linear streaks running across the full width of the inner enamel epithelium, in contrast to the completely unstained layer of the inner enamel epithelium which showed no enzyme activity. He stated that these streaks correspond to the "kionoblast" in position. In 1956, Symons re-interpreted his previous findings in view of the confirmation of the presence of the "kionoblast" and concluded that they appear to form a network around the
ameloblasts and that they connect directly with the "honey-comb" matrix around the developing enamel prisms. This agrees with his previous suggestions (1955a) that the kionoblasts may manufacture the interprismatic substance of the enamel (the "honey-comb" matrix representing the developing interprismatic matrix). Ten Cate (1956) also confirmed the presence of the "kionoblast" among the inner enamel epithelium. Insinuating cells of the stratum intermedium between the inner enamel epithelium were thought by Ten Cate (1961) to represent stages in the ontogeny of the "kionoblast" (Symons, 1955a, b, 1956; Ten Cate, 1956). According to Symons (1955b), "kionoblasts" exhibit high alkaline phosphatase activity, in contrast to the insinuating cells which were found to be devoid of the enzyme (Ten Cate, 1961). This chemical difference between the "kionoblast" and the insinuating stratum intermedium cells led Ten Cate (1961) to believe that they are not identical. It is generally accepted that the stratum intermedium cell is histochemically characterized by its high concentration of alkaline phosphatase, whereas the inner enamel epithelium is devoid of the enzyme. Ten Cate (1961) explained this by postulating that the insinuating cell loses its phosphatase content to become an ameloblast.

During this investigation, in one case only, in sections stained with Heidenhain haematoxylin and eosin,
appearances like those of the so-called "kionoblasts" were observed among the inner enamel epithelium as intensely stained cells or nuclei. In addition, the same picture was observed among the cells of the oral epithelium, especially the Malpighian layer, as well as among the outer enamel epithelium and the stratum intermedium of the enamel organ (Fig. 53a, b), an observation which has not been mentioned before. At the beginning, I thought that these pyknotic cells among the inner enamel epithelium were the "kionoblasts", but after examining serial sections prepared by different technical staining methods (Aoyama method, Heidenhain haematoxylin, Held, Kolatchev-Nassonov, Feulgen) all the nuclei were of the same intensity and the cells of the same size, i.e., there was no marked difference between them which excluded the possibility of the occurrence of a different cell among the inner enamel epithelium. I think that this odd case might be due to either: (1) The staining solution, Heidenhain haematoxylin, sometimes makes a scum on the surface, and if not discarded gives uneven staining, or (2) bad fixation or embedding. In this particular case, I was unable to obtain a good series which proves that there was something wrong during the preparation of the block.

Symons (1955a), using one of the methods applied in this study, namely the Feulgen method, stated that in the early stages there was only a slight difference between
the "kionoblast" and the inner enamel epithelial nuclei, and that in later stages all the nuclei of the inner enamel epithelium were alike.

Of all the techniques used in this study, the Feulgen method, being a chemical reaction, offers more proof against the presence of any type of different cells among the inner enamel epithelium, and lends further support to the fact that what is seen with other techniques between the inner enamel epithelium are not cells. Apart from the Feulgen preparations, linear streaks, described by the writer as the cementing substance, were observed between the inner enamel epithelium, which pass along the full width of the layer, or might be interrupted and discontinuous. These streaks are apparent from the time of the first differentiation of the inner enamel epithelium, i.e., when the nuclei are at different levels and starting to be arranged in the basal end (Fig. 58a, b), which might account for the discontinuity of the linear streaks, until enamel matrix production has begun (Fig. 58d). I have shown that during enamel matrix formation these streaks, (i.e., the cementing substance) appear to be continuous with the interprismatic substance. It is generally accepted that these structures being intercellular to the ameloblasts are related to the formation of the interprismatic substance and apparently produces the "honey-comb" pattern, which is
the same as that of the interprismatic substance.

From the above discussion it is clear that Jasswoin, Symons and the writer, all agree that a structure exists between the ameloblasts which is related to the formation of the interprisms. However, the difference in opinion lies in the fact that both Jasswoin and Symons believe that these structures are cells morphologically and histologically different from the ameloblasts, while the writer believes that they are not cells, but are the intercellular spaces filled with a secretion elaborated by the stratum intermedium (probably with alkaline phosphatase), to be contributed to the formation of the enamel matrix. The writer finds support to this supposition from (1) the fact that stratum intermedium as well as these streaks both show high alkaline phosphatase activity (Symons, 1955b, 1956); (2) these streaks are so thin in comparison with the ameloblasts, and with no cellular details at all; (3) Symons stated that these cells occurred in groups or singly and are connected to each other by delicate processes, i.e., not alternating with the ameloblasts, in which case, it would not be easy to accept that the kionoblasts are responsible for secreting the interprismatic substance; (4) none of the electron photomicrographs has shown any difference in dimensions or cytology among the inner enamel epithelial cells.
In conclusion, one can say that by the different technical methods used in this investigation, darkly stained streaks exist between the ameloblasts. These were considered as the intercellular spaces between the ameloblasts, filled with some sort of secretion which appears to originate from the stratum intermedium layer and pass between the cells to be deposited in the enamel matrix region. These streaks were, however, observed to be continuous with the interprisms.

**Characteristics of Ameloblasts:**

Reith (1959, 1960) reported that the ameloblasts are close to each other with a minimum of extracellular space. The present observations (Fig. 62a, b) are in agreement with this statement. Reith also stated that the terminal bars seen with the light microscope (Orban, Sicher and Weinmann, 1943) are present at the junction of the ameloblasts with Tomes' process. These structures were observed in this study and were described as the cementing substance which appears at the distal ends of the ameloblasts continuous with the interprisms.

In this study, small granules at the formative (distal) end of the ameloblasts were observed and these were seen to fuse to form larger bodies. It is suggested that these granules represent specific secretory activity. These rounded bodies have been previously noted by Hertwig (1872)
who introduced the term "calcospherites", and by Williams (1896) who called them "globules" and suggested his "globular theory of enamel formation". The first nomenclature, "calcospherites", was adopted by Mummery (1924), Andrew (1919), Lams (1921) and Noyes, Schour and Noyes (1938). Noyes et al. stated that "the calcospherites" are formed by the fusion of minute "drop-like" pre-enamel granules, which becoming larger as they reach Tomes' processes, are deposited one upon the other in the form of a large calcospherite to form the enamel prism. On the other hand, Saunders, Nuckolls and Frisbie (1942), Marsland (1951) and Symons (1962) upheld the term "globule". Reith (1960) stated that: "the wide variety of large granules is hard to interpret". However, I think that they are the result of running together, i.e., fusion of smaller secretory granules.

An examination of the sections shows that some cells reach full granulation and then enter upon degranulation, as in the normal secretory cycle, but that others obviously actively produce and discharge their granules with little accumulation of a cytoplasmic store. Quite obviously, these cytoplasmic differences are manifestations of changes in the cell to be associated with its secretory function. This is supported by Severinghaus' statement (1957) that "the secretory cells naturally go through cycles of accumulation and loss of cytoplasmic granules".
One cannot ascertain the precise origin of the vacuoles seen in the distal ends of the cytoplasm of the ameloblasts, which were thought to be the first indication of Tomes' processes (Nuckolls et al. 1943, 1947); but the relationship of the granules to the vacuoles suggest that the vacuoles are also a part of the physiologic activity of the ameloblasts. Covell and Scott (1928) described in the cytoplasm of nerve cells granules of variable size and vacuoles, some of the former being usually contained in the latter. They suggested that the granules might develop in a vacuole by a process such as condensation, or the vacuole might form simply through the adsorption of water.

The exact role of the ameloblasts in enamel formation is a matter still under consideration. Several authorities have expressed the opinion that amelogenesis in rodents is entirely an intracellular process (Watson and Avery, 1954; Quigley, 1959a; Lenz, 1959; Frank and Sognnaes, 1960). These authors reached this conclusion because they were unable to see a cell membrane between the cytoplasm of the distal end of the ameloblast and the newly formed enamel matrix in their preparations. Consequently, they denied the presence of the interprismatic substance. The evidence provided in this investigation favours Fearnhead's (1960) and Watson's recent results (1960), who now support the concept of extracellular enamel formation, and
Fearnhead's view (1961), that a granular "precursor substance" is synthesized within the cell and this is then discharged into the extracellular region where the granules undergo fibrillogenesis and mineralization, and that a cell membrane separating the ameloblasts from the newly formed enamel is present. Nylen and Scott (1958, 1960) were able to demonstrate, not only clear cell membranes in the region of Tomes' processes, but also the intercellular deposition of matrix fibrils.

In conclusion, electron micrographs (Nylen and Scott, 1958, 1960; Reith, 1960; Watson, 1960; Fearnhead, 1961) leave no doubt about the existence of a cell membrane between the newly formed enamel and the cytoplasm of the ameloblast.

**Role of Stratum Intermedium in Enamel Formation:**

The stratum intermedium is usually regarded as a layer whose functions are closely connected with the development of enamel and with the processes of calcification (Orban, 1960 and Schour, 1953). Evidence that the stratum intermedium is responsible for the elaboration of some elements which contribute in the formation of the fully formed matrix was obtained during this investigation. The stratum intermedium cells showed similar characteristics to those of the ameloblasts. The Golgi apparatus of the cells adjacent to the ameloblasts was seen polarized towards the latter cells,
suggesting that they have changed polarity, a feature of secretory cells which suggests that the stratum intermedium cells might have a secretory function. This is supported by the fact that the cementing substance which is continuous and forms the interprisms, is observed to originate from the stratum intermedium layer. In addition, during amelogenesis (enamel matrix formation and maturation), many granules of different sizes were seen in these cells and in between them, and in the spaces between them and the ameloblasts. These granules were observed in sections prepared by different methods, i.e., as Feulgen-positive red granules of poly-saccharides, as sudanophil granules, and as clumps of argentophil material.

Previous findings provide some support for this view, although the precise extent to which the stratum intermedium cells participate in the formation of the enamel matrix does not appear to be fully appreciated (Gottlieb, 1941; Jasswoin, 1924; Kreshover, 1944; Prenant, 1921; Williams, 1896, 1925). Santone (1935) observed these specific granulations in the cells of the stratum intermedium. He was of the opinion that their presence might explain an important function, either in the nourishment of the ameloblasts, or they might be regarded as true secretory cells, thus contributing in part in the formation of enamel.

In the present study it has been observed that
the stratum intermedium cells overlying the ameloblasts in the future enamel-free areas remain squamous throughout the developmental period, also the ameloblasts in this region never attain the maximum height of the rest of the layer. It is suggested, therefore, from the difference in height between the ameloblasts and the very thin layer of enamel secreted by the short cells, that some organizing substance is released by the cubical stratum intermedium cells, which is capable of initiating the formation of enamel by the very tall ameloblasts. Moreover, the stratum intermedium seems to be essential to enamel formation since it is absent in that part of the tooth germ which outlines the roots. Thus, it might be concluded that the stratum intermedium cells play an important role in the formation of enamel, a conclusion which is in agreement with Marsland (1951) and Suga (1959).

The stratum intermedium layer is known to be particularly rich in phosphatase (Bevelander and Johnson, 1945, 1949; Engel and Furnta, 1942; Gomori, 1943; Greep, Fischer and Morse, 1948; Kabat and Furth, 1941; Morse and Greep, 1947; Pourtois, 1960; Ten Cate, 1961, 1962; Symons, 1956; Avery, 1954; Sasso et al., 1957). Therefore, it might be assumed that these cells are concerned with the calcification of the matrix. This is supported by the presence of round bodies and granules in the stratum inter-
medium cells at the end of matrix production which might give evidence of continued elaboration of certain compounds, probably calcium. In other words, that the stratum inter-medium layer is rich in phosphatase, would tend to support the theory that they are actively involved in the process of calcification.

Radial Cells:

Sometimes the bundles of Korff's fibres are so thick that they overlap the odontoblast cell or lie on part of it, which might give the impression of the radial cells. But, even in these cases, one can see clearly the origin of these fibres deep in the subodontoblastic layer.

Symons (1955b and 1956) has proved that the "radial cell" and the related Korff's fibres show heavy alkaline phosphatase activity, similar to the subodontoblastic zone, from which Korff's fibres arise, while the odontoblasts have very marked content of RNA. Thus it would appear that Symons' "radial cell" is nothing but bundles of Korff's fibres (and other fibres) which pass between the odontoblasts, since both give the same reaction to alkaline phosphatase activity. A fact which lends further support to this supposition comes from Symons' statement that "it is not possible to determine the fate of this cell at a late stage in dentine formation since at that time all the cells of the odontoblast layer become similar in shape and staining
reaction". However, I have observed these darkly stained bundles among the odontoblasts in later stages. Another statement was made by Symons: "Frequently the radial cell is of greater length than the odontoblast, ..., the cell appears to show branching at both dentinal and pulp ends, the former enters the substance of the dentine, whereas the latter branches link up with the deeper pulp fibres".

The presence of a cell of different morphological appearance called the "radial cell" among the odontoblasts, was first described by Jasswoln (1924), who stated that both the radial fibres (of von Korff) and the tangential fibres (of von Ebner) in the dentine are produced by the "ectoplasm" of the "radial cell". Moreover, he claimed that some of these cells can transform into odontoblasts. The presence of the "radial cell" amongst the odontoblasts of the developing tooth has been confirmed by Symons (1955a, c) who has shown that the radial cells are responsible for the formation of the fibres of the dentine matrix, apart from the element contributed by the von Korff fibres. In 1956, he reinterpreted his previous description of the "radial cell" and has shown that this cell is connected to the dentine matrix and to certain cells of the pulp lying beneath the odontoblast layer by fibre-like branches. It is slender and stains more deeply than the rest of the odontoblasts.

I have observed that in Feulgen preparations all
the nuclei were of the same intensity and all the cells were more or less of the same size, and even the intercellular spaces, where Korff's fibres pass were of the same dimensions. Symons (1955a) made a similar statement with Feulgen method. As mentioned before, Feulgen technique is a good test for the detection of any difference which might be present. However, with other staining methods used in the present work, only the Korff's fibres which appeared to emerge from the zone of pulp tissue just beneath the odontoblast layer, stained more intensely, all the cells were alike. In my opinion, I would explain this feature as follows: Subodontoblastic fibres, in approaching the odontoblast layer, unite (or come closer) some of them form large bundles and pass between the odontoblasts and finally they separate again as they reach the predentine where they spread and form the dentine matrix. A similar description is generally accepted in describing how Korff's fibres emerge from the subodontoblastic zone, corkscrewing between the odontoblasts and finally unravel fan-like in the predentine-dentine matrix.

Much less clearly expressed views have been put forward by others in support of the role of a non-odontoblast cell in dentine formation. Mummery (1892, 1924) suggested that certain cells, smaller than the odontoblasts and related to the connective tissue fibres, play a part in dentine development. Though he described these cells as
mingling with the odontoblasts, he did not state that they are arranged in a definite layer. Hopewell-Smith (1919) stated that certain round cells of the pulp of an osteoblastic nature play an important part in the production of dentine. Of the electron microscope studies of the odontoblasts no indication of the presence of any difference among these cells was pointed out.

Finally, I conclude that these darkly stained areas between the odontoblasts are occupied by fibres which arise from the subodontoblastic layer since no cell boundaries nor nuclear or cytoplasmic details could be detected.

**Korff's Fibres:**

Using different techniques, (among which was Gomeri's method for Korff's fibres and Power's method for nerve fibres), I have observed that there were numerous simple fibrillae originating from pulpal cells of the subodontoblastic layer, which reached the dentinal matrix after corkscrewing between the odontoblasts. The majority of the fibrillae were quite simple, but among them there were some that were intertwined like ropes, and some that issued off shoots uniting with the neighbouring fibrillae. Besides these were several fibrillae which were united together in the form of fibrillous bundles, ramifying or spreading fan-like as they approach the predentine. Similarly, Shibata (1927), using a different method, gave a similar description.
but he concluded that his observed fibrillae in the pulp "do not correspond to what was believed to be nervous fibrillae by Mummery in their shape and chemical nature, but rather to Korff's dentinal matrix primitive fibres". He believed that these fibrillae are the "lattice fibres", of a similar nature to the colloid fibres, and that during the formation of dentinal matrix some of the "lattice fibres" turned into the colloidal form or into "Korff's fibrillae" ramifying longitudinally among dentinal matrices and after contribution to the formation of calcification as its foundation, they remained buried there as dentinal primitive fibres. He thus agreed with Korff's view on the whole, that odontoblasts mainly serve to participate in the formation of dental fibrillae and are merely subordinate to the dentine formation. In Shibata's view, the so-called "lattice fibres" that are widely believed to be found in the pulp, especially in the odontoblast layer, are a kind of connective tissue and have the same nature as precolloidal fibres; and that Korff's fibres originally exist as the lattice fibres and then they turn into the colloid fibres.

In the present investigation, comparison of the findings shown by the three silver methods (Aoyama, Power's and Gomeri's) and represented in Figs. (40a, c, d, 50a, b, c and 51a, b), show a striking resemblance in the origin, position and form of these fibres. However, I cannot state
definitely whether these fibres, are two different types: nerve fibres and Korff's fibres, or whether they are one and the same thing. Possibly both are of the same nature reticulin or collagen fibres.

The presence of some granules in the predentine and of granules and vacuoles in the odontoblasts at that end, which were observed during this investigation (Fig. 62c) might be interpreted as indicating that some of the elements that go into fibril formation arise in the odontoblasts.

An unknown spherical body were described by Nylen and Scott (1958) as occurring in the Golgi zone in young odontoblasts. They have also described two groups of fibrils of typical collagen. They stated that the first fibrils formed make up the so-called Korff's fibres, which constitute the major component of the predentine. These fibrils are found between the odontoblasts terminating in a fan-like arrangement in the region between the ends of the cells and the future dentine enamel junction. In later stages when the fibrillar basis of the predentine is established the Korff's fibres pass as compact bundles of fibrils between the odontoblasts through the predentine. This is a generally accepted view. The collagenous portion of the developing dentinal matrix has been investigated quite extensively (Watson and Avery, 1954; Lenz, 1958; Nylen and Scott, 1958). In 1960, Nylen and Scott observed that in areas close to the
odontoblasts large amounts of interfibrillar material and relatively few fibrils were seen. This observation led them to suggest that fibrils arise from an afibrillar substance in the subodontoblastic region, and that fibril synthesis occurred extracellularly in situ, and not from the Korff fibres which run in discrete compact bundles between the odontoblasts. Finally, they concluded that "the obvious evidence of the continuous rise in cellular activity as reflected in the increase in cytological detail, with the progression of dentine formation suggest that the odontoblasts participate more directly in the development of dentine matrix than has been previously accepted. These findings may be taken to indicate that the cells are responsible for the synthesis and secretion of the material from which the fibrils and the investing substance of dentine arise". They added that "to this consideration, supporting evidence from histochemical studies has already been published by them in 1958".

These conclusions are rather confusing. One cannot deny the participation of Korff's fibres and other subodontoblastic fibrils in the formation of dentine matrix. In addition, I quite agree that the odontoblasts play an important part in this process, by synthesizing materials which are converted into fibrils either intracellularly or extracellularly after the addition of some substance from
the subodontoblastic matrix, and that these granules may function in the maturation (calcification) of the dentine.

In support of the present observations and conclusions, Stewart (1961) described granules and vacuoles in the more mature odontoblasts. He thought that the granules represent similar bodies as those mentioned by Nylen and Scott (1958). However, Stewart is of the opinion that the vacuoles and granules represent specific physiologic activity in the odontoblasts, and that the migration of the granules into the peripheral dentine through the "odontoblast process" is a function of the maturation of dentine. He stated that the origin of the granules may be explained, as reported by Palay (1958), on the basis of specific secretory activity of the mitochondria.

Although his explanation might be accepted, one must not ignore the Golgi apparatus which shows different forms during dentine production and calcification and the part which it might play in these processes. However, both the Golgi apparatus and the mitochondria are regarded as being associated with the secretory activity of the cells.

Finally, the cytoplasm of the odontoblast is clear compared with that of the ameloblast, and thus it is concluded that the structure of the mature ameloblast is very complicated. This is supported by Quigley (1959).
Odontoblast Process:

The presence of the odontoblastic processes erroneously called "Tomes' processes" in the dentinal tubules was not confirmed in this study. The present observations agree with those of James (1957) and with Tomes' original work. J. Tomes (1856) did not state that the fibres sometimes observed in the dentinal tubules are odontoblastic cytoplasmic extensions or processes. However, he explained these appearances by assuming that "the fibril consists of a sheath containing a semi-fluid matter, similar to the white fibrilla of nerves", and that this might be the reason why the tubules sometimes appear transparent.

The precipitation of silver in the dentinal tubules and its lateral branches described in this study, had been demonstrated by Zander and Smith (1945) and by Powers (1950) who had presented figures resembling exactly those of the present work (Fig. 47a, b, c).

GENERAL DISCUSSION

The present discussion will centre on the interrelationship between the cytoplasmic organelles (Golgi apparatus and mitochondria) and the cytoplasmic inclusions (fats, carbohydrates and other secretion granules).

That the Golgi apparatus and mitochondria are concerned with cellular metabolism is an accepted statement.
There is reason for believing that the Golgi apparatus contains a carbohydrate, since the Golgi zone gave a positive reaction with Feulgen technique. It has often been suggested that the Golgi apparatus contains a lipid capable of reducing silver nitrate. In support of this view I have found that the sudanophilia occurs in the Golgi zone. There is a general agreement that the basal end of the ameloblast is occupied by the mitochondria; from their chemical composition, it is evident that they contain appreciable amounts of fatty material which is characterized by reducing osmic acid and silver nitrate, and by staining with Sudan Black. This might account for the presence of many small osmiophilic granules in this region (Beams and King, 1933; Saunders et al., 1942; Irving, 1958); the argentophile clumps (Jasswol, 1924; present work); and the sudanophil granules demonstrated in this area (Irving, 1958; present work). This might suggest that the mitochondria give off material which passes to the Golgi apparatus, which in turn adds other substances to it or concentrates it into droplets before being discharged. There are some facts indicating the participation of these organelles in the metabolism of fats and carbohydrates.

By comparing the present results, some interesting features emerged. It has been observed that the sudanophil granules (lipid bodies) and the Feulgen-positive granules
(polysaccharides), some of which were surrounded by a Feulgen-positive shell, are present in the same regions. This led to the suggestion that there is a relation between these two substances. It is known that many tissues contain substances which have a very reactive aldehyde group. This generally forms part of a fatty molecule. It is also known that under certain conditions, fat droplets are broken down into carbohydrates. Danielli (1953) noticed that in the liver, when the cells contained fat droplets, each droplet appeared to be surrounded by a spherical shell containing a high concentration of aldehyde. Thus, he concluded that there appeared to be an association between fat droplets and sites of high aldehyde activity. Therefore, one might assume that some of the Feulgen-positive granules are fat droplets. Montagna and Noback (1947) and Wislocki and Sognnaes (1950) have shown that Sudan Black, which is more commonly used for visualizing lipid material, will stain some acid mucopolysaccharides (granules of the mast cells). It would appear probable from the similarity of appearance of Sudan Black and Feulgen preparations (both of which stain the same regions) that an acid mucopolysaccharide is being stained. A similar observation was made by Irving (1958b, 1959) who used Sudan Black and toluidine blue and found that both stain the same region. Thus he concluded that the nature of the staining material seemed to be associated
with mucopolysaccharides, in other words, mucopolysaccharides
might be involved in calcification and "there is strong
evidence for this view (Boyd and Neuman, 1951; Sobel and
Burger, 1954; Belanger, 1954)." The fact that the sudano-
phil material is concerned with calcification is convincingly
shown by the changes seen in rickets (Irving, 1959). In
some respects the above results are confusing. The chief
difficulty in the present investigation was the lack of
histochemical methods for specific mucopolysaccharides, for
Since both the sudanophil substance and the polysaccharides
it is known that Feulgen method is not selective and stains
have been shown to occur in the Golgi region, it
several mucopolysaccharides. Irving (1960) described a
seems probable that both substances might be connected
method of Sudan Black which appeared to be specific for a
substance, probably of acid mucopolysaccharide nature. Thus
it appears that Feulgen stains mucopolysaccharides including
one stained by Sudan Black, which can be regarded as an
acid mucopolysaccharide. However, it seems that the following
can be safely concluded: the sudanophil substance similar to
the polysaccharides, is connected with calcification,
especially in the early stages, since it is found in the
dentine–predentine junction and in young enamel where it
begins to be acid soluble.

Dalton and Felix (1957) in their explanation of
the Golgi function during cellular activity stated: "The
Golgi complex thus may serve as a hydrostatic mechanism for
removing or segregating water and possibly other fluids
during the process of absorption on the one hand, and during the maturation of specific secretory products on the other. Such a function would explain the relative hypertropy of the apparatus during the periods of synthetic activity.

Emel (1945) observed that alkaline phosphatase is particularly abundant in the region corresponding to the Golgi zone. Similarly, Deane and Dempsey (1945) have found dense aggregations of alkaline phosphatase in the Golgi region of some cells lining the intestinal villus in a number of animals including the rat, mouse, bat, guinea pig, rabbit, cat, pig and monkey. They have also found acid phosphatase concentrated in the Golgi zone of the intestinal cells of these animals. Thus it has been suggested that alkaline phosphatase might occur in the Golgi apparatus (Bourne, 1951).

As mentioned above, the Golgi zone showed many granules of polysaccharides. Leblond (1950), has pointed out the possibility that mucopolysaccharides may "play a role in the elaboration and discharge of secretion material". Studies on the differentiation of the chick and the mouse duodenum have shown a close correlation between the development of a PAS reactive substance (polysaccharide) and of alkaline phosphatase. Moog and Wegner (1952) have shown that neutral polysaccharides are present in all cytoplasmic and extracellular sites that contain a high alkaline phosphatase activity. These findings led them to suggest that these two
materials are linked in function. Thus they concluded that a mucopolysaccharide is usually concomitant with sites that are rich in alkaline phosphatase. One might, therefore, assume that the functional phosphatase molecule is partly carbohydrate. Evidence for this is given by Schmidt and Thannhauser (1943) who prepared a powerful purified phosphatase from calf intestine which contained 20% carbohydrate. From this it is clear that a relationship between polysaccharides and alkaline phosphatase may be defined in functional terms. A part of such definition has already been advanced by Gersh (1949), who suggested that the polysaccharide component of the Golgi apparatus may serve as a structural support for enzymes which are thought to be localized in the Golgi region.

Many histochemical studies on the distribution of glycogen polysaccharides and alkaline phosphatase in teeth are found in the literature and the role of these substances in calcification is discussed. Nuckolls et al. (1947) in their discussion of the biochemistry of mineralization concluded that calcification would involve the degradation of the polysaccharide to glucose and the uptake of inorganic phosphate to form glucose-6-phosphate and that the enzyme is considered to act in a specific way to liberate inorganic phosphate ions. The studies of Gersh and Catchpole (1949) emphasize the possible enzymatic role of mucopolysaccharides.
in tissues, and Cobb (1949) has suggested that the depolymerization of the carbohydrate complex may be associated with an increase in reactive groups which combine with calcium.

Finally, I think one can accept satisfactorily Moog and Wegner's explanation (1952), based on Bradfield's theory (1950, 1951) of how alkaline phosphatase and mucopolysaccharides participate in the formation of fibrillar protein. The important consideration, therefore, is that polysaccharides and phosphatase are necessary for the calcification mechanism.

Chromosomes give a strongly alkaline phosphatase reaction, and in the salivary glands of Drosophila, it has been shown that the enzymes are concentrated in the Feulgen-positive bands (Danielli and Catcheside, 1945; Krugellis, 1946). In isolated nuclei a number of enzymes, particularly alkaline phosphatase, have been shown to be present (Dounce, 1943; Richter and Hultin, 1949). Therefore, the nucleus may be considered the site of origin of the enzyme which may then diffuse outwards from the nuclear membrane to operate within the cytoplasm (Hughes, 1952). To this suggestion, I might add, that the alkaline phosphatase diffused from the nucleus, becomes incorporated within the Golgi apparatus, to be released when necessary during the functional activity of the cells.
The relationship between RNA and alkaline phosphatase in developing teeth has been examined by Wislocki and Sognnaes (1950), Johnson and Bevelander (1954), Symons (1956) and Pourtois (1961). From their studies it is concluded that the distribution of RNA is the reverse of that of alkaline phosphatase. Cells rich in RNA may be taken to be actively engaged in protein manufacture (Caspersson, 1947). However, alkaline phosphatase is concerned in the manufacture of RNA (Bradfield, 1949, 1950) and in the production of fibrous protein (Bradfield, 1949, 1950; Jeener, 1947, 1948). This explains why during matrix production the ameloblasts contain a high concentration of RNA and no alkaline phosphatase, indicating that the cells are engaged in the manufacture of protein, while during maturation, the cells show alkaline phosphatase activity indicating that they are producing fibrous protein.

The chemical nature of the alkaline phosphatase has been reviewed by Moog (1946), who showed that they are proteins activated by metal ions. Cloeteus (1941) proved that alkaline kidney phosphatase is strongly bound to zinc. It would be interesting if an accurate information of the presence of this enzyme in the nuclei of the tooth germ layers could be obtained, and if possible to which metal ion is it bound? It might be calcium. If this is solved, it might throw more light on the process of calcification in teeth.
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