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Calrentinin-Immunoreactivity during Odontogenesis in the Rat

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CALRETININ-IMMUNOREACTIVITY

DURING ODONTOGENESIS IN THE RAT.

Dharmesh Mistry

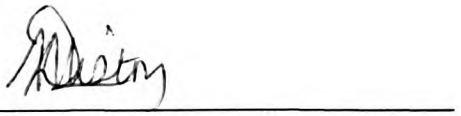
A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of

Master of Science in Dentistry

February, 2000.

DECLARATION

I, Dharmesh Mistry declare that this research report is my own work. It is being submitted for the degree of Master of Science in Dentistry to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



Signature of Candidate

4th day of February, 2000.

DEDICATION

This work is dedicated to my family for their support, patience and encouragement.

PUBLICATIONS AND PRESENTATIONS

None.

ABSTRACT

Calretinin is a 29kD calcium-binding protein that is abundantly expressed in the central and peripheral neural tissues. Calretinin expression outside the nervous system has been less extensively characterised. The aim of this study was to determine the expression of calretinin during various stages of odontogenesis in the rat (*Rattus Norvegicus*).

Five categories of embryonic (E) and post-natal (P) animals at various ages (E17, E18, E20, P0, and P7) both male and female were used to represent the various stages of tooth development (tooth bud, cap, early bell, and late bell stages).

The heads of the experimental animals were harvested at the appropriate time period and fixed in 10% phosphate buffered formalin at room temperature. No decalcification was used in the experimental tissues. Each head was cut in half mid-sagittally, and one half of the specimen was then further divided in three places coronally approximately in the location of the dentition of the rat. Haematoxylin and eosin stained sections were prepared and studied to locate the developing tooth germs. Selected sections were immunohistochemically stained using the streptavidin-biotin-complex [StreptABC (Dako)] immunoperoxidase technique with calretinin antiserum (Zymed Laboratories Inc.) at a concentration of 1:25, after antigen retrieval using microwave boiling. Positive and negative controls were utilised in the form of normal brain tissue and neurolfactory epithelium.

The results of this study show that calretinin is mainly expressed in oral ectoderm derived tissues during odontogenesis in the developing enamel organ in the rat. It is consistently expressed in the dental lamina (DL), outer enamel epithelium (OEE), stellate reticulum (SR) and stratum intermedium (SI) throughout all the stages of tooth development studied. In the

inner enamel epithelium (IEE) and ameloblasts (AB) calretinin expression shows both a temporal and spatial distribution depending upon the degree of differentiation of the cells. In the inner enamel epithelium staining intensity and distribution increases with advancing differentiation reaching a maximum when pre-ameloblasts are formed. Thereafter staining decreases in intensity and distribution so that in mature ameloblasts it is restricted to the cytoplasm and is only weak to moderate in intensity. In addition some staining of pre-odontoblasts occurred in the early developmental sections, but this disappeared in the maturing odontoblasts which were negative.

Calretinin is not expressed in dental papilla, dental sac and ectomesenchyme which showed no staining throughout the period of tooth development studied.

Calretinin has been shown to be widely distributed in tooth germs during odontogenesis. Its role in odontogenesis remains to be determined. Correlation of these results with the results of previous studies which have shown calretinin expression in ameloblastomas but not in other odontogenic cysts will lead to a better understanding of the potential role of calretinin as a specific immunohistochemical marker of ameloblastic epithelium.

ACKNOWLEDGEMENTS

I would like to convey my gratitude and appreciation to the following people:

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ABBREVIATIONS USED IN FIGURES

oe	Oral epithelium
dl	Dental lamina
em	Ectomesenchyme
oee	Outer enamel epithelium
iee	Inner enamel epithelium
dp	Dental papilla
sr	Stellate reticulum
si	Startum intermedium
pob	Pre-odontoblasts
ob	Odontoblasts
ab	Ameloblasts
cl	Cervical loop
tp	Tomes processes
E	Enamel
D	Dentine
P	Predentine
M	Molar
I	Incisor

CHAPTER 1

1.0 INTRODUCTION

Calretinin is a calcium-binding protein of 29kD. It is a member of the large family of E-F hand proteins, to which S-100 protein, calbindin 28kD and calmodulin also belong. E-F hand proteins are characterised by a peculiar amino acid sequence that folds up into a helix-loop-helix motif, which acts as the calcium-binding site, and calretinin has six such E-F hand stretches, although other lower molecular weight forms resulting from alternative splicing have been described (1). The calretinin gene was initially isolated from a cDNA clone from the chick retina and shows 60% homology with the calbindin gene (2, 3, 4). Calretinin is abundantly expressed in central and peripheral neural tissues, particularly in the retina and in neurones of the sensory pathways (5, 6). Conversely, calretinin expression outside the central nervous system has been less extensively characterised. Initially calretinin expression was only described in epithelial cells of the guinea pig ear and interstitial cells of the rat testis. More recently however it has been described in mesothelial cells and in such diverse tissues as the pilar infundibulum, eccrine glands, convoluted tubules of the kidney, Leydig and Sertoli cells of the testis, epithelium of the rete ovary, adrenal cortex, epithelial cells of the thymus, adipocytes, muscle spindle, and neurolfactory epithelium (7, 8). As far as tumours are concerned a recent study has established calretinin as a definitive marker for mesotheliomas but in this study calretinin was also expressed in some carcinomas and adenocarcinomas of lung, breast, pancreas and ovary (7). There was only partial correlation between staining of normal tissue and their neoplastic counterparts. In some tumours calretinin immunoreactivity appeared to be acquired following neoplastic transformation (7).

Calretinin expression has also recently been studied, in our department, in odontogenic tumours and cysts (9). It has been demonstrated that calretinin is expressed both in unicystic and conventional ameloblastomas, but not in various other odontogenic cysts. This observation may provide a reliable and easily reproducible method for distinguishing ameloblastic tissues from other odontogenic epithelia (9).

No other study of calretinin-immunoreactivity has been carried out in normal, cystic or neoplastic epithelial odontogenic tissues. Ichikawa, *et al.* have studied dental tissues but their studies have concentrated on neural elements in teeth and oral tissues in the rat (10, 11, 12). They demonstrated that calretinin immunoreactivity was distributed in the entire size range of the trigeminal ganglion neurones. It was also observed in the nerve fibres surrounding neuronal cell bodies in autonomic ganglia, and nerve endings in the lip, tongue, incisal papilla, soft palate, pharynx and epiglottis. In the tooth pulp myelinated and unmyelinated calretinin immunoreactive fibres were seen which projected as varicose terminals to the subodontoblastic and odontoblastic layers.

Associated calcium binding proteins, calbindin 10kD and calbindin 28kD, have been demonstrated in the rat during odontogenesis in both incisors (13, 14) and molars (15). Further reference to these studies will be made in the discussion.

CHAPTER 2

2.0 LITERATURE REVIEW

Advances in the unravelling of amino acid sequencing and three-dimensional structure have led to the description of two families of calcium-binding proteins, the EF-hand homologue family and the annexin family. The EF-hand motif consists of two alpha helices, "E" and "F", joined by a Ca^{2+} binding loop. EF-hands have been identified in numerous calcium-binding proteins by similarity in amino acid sequence and confirmed in some crystal structures. Functional EF-hands always seem to occur in pairs. The EF-hand homologue family contains more than 200 different calcium-modulated proteins, which have a broad range of functions. Among them, are calmodulins, troponin C, myosin regulatory light chain, parvalbumin, S-100 proteins and calbindins 9- and 28kD. The most striking feature of the EF-hand family is the ability to modulate the activity of a number of enzymes (16). The EF-hand homologue family can be further divided into two groups:

- Group 1: Proteins having four or less EF-hand domains such as calmodulin (Cd), myosin light chain (MLC_1, MLC_3, MLC_2), parvalbumin, calpin, calbindin 9kD; and
- Group 2: Proteins with six EF-hand domains such as calbindin 28kD and calretinin.

Several authors have proposed that the proteins of the first group are all derived from a four-domain ancestor resulting from two successive duplications of a single EF-hand domain (17, 18, 2). They have proposed that calbindin 28kD would arise from a

duplication followed by a triplication, of a single EF-hand domain. This proposal can be best understood using the diagrammatic representation below (17).

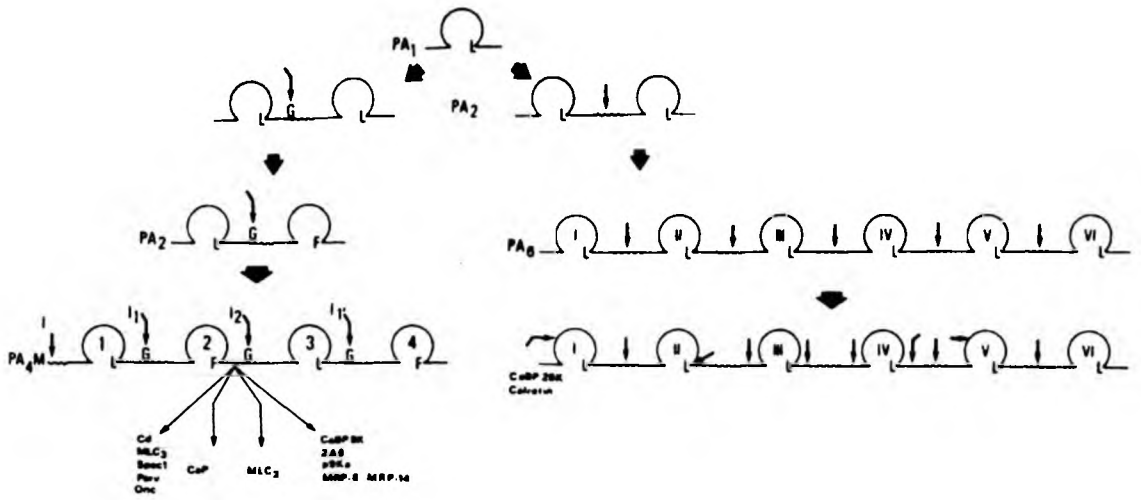


Figure 1: Schematic outline of hypothesis for the evolution of different lineage of the EF-hand calcium-binding protein family. Each calcium-binding site is represented by a loop flanked by two straight lines for the two helices E and F. Roman numerals indicate the number of the sites in the present day proteins, the Arabic numerals indicate those of the proposed ancestral proteins – PA_1: one-site primordial ancestor, PA_2: two site primordial ancestor, PA_4: four-site primordial ancestor (From 17).

On the other hand the annexins are a family of calcium-binding proteins each of which possesses four to eight homologous internal repeats which bind calcium and phospholipids in the presence of calcium (19). The proteins are present within a wide range of tissues and cell types, with each cell type having all or a subset of the proteins. The proteins are localised on the inner surface of the plasma membrane associated with the cytoskeleton and in some cases also with intracellular structures. Some members are major substrates for tyrosine and serine kinase. The precise function of the annexin

proteins are unknown but they are likely to play an important role in cellular regulation, inhibition of phospholipase A2, membrane-cytoskeletal linkages, control of membrane fusion events in exocytosis, and the regulation of cell surface receptors (20). An example is Annexin V (19).

2.1 Functions of Calcium-binding proteins.

The biological role of calretinin and other calcium-binding proteins is poorly understood. Calcium ions clearly play a vital role in cell metabolism and exert their effect in part via interactions with a wide variety of intracellular calcium-binding proteins. A few of these proteins are present in all cells, whereas the vast majority are expressed in a tissue-specific fashion. The physiological functions of a few of these proteins are achieved via calcium-dependent interaction with other proteins, thereby regulating their activity. Their major role is assumed to be buffering, transport of Ca^{2+}, and regulation of various enzyme systems. Since cellular degeneration is accompanied by impaired Ca^{2+} homeostasis, a protective role for calcium-binding proteins in certain neurone populations has also been postulated (21, 22).

The ubiquitous intracellular distribution of parvalbumin, calretinin, and calbindin would be consistent with a role in electrical transmission. The role could involve interacting with other proteins or facilitating calcium diffusion, or providing short-term buffering for local calcium concentrations in the cytosol (17).

Billing-Marczak, *et al.* (23) postulate that the presence of calretinin correlates with an increased survival ability of cells under pathological conditions connected with increased intracellular calcium levels. Not all studies confirm this hypothesis indicating that the presence of calretinin does not protect cells against calcium

overload and that calretinin does not act as an intracellular buffer. They conclude that calretinin acts as a calcium modulator rather than a calcium buffer.

Gotzos, *et al.* (24) have shown that human tumour cells of epithelial origin express calretinin. They demonstrated calretinin immunoreactivity at precise moments of the cell cycle and, in particular, in the phase G1 and during mitosis. During mitosis calretinin was localised both in the cytoplasm and in the mitotic spindle. Also calretinin was found after prophase and until telophase. In the spindle apparatus, calretinin is already present in cells in prometaphase and persists in all succeeding mitotic phases. It is associated with the kinetochore microtubules but, in contrast to calmodulin, also with the polar mitotic phases. The role that calretinin plays in well-defined moments of the cell cycle of these cells is as yet unknown, but according to Gotzos, *et al.* (24) calretinin in collaboration with other molecules intervenes in the dynamic phenomena regulating the separation of the chromosomes. In a later paper Gotzos, *et al.* (25) showed that rapidly proliferating cell lines expressed calretinin, whereas the protein was absent from cell lines with a low multiplication rate, hence suggesting a possible role for calretinin in maintaining the proliferative cycle of tumour cells.

2.2 Localisation of Calcium-binding proteins.

All proteins of the superfamily are located in the cytosol, and calbindin and parvalbumin immunoreactivities are detected throughout many of the respective positive neurones, in dendrites and axons as well as cell bodies (17). Rogers, *et al.* (4) have confirmed that both calretinin and calbindin are generally present throughout the cytosol of cells that contain them. On the other hand Buffa, *et al.* (26) have reported immunoreactivity to be cytoplasmic and diffuse, but point out that in other studies it

showed a preferential localisation for the nuclei; and in one both nuclei and cytoplasm reacted with the antiserum. Billing-Marczak, *et al.* (23) have stated that calretinin has a general neuronal localisation and is predominantly a cytosolic protein.

2.3 Methods used to localise calretinin

Several methods have been used to demonstrate the presence of calretinin, the most frequently used being immunohistochemistry using the avidin-biotin-peroxidase complex (ABC) method or the peroxidase anti-peroxidase (PAP) method (7, 8, 10, 11, 15, 24, 25, 27, 28, 29,30). Other methods used have included immunofluorescence (10, 31, 32), in situ-hybridisation (18, 30), immunoprecipitation using in vitro translation (17), reverse transcriptase-polymerase chain reaction (RT-PCR) (25), sandwich ELISA technique (33), western blot analysis (25, 27, 28, 29, 30), and immunoelectronmicroscopy (12).

2.4 Overview of tooth development (odontogenesis) in the rat.

The dentition of the rat is monophydont (one set of teeth). It consists of one incisor and three molars in each quadrant of the jaws and combines two types of teeth in one animal. The incisors are situated anterior to the molars in the upper jaw and extend below and beyond the molars in the lower jaw. The incisors grow, calcify and erupt continuously throughout the life of the animal and therefore show in one tooth the complete life cycle of tooth development from inception to maturity.

The incisor has no root but consists of a powerful crown which may be divided longitudinally into two parts, the labial or convex portion covered with enamel and the remaining lingual or concave and lateral portions covered with cementum. The curved dentine cone constitutes the bulk of the tooth is compressed medio-laterally and has a slight lateral shear. During function the apex of the cone is worn away by attrition. The

alternate backward and forward movement of the jaws in gnawing produces a much more rapid wearing of the relatively softer cementum and dentin on the lingual side than of the very hard enamel of the labial surface, and results in a sharp chisel-shaped edge. The rat molars lack enamel on the tips of the cusps (34).

The chronology of tooth development in the rat is summarised in Table 1 and is depicted diagrammatically in Figures 2 and 3, and histologically in Figures 4 to 9.

Developmental process	**Incisors**	**Molars**		
		M_1	**M_2**	**M_3**
Initiation (1st appearance of dental lamina)	14th day in-utero	13th day in-utero	14-15th day in-utero	20th day in-utero
Histodifferentiation of Ameloblasts begins	18-19th day in-utero	20th day in-utero	21-22nd day in-utero	8-10th day
Apposition of Dentine begins	20-21st day in-utero	20-21st day in-utero	1-2nd day	13-14th day
Crown completed (Amelogenesis ends)	Growth continues	11th day	13th day	21st day
Bifurcation of roots begins	None present	14-16th day	16-18th day	24th day
First appearance in oral cavity	About 10th day	19th day	22nd day	35th day
Functional occlusion	16th day	25th day	28th day	40th day
Beginning of pigmentation	28-30th day	No pigmentation		

Table 1: Chronology of the development of the dentition of the albino rat. (From 34)

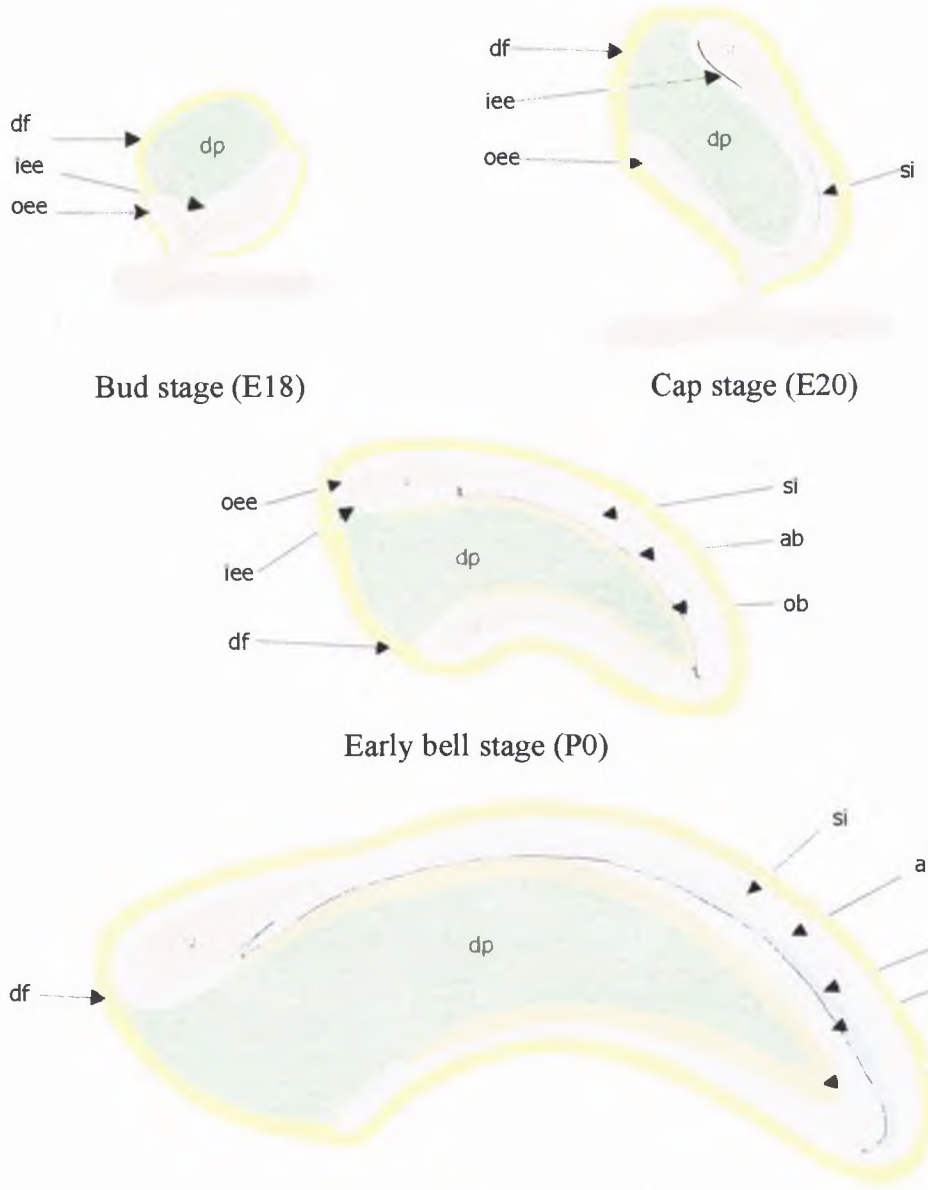


Figure 2: Schematic representation of the stages of incisor tooth development analysed. Embryonic day 17 and 18 (E17 and E18) and post-natal day 0 and 7 (P0 and P7) represents sagittal sections. At E18 the epithelium has formed a bud and dental mesenchyme has condensed around it. At E20 (cap stage) histodifferentiation has started. The mesenchyme has segregated to dental papilla and dental follicle (sac). At P0 (early bell stage) the morphology of the tooth is evident. At P7 (advanced bell stage) the complete form of the incisor tooth can be seen (Adapted from 35).

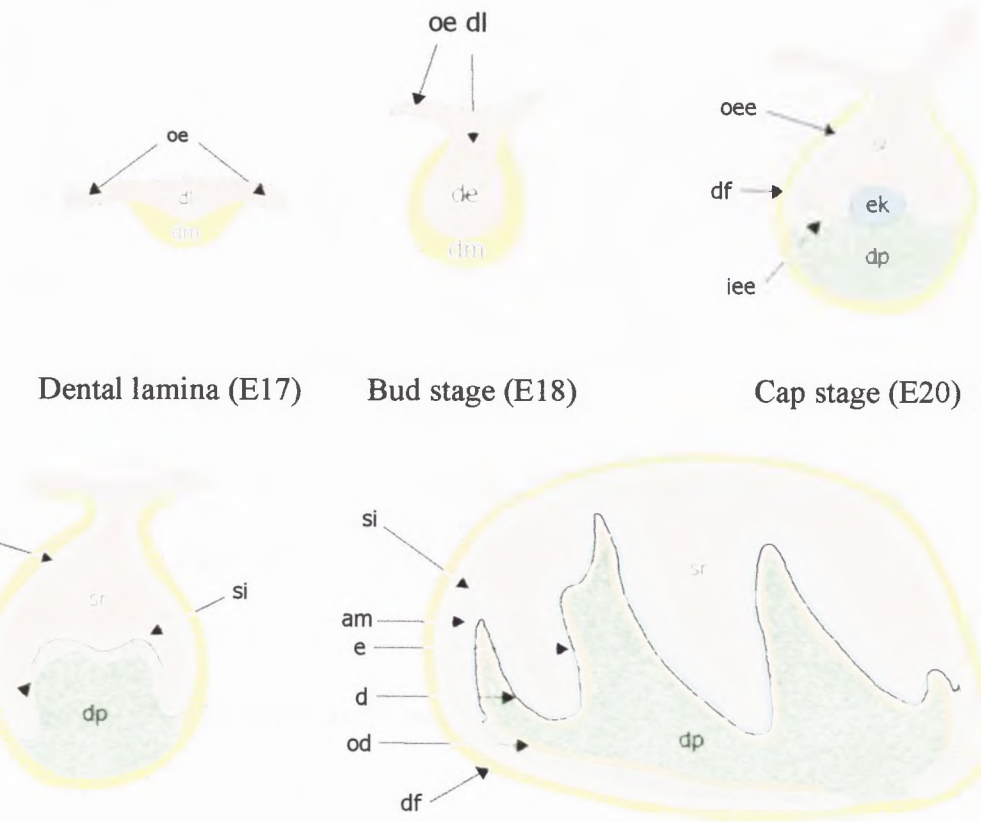


Figure 3: Schematic representation of the stages of molar tooth development analysed. Embryonic day 17 (E17) to post-natal day 7 (P7) represents coronal sections. In E17 rat embryo the oral epithelium has thickened and forms the dental lamina. At E18 the epithelium has formed a bud and dental mesenchyme has condensed around it. At E20 (cap stage) histodifferentiation has started and the enamel knot has formed. The mesenchyme has segregated to dental papilla and dental follicle (sac). At P0 (early bell stage) the morphology of tooth cusps are evident. At P7 (advanced bell stage) the complete cusp pattern of the molar tooth can be seen (Adapted from 35).

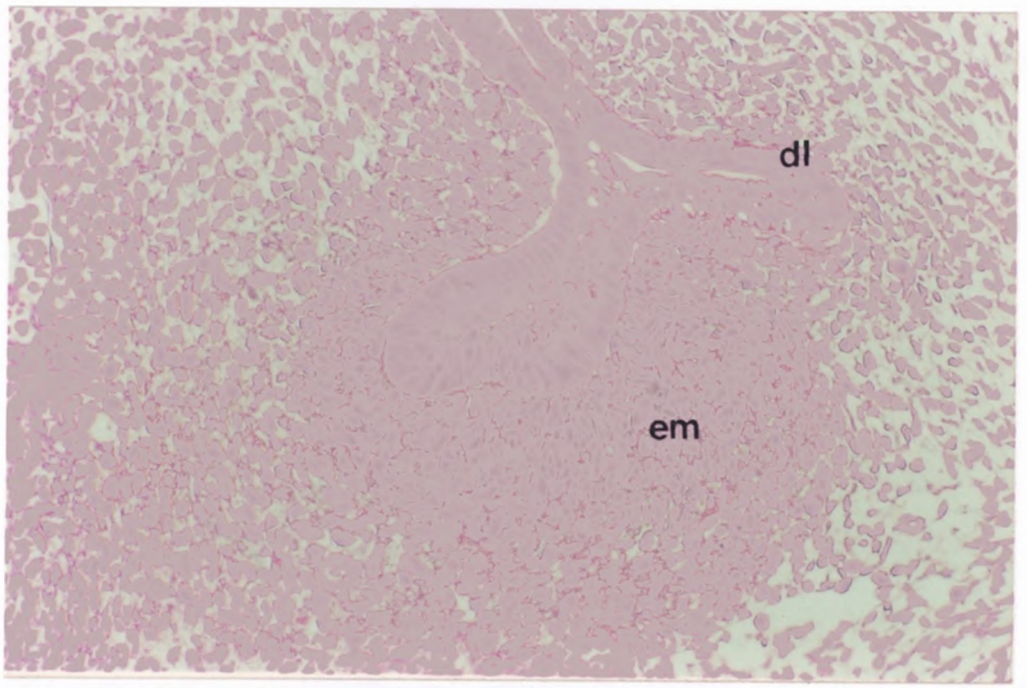


Figure 4: A tooth germ in the bud stage of tooth development showing ectomesenchyme and dental lamina. Haematoxylin and Eosin x 256.

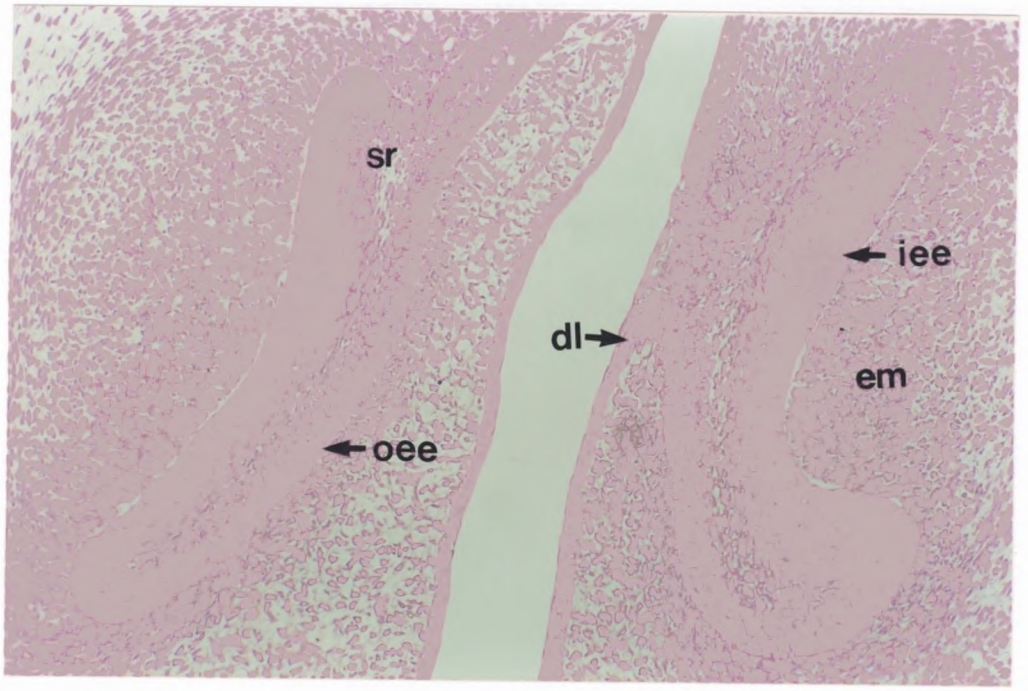


Figure 5: Molar tooth germs in the early and late cap stage of tooth development. Haematoxylin and Eosin x 160.

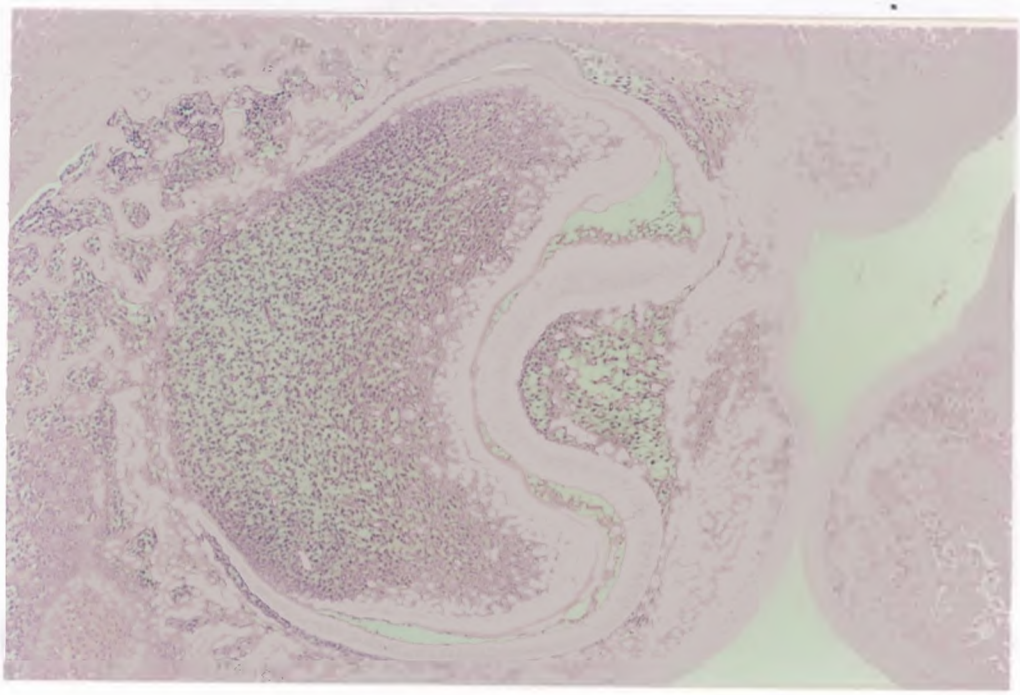


Figure 6: A coronal section of a molar tooth germ in the bell stage of tooth development. Haematoxylin and Eosin x 100.

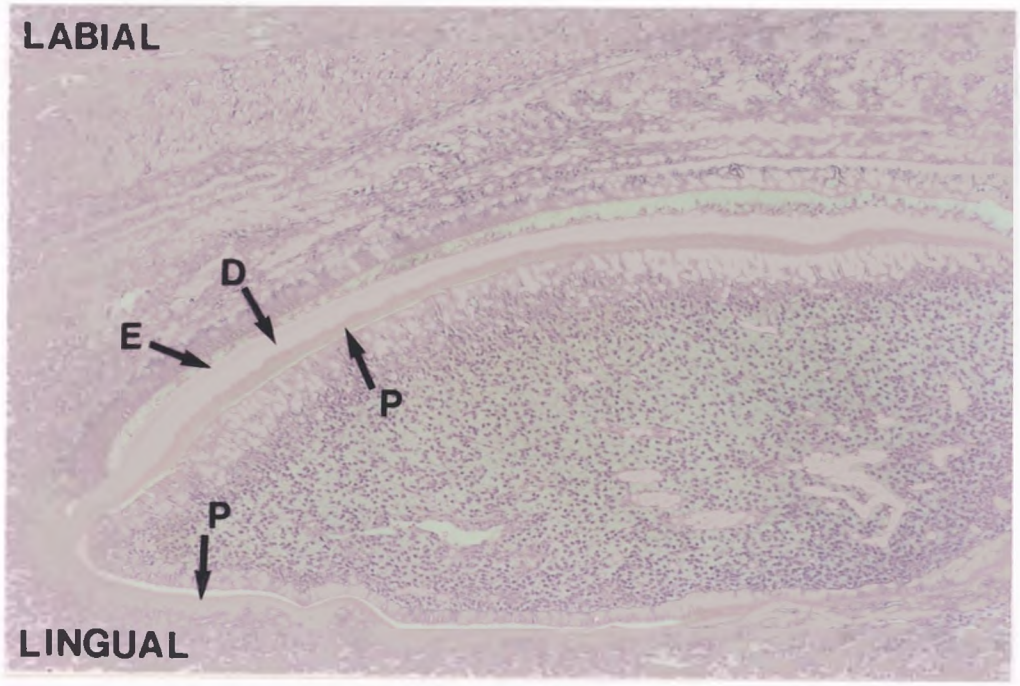


Figure 7: A sagittal section of an incisor tooth germ in the bell stage of tooth development showing deposition of enamel, dentine and predentine on the labial aspect of the tooth, and only predentine and dentine on the lingual aspect of the tooth. Haematoxylin and Eosin x 100.

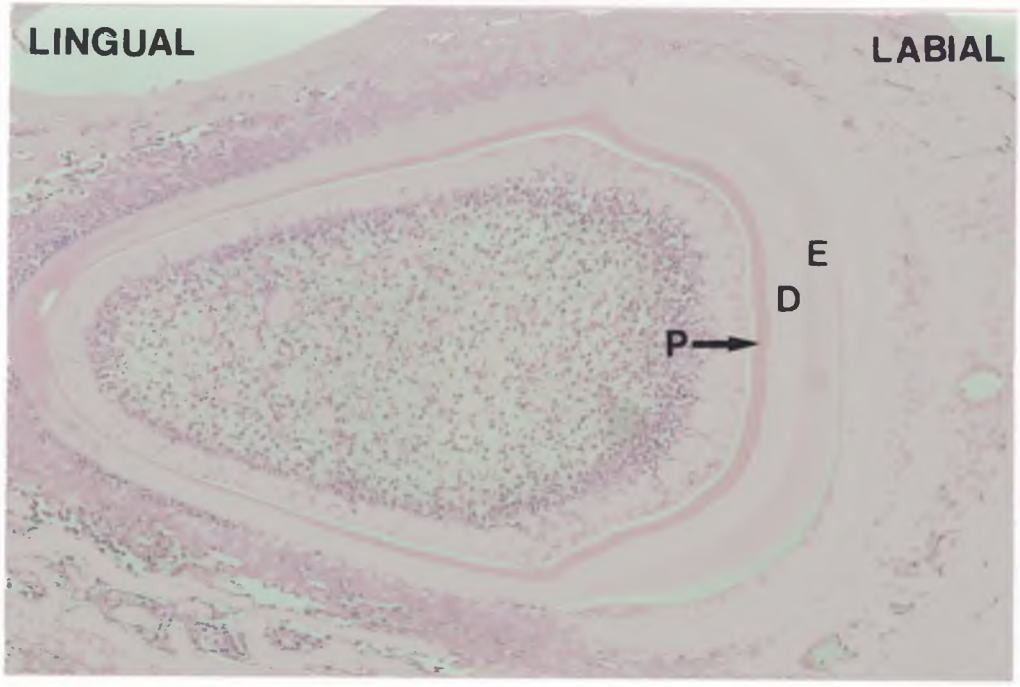


Figure 8: A coronal section of an incisor tooth germ in the bell stage of tooth development showing deposition of enamel, dentine and predentine on the labial aspect of the tooth, and only predentine and dentine on the lingual aspect of the tooth. Haematoxylin and Eosin x 160

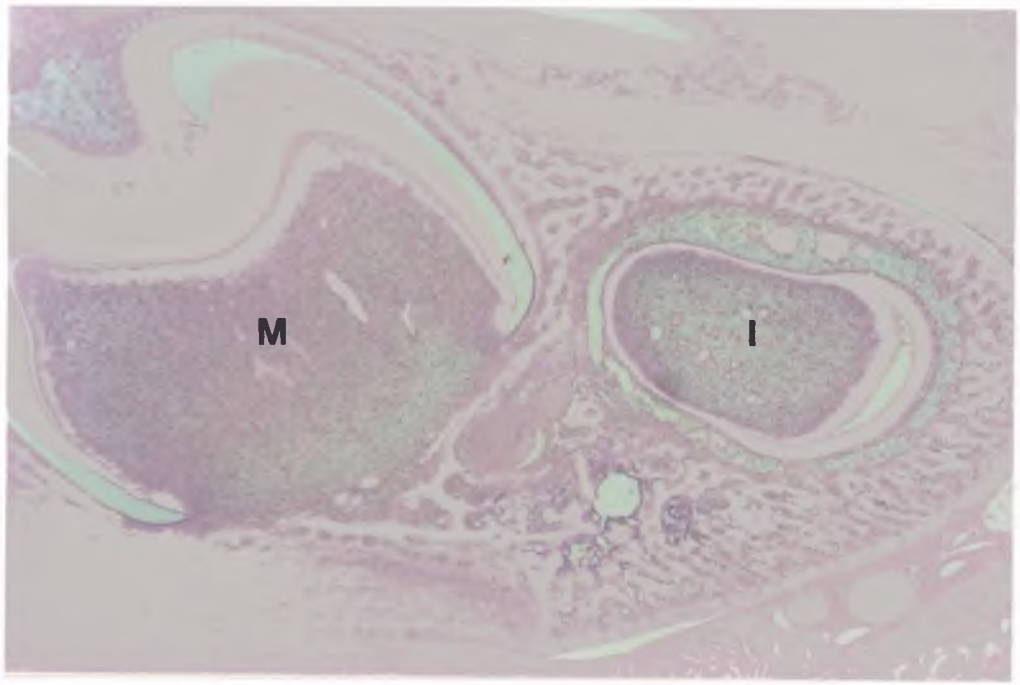


Figure 9: A coronal section of an incisor and molar tooth germs in the mandible showing the positional relationship of the incisor and molar teeth, i.e. the incisor tooth develops beneath the developing molar teeth. Haematoxylin and Eosin x 100.

CHAPTER 3

3.0 AIMS AND OBJECTIVES

As already mentioned recent studies in our department have demonstrated that the ameloblastic epithelium in both conventional and unicystic ameloblastomas expressed calretinin while the epithelial lining of other odontogenic cysts did not (9). The results of these studies suggested that calretinin might be a specific marker for ameloblastic epithelium and may have an important role to play in the differential diagnosis of both cystic and solid odontogenic tumours (9).

Since odontogenic tumours are known to recapitulate normal odontogenesis we decided to investigate whether calretinin was expressed in odontogenic tissues during normal tooth development in the rat. This would allow comparison with the calretinin expression observed in ameloblastomas thus providing a greater understanding of the potential use of this immunohistochemical marker in the diagnosis of odontogenic lesions.

CHAPTER 4

4.0 MATERIALS AND METHOD

4.1 Pilot study and determination of protocol for staining and immunohistochemical staining techniques.

The relevant forms were submitted and approval from the Animal Ethics Screening Committee of the University of the Witwatersrand was obtained prior to the commencement of this study. Approved AESC clearance certificates and modification and extensions clearance certificates are in Appendix A.

The experimental animal used in this study was the Norwegian rat (*Rattus Norvegicus*) of the Sprague-Dawley strain. A pilot study was done in which tissue was harvested from a single rat at birth, i.e. postnatal day 0 (P0). The tissue was fixed in 10% phosphate buffered formalin at room temperature for 48 hours. Different methods were used to determine the best techniques for the immunolocalisation of calretinin. Briefly each technique used in the pilot study will be discussed with the results obtained for each. Tissues were processed and stained without prior decalcification and the results compared with tissues that had been decalcified.

4.1.1 Surface decalcification

The paraffin embedded blocks were surface decalcified with 5% Nitric acid for approximately 20 - 30 minutes. This surface decalcification would only decalcify approximately 20 μm of the embedded tissue. Serial sections were than cut and stained using the method outlined in 4.3 for calretinin immunostaining. The results showed that calretinin immunostaining was not affected by the decalcification

process. The disadvantage of this method was that the decalcification was not uniform throughout the sections.

4.1.2 Decalcification using formic citric acid

After fixation in 10% phosphate buffered formalin for 48 hours at room temperature the tissue was decalcified in formic citric acid (90ml Formic acid, 1000 ml distal water, 80ml HCl (32%), and 10g Sodium citrate) for approximately 2 days until the end-point was reached which was determined using saturated ammonium oxalate, i.e. when saturated ammonium oxalate is added to 5ml of decalcifying solution, this solution turns milky if calcium is still present in the tissue being decalcified. If no calcium is present the mixture remains clear. The tissues was then thoroughly washed in tap water to remove any trace of the decalcification agent and then routinely processed and immunostained as outlined in 4.3. The results showed that the intensity of the staining of calretinin positive cells decreased with no effect on the level of background staining when compared with undecalcified sections.

4.1.3 Decalcification using EDTA and citric acid.

After fixation in 10% phosphate buffered formalin for 48 hours at room temperature the tissue was decalcified for approximately 2 weeks at a pH of 7.1 - 7.2 in 0,50mol/L EDTA and 0,28mol/L citric acid. The tissue was washed in tap water to remove any trace elements of the decalcification agent and then routinely processed and immunostained as outlined in 4.3. The results showed a decreased intensity of calretinin immunoreactivity, when compared with the undecalcified sections. In addition the amount of background staining present was excessive.

4.1.4 The use of normal rat serum to replace normal goat serum in the immunostaining process to eliminate excess background staining.

In the immunostaining protocol normal goat serum was replaced with normal rat serum in an attempt to decrease the excessive background staining that was seen on the sections. The results in fact showed increased background staining in those sections in which rat serum was used probably because of the fact that the normal rat serum was not purified.

4.2 Specimens / Experimental procedure

Five categories of animals at various ages, and both male and female, were used for histological processing and examination, to represent the various stages of tooth development. Depending on the yield a minimum of 10 animals were used in each category.

Category A: 10 foetal rats (Embryonic day 17) representing bud and early cap stages of odontogenesis.

Category B: 10 foetal rats (Embryonic day 18) representing bud, early and late cap stages of odontogenesis.

Category C: 10 foetal rats (Embryonic day 20) representing late cap and early bell stages of odontogenesis.

Category D: 10 0-day-old rats (Postnatal day 0) representing early bell stage of odontogenesis.

Category E: 10 7-day-old rats (Postnatal day 7) representing late bell stage of odontogenesis.

The foetal rats were harvested from time-mated (vaginal plug considered as day 0) mature females representing the different categories as mentioned above. Each pregnant female rat was euthanased using a lethal dose (2ml) of sodium pentobarbitone (Centaur Lab, EUTHA-NAZE ®) injected into the liver to take effect. Thereafter the foetal rats were dissected and were fixed for 48 hours in a solution of 10% phosphate buffered formalin at room temperature. Once fixed the heads of the embryos were dissected and harvested for histological processing and examination as outlined below.

Similarly each of the 0-day-old and 7-day-old rats were euthanased using a lethal dose (0.2 to 0.5ml) of sodium pentobarbitone (Centaur Labs, EUTHA-NAZE ®) injected into the heart to take effect. The heads only were harvested and fixed for 48 hours in a solution of 10% phosphate buffered formalin. Once fixed the heads were histologically processed and examined as outlined below.

No decalcification was used in the experimental tissues because in the pilot study decalcification adversely affected calretinin staining intensity and background staining (4.1.1, 4.1.2, 4.1.3).

Each head was cut in half mid-sagittally, as shown in figures 10 to 12. One half of the specimen was then further divided in three places coronally as illustrated in figure 12 approximately in the location of the dentition of the rat. Both the mid-sagittal and coronal sectioning represented the tooth bearing areas in the jaws and included the control tissue.

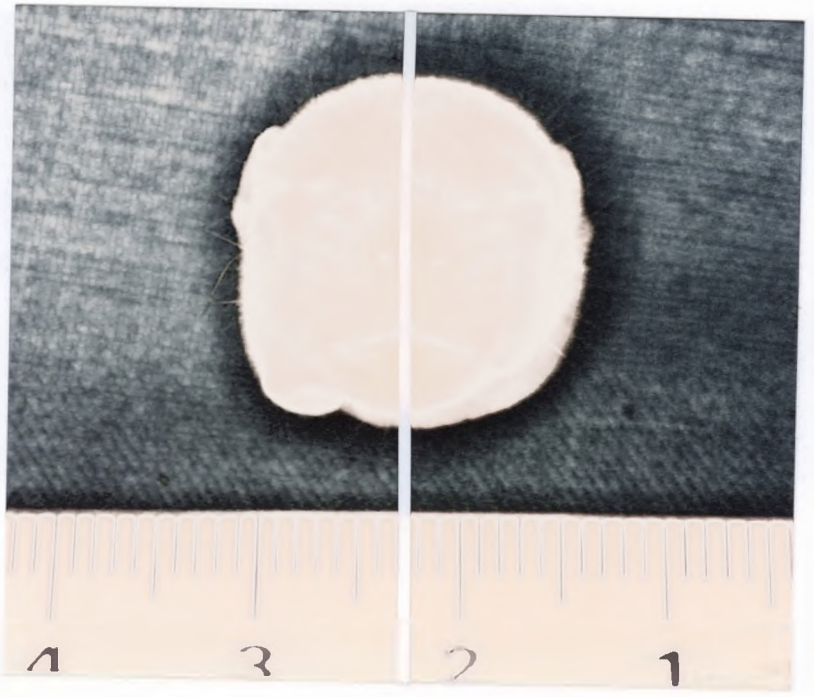


Figure 10: 7 day old specimen showing plane of mid-sagittal cut.

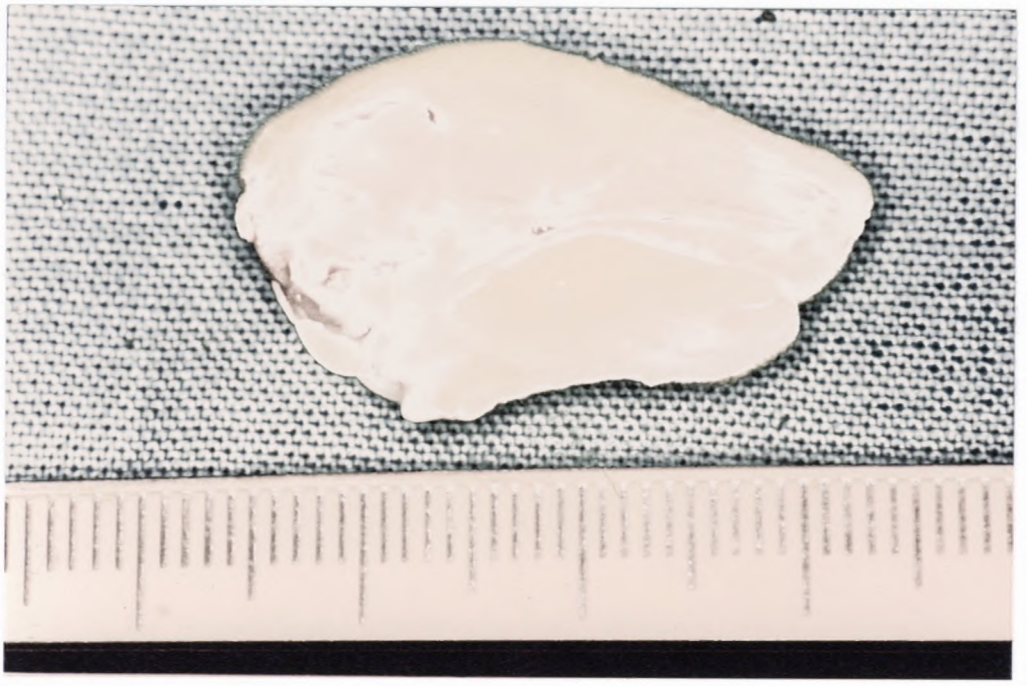


Figure 11: 7 day old specimen showing one half of the mid-sagittally cut head and jaws that was embedded to locate teeth in this plane.

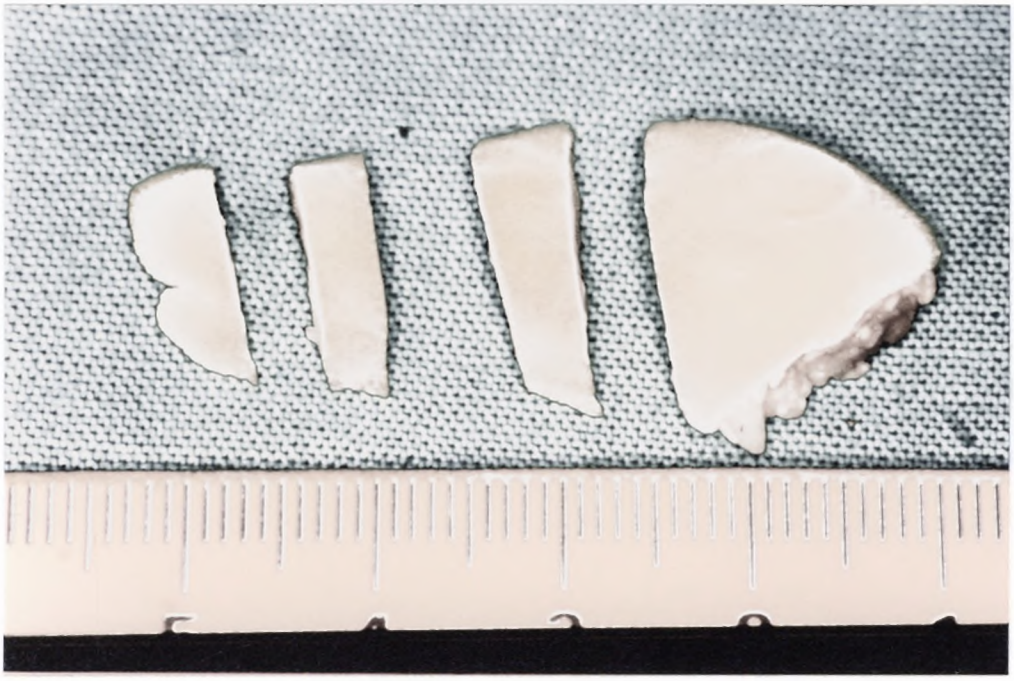


Figure 12: 7 day old specimen showing one half of the mid-sagittally cut head and jaws that has been further sectioned coronally at approximate location of the tooth bearing areas. Each coronal section was than embedded.

After paraffin wax embedding serial sections 4μm thick were cut and stained with Haematoxylin and Eosin using standard techniques.

These sections were examined microscopically to locate the tooth bearing regions. Selected sections were immunohistochemically stained with calretinin antiserum using the streptavidin-biotin-complex [StreptABC (Dako)] immunoperoxidase technique.

4.3 Immunohistochemistry

Immunohistochemistry was performed on deparaffinised 4μm thick sections using polyclonal rabbit anti-calretinin (Zymed Laboratories Inc.)

Calretinin immunopositivity was demonstrated using the streptABC immunoperoxidase technique on freshly cut 4μm sections that had been placed onto 3-aminopropyltriethoxysilane (APES) coated glass slides at room temperature overnight.

In brief, deparaffinised sections (Xylene, 2 x 5 minutes) were microwaved in 0,01 M citrate buffer (pH 6.0) for 2 x 5 minutes (800W, medium power). When cool (after 20 minutes), sections were quenched in 3 % methanol hydrogen peroxide for thirty minutes, washed in water and rinsed in phosphate-buffered saline (PBS, pH 7.6), incubated with normal goat serum (1:5) for 20 minutes and then treated with the primary anti-serum (1:25) at room temperature for one hour. The sections were then incubated with biotinylated secondary antibody for 30 minutes. After washing with phosphate-buffered saline (PBS, pH 7.6), the sections were further treated with strept ABC for 30 minutes at room temperature. The sections were then washed with phosphate-buffered saline. Bound peroxidase was visualised with a 3,3' diaminobenzidine hydrochloride (DAB,Sigma) for five minutes resulting in a brown reaction product. Sections were counterstained with Meyer's haematoxylin (5 minutes) and mounted. All reagent dilutions were performed in 3% bovine serum albumin factor V (BSA, Boehringer Mannheim).

Optimum dilutions, times and temperatures were determined in the preliminary pilot study.

Appropriate positive controls in the form of neurolfactory epithelium (Figure 13A) and normal brain tissue (Figure 13B) were utilised. Negative controls consisting of the same

tissues as well as serial adjacent sections of the developing tooth germs were achieved by substituting the primary specific antibody with non-immune serum.

4.4 Evaluation

The presence and distribution of immunohistochemically stained cells and the intensity, pattern and localisation of staining, were determined using conventional light microscopy. The tissues that were evaluated for calretinin were all the epithelium and mesenchyme involved during odontogenesis, i.e. oral epithelium, dental lamina (DL), ectomesenchyme, outer enamel epithelium (OEE), inner enamel epithelium (IEE), dental papilla, dental follicle, stellate reticulum (SR), stratum intermedium (SI), ameloblasts (AB) at all the different stages, and odontoblasts (OB).

The presence or absence of calretinin was evaluated in the cells mentioned above. If calretinin was present an intensity grading system of light, moderate or intense was used. Furthermore the localisation of the calretinin staining was evaluated as being either single, focal, or diffuse among the cells, and also whether the staining was nuclear, cytoplasmic or both.

The staining intensity and distribution pattern of calretinin immunostained cells was compared to the neurolfactory epithelium and brain, which were used as our internal controls in this study. The neurolfactory epithelium, Figure 13A, and brain cortical cells, Figure 13B, stained intensely with a diffuse distribution pattern.

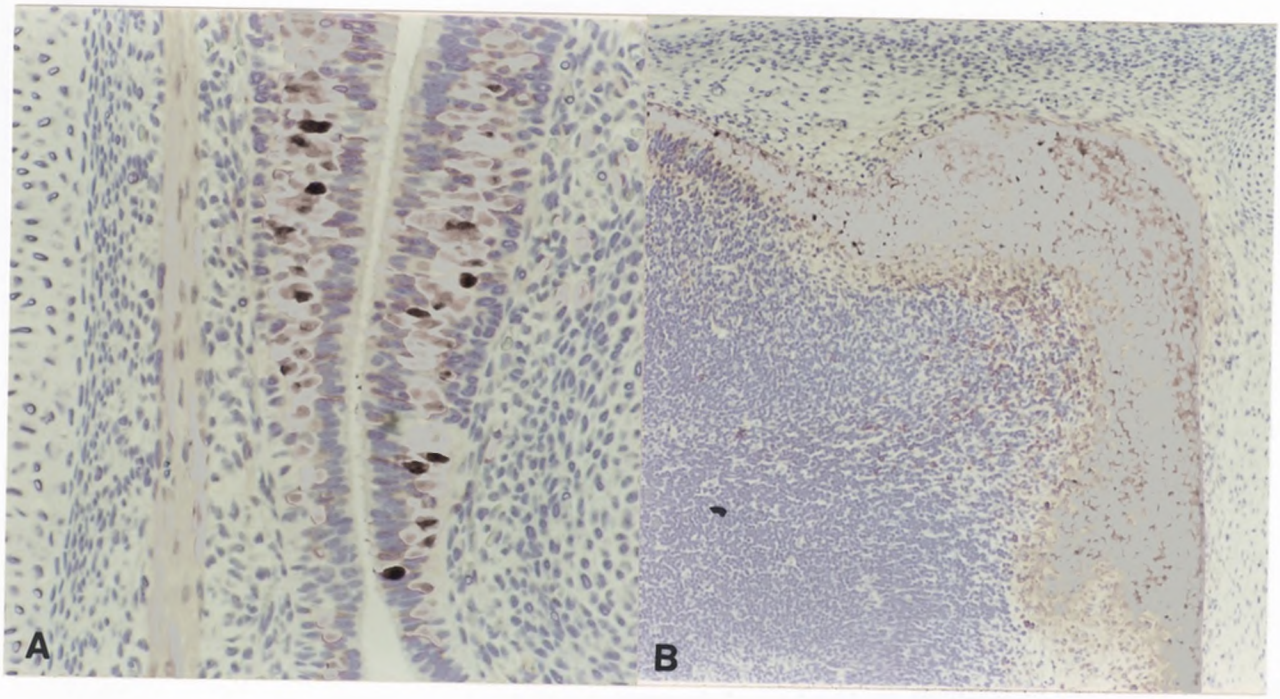


Figure 13: Photomicrographs showing positive control tissues, neurolfactory epithelium (A), Calretinin x 256, and brain (B), Calretinin x 100, showing calretinin immunostaining. Calretinin x 100. Counterstained with Meyer's Haematoxylin.

CHAPTER 5

5.0 RESULTS

The raw data is contained in Appendix B, while the frequency of calretinin immunostaining in incisors and molars at various stages of tooth development in the different age groups is summarised in Appendix C.

In the text only a descriptive and graphical analysis of the results is provided for each of the odontogenic tissues analysed. In addition no distinction has been made between molar and incisor tooth germs. This format was decided upon since any attempt to statistically analyse the results will inevitably result in misrepresentation because of:

a. Overlap between various stages of odontogenesis,

b. Presence of many more developing molar teeth than incisor teeth in the sections examined,

c. Not all odontogenic tissues being represented in all specimens examined,

d. Different planes of orientation of the developing teeth,

e. Processing artefacts,

f. The number of developmental stages exceeding the total number of specimens examined because in some specimens more than one stage of tooth development is represented.

In all of the tissues studied calretinin was expressed in both the cytoplasm and in the nucleus.

In this descriptive and graphic report the term specimen refers to the number of tooth germs in which the tissues under discussion were actually represented and not the total number of animals.

5.1 Oral epithelium

No calretinin immunostaining was seen in the oral epithelium from the tooth bud (E17) to late bell (P7) stages of tooth development (Figures 15 and 19).

5.2 Dental Lamina

Calretinin immunostaining of the dental lamina was first noticed in a few specimens in the early cap stage (E17) of tooth development. Positive cells were also seen in approximately half of the specimens in the cap (E18) and late cap (E20) stages, and the majority of the specimens in the early bell (P0) and late bell (P7) stages of tooth development (Figure 14). Staining varied from weak to intense, and was always focally distributed particularly in the basal cells located at the periphery of the lamina (Figure 15).

5.3 Ectomesenchyme

No calretinin immunostaining was seen in the ectomesenchyme at any stage of tooth development (Figures 16 and 17).

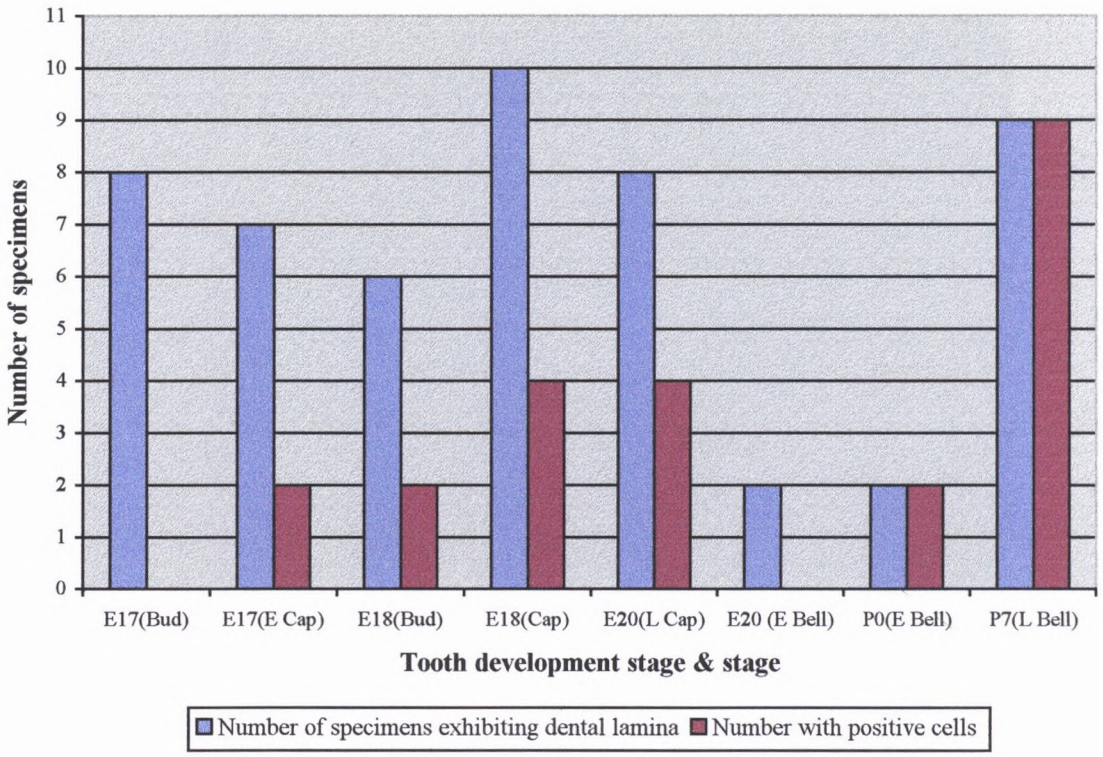


Figure 14: Histogram showing the frequency of dental lamina immunopositivity.

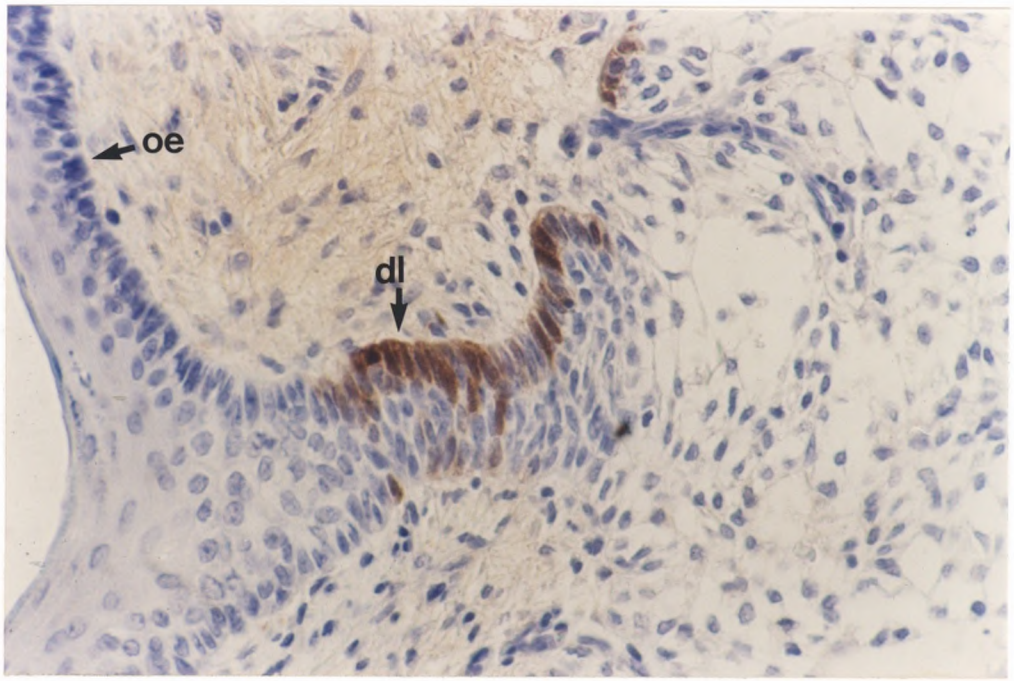


Figure 15: A section of a tooth germ (P0) showing positive calretinin immunostaining of the dental lamina and negative staining of the oral epithelium. Staining of the mesenchyme was interpreted as background. Calretinin x 400. Counterstained with Meyer's Haematoxylin.

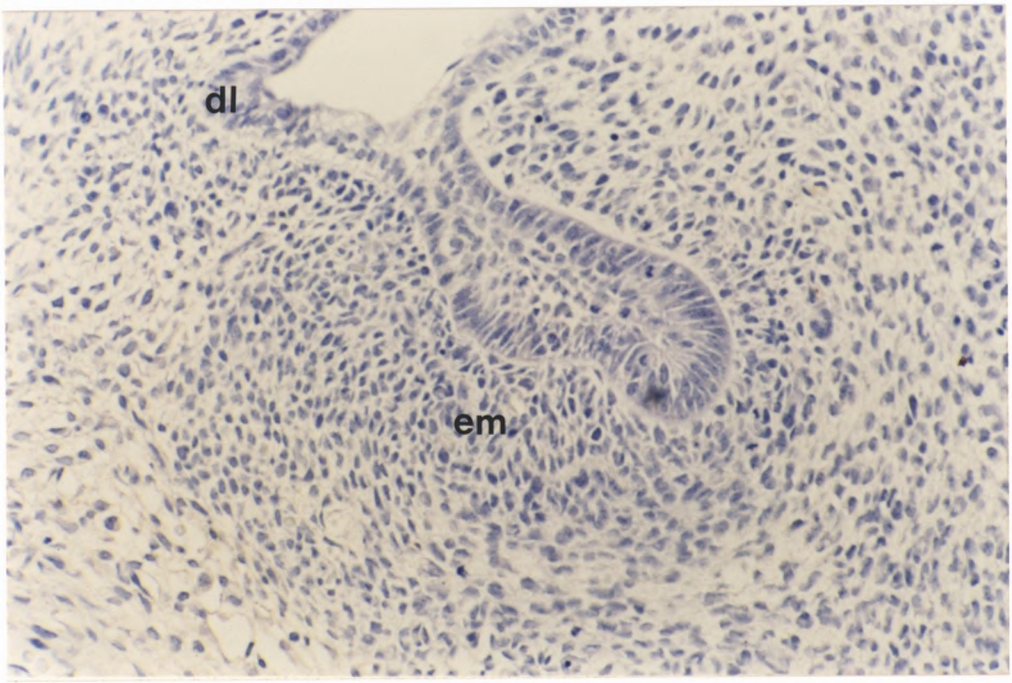


Figure 16: A tooth germ in the bud stage of tooth development (E17) showing negative calretinin immunostaining of the ectomesenchyme and dental lamina. Calretinin x 256. Counterstained with Meyer's Haematoxylin.

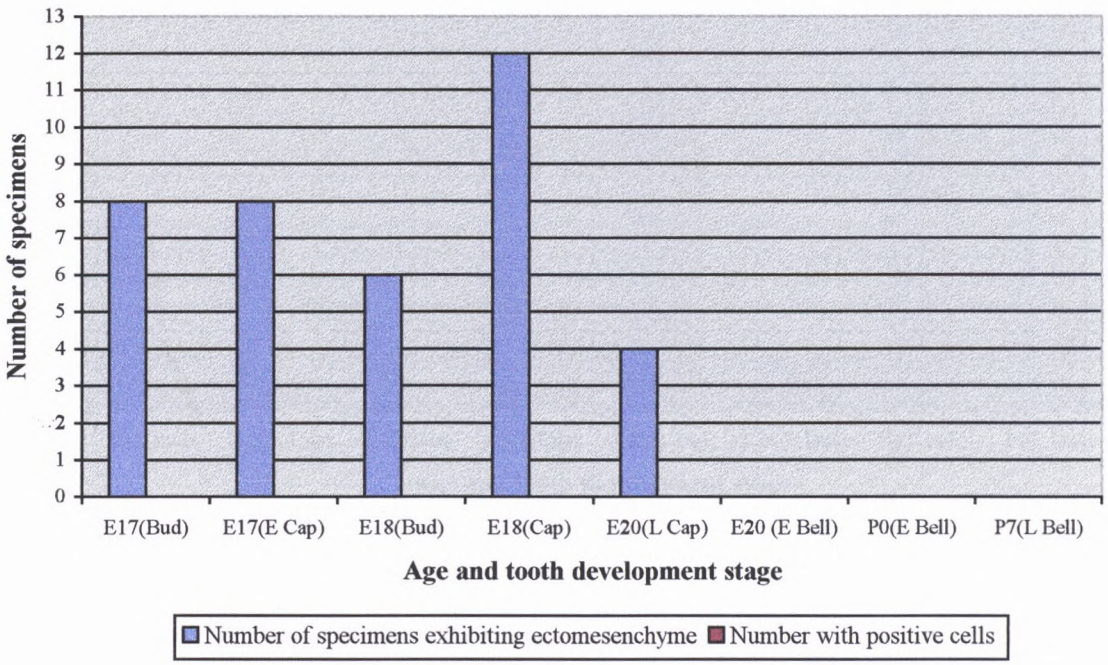


Figure 17: Histogram showing the frequency of ectomesenchyme immunopositivity.

5.4 Outer enamel epithelium (OEE)

In the outer enamel epithelium (OEE) immunopositive cells were first seen in only one specimen in the early cap (E17) stage. In the cap (E18) and late cap (E20) stages approximately half of the specimens showed immunopositive cells, while in the early bell (P0) and late bell (P7) stages the majority of the specimens showed positive cells (Figure 18). It was interesting to note that as tooth development progressed into the advanced stages the staining intensity increased, i.e. from weak to moderate in the early cap (E17) and late cap (E20) stages to moderate to intense in the early bell (P0) and late bell (P7) stages of tooth development. The staining pattern was once again focally distributed, and tended to be concentrated in that part of the OEE where it is continuous with the dental lamina (Figure 19, 20 and 23).

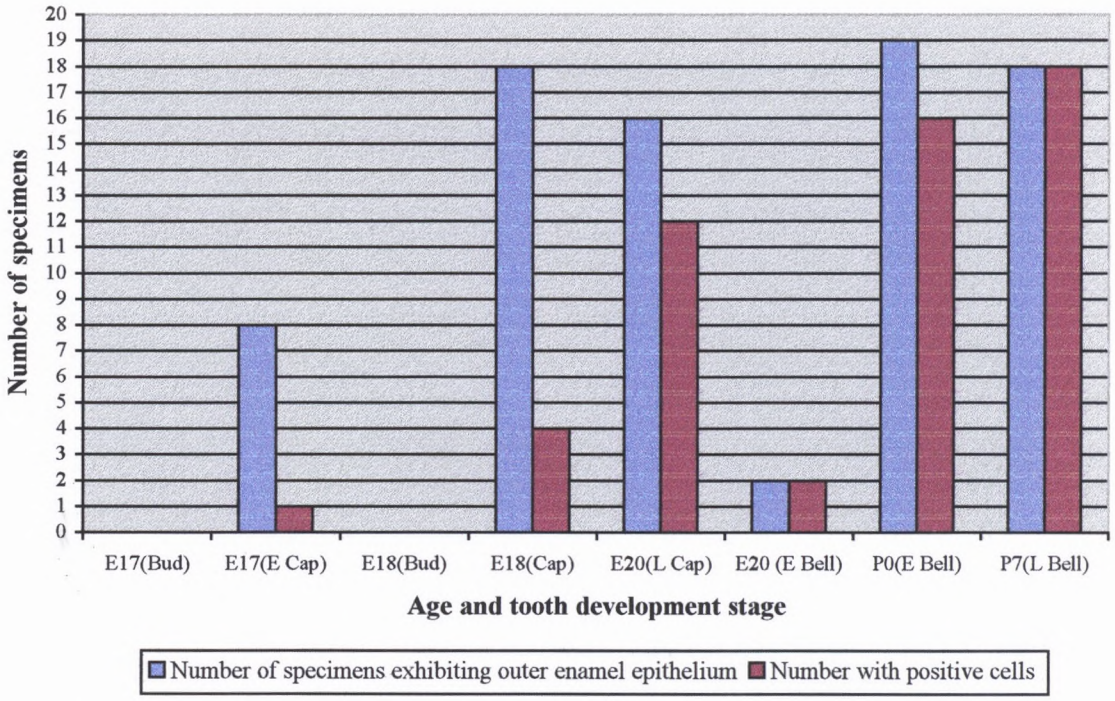


Figure 18: Histogram showing the frequency of outer enamel epithelium immunopositivity.

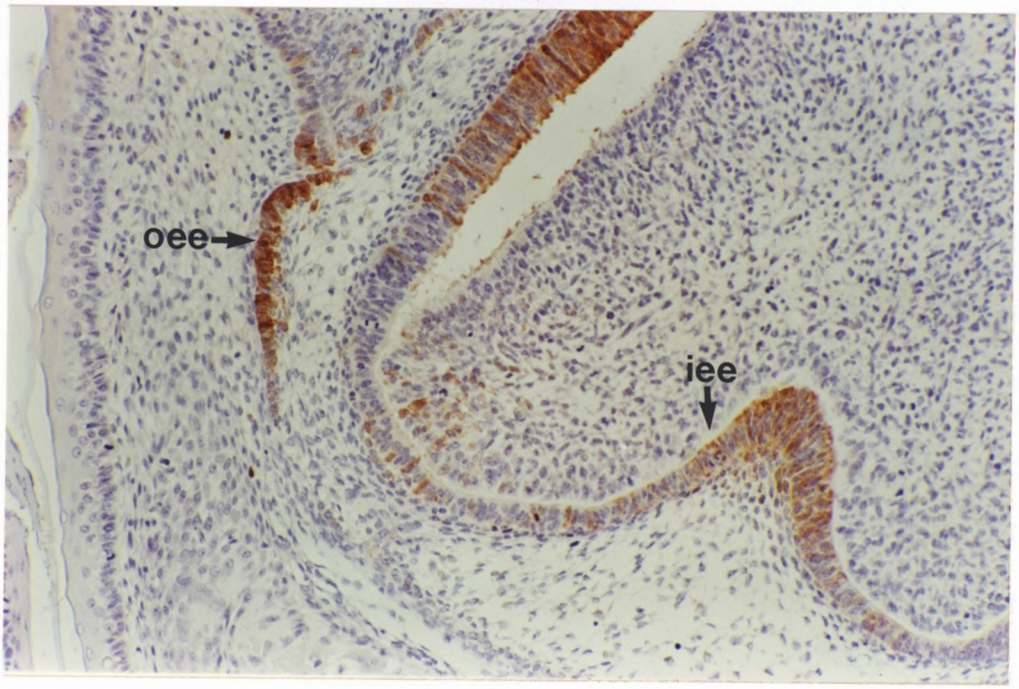


Figure 19: A tooth germ of a P0 day specimen showing positive calretinin immunostaining of the outer enamel epithelium and inner enamel epithelium. Calretinin x 160. Counterstained with Meyer's Haematoxylin.

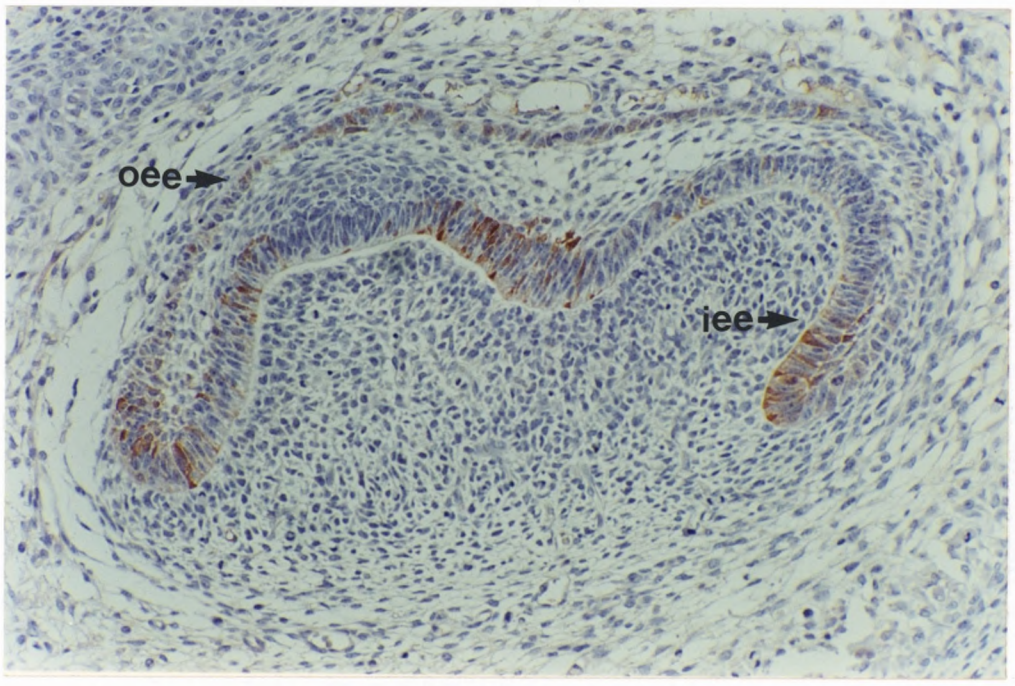


Figure 20: A molar tooth germ of an E20 day old specimen showing positive calretinin immunostaining of inner enamel epithelium and outer enamel epithelium. Calretinin x 256. Counterstained with Meyer's Haematoxylin.

5.5 Inner enamel epithelium (IEE)

Calretinin immunostaining was noticed in the inner enamel epithelium (IEE) in most of the specimens in the late cap (E20) and early (P0) and late (P7) bell stages of tooth development (Figure 21). Staining intensity varied from weak in the late cap (E20) to moderate in the early bell (P0) stages of tooth development. Some specimens in the early (P0) bell stage of tooth development showed intense staining which was a common finding for the IEE in the late bell (P7) stage of tooth development. The distribution pattern of the immunostained cells was focal which were concentrated in regions of ameloblast differentiation (Figures 22, 23, 24 and 25). In some sections it could be clearly seen that those parts of the IEE situated in the vicinity of the future cusp tips showed no calretinin staining (Figure 37). This correlates well with the absence of enamel over the cusp tips in the mature teeth.

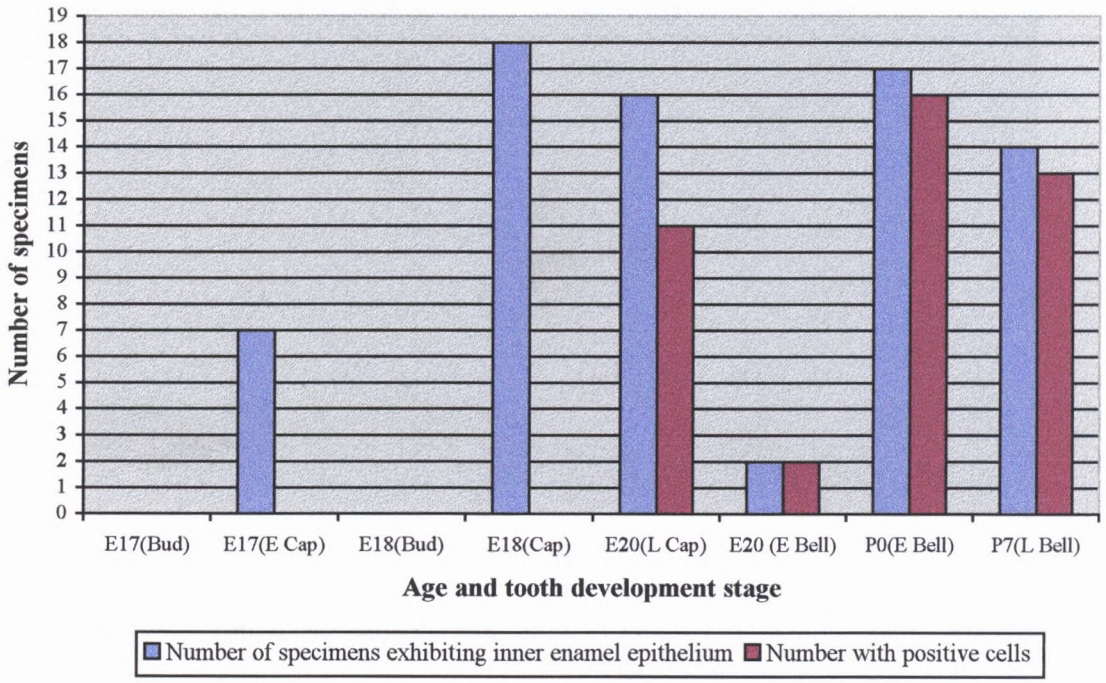


Figure 21: Histogram showing the frequency of inner enamel epithelium immunopositivity.

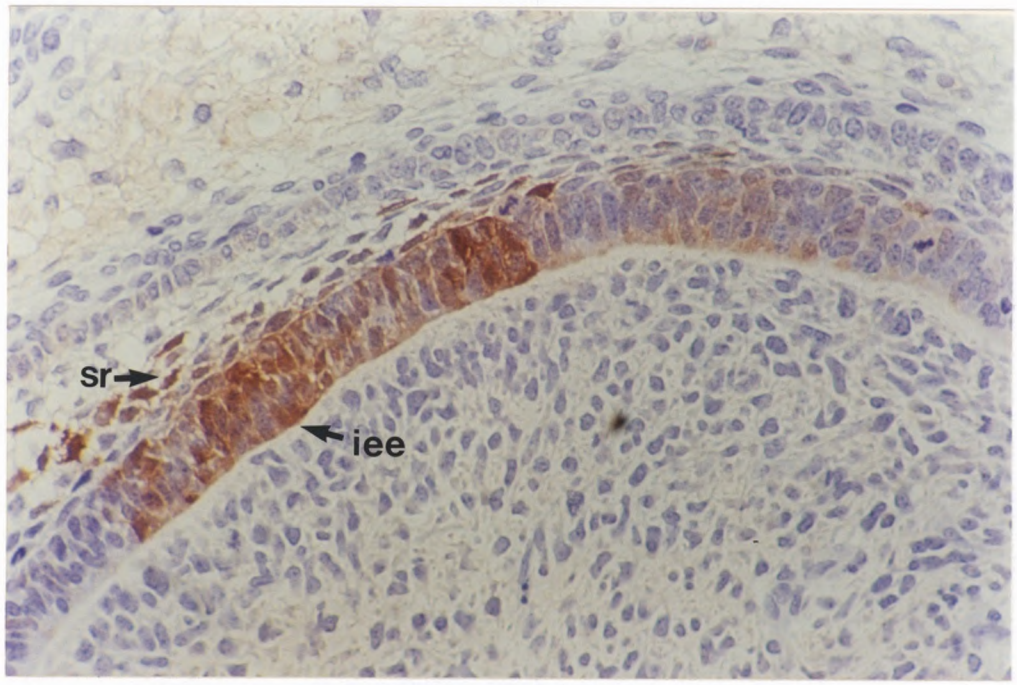


Figure 22: A tooth germ in the early bell stage of development showing positive calretinin immunostaining of inner enamel epithelium and stellate reticulum. Calretinin x 400. Counterstained with Meyer's Haematoxylin.



Figure 23: A coronal section of a molar tooth germ in early bell stage of development showing positive calretinin immunostaining of inner enamel epithelium, outer enamel epithelium and pre-odontoblasts. Epithelium island (arrowed) at the lower end of the dental papilla is derived from the cervical loop. Calretinin x 160. Counterstained with Meyer's Haematoxylin.

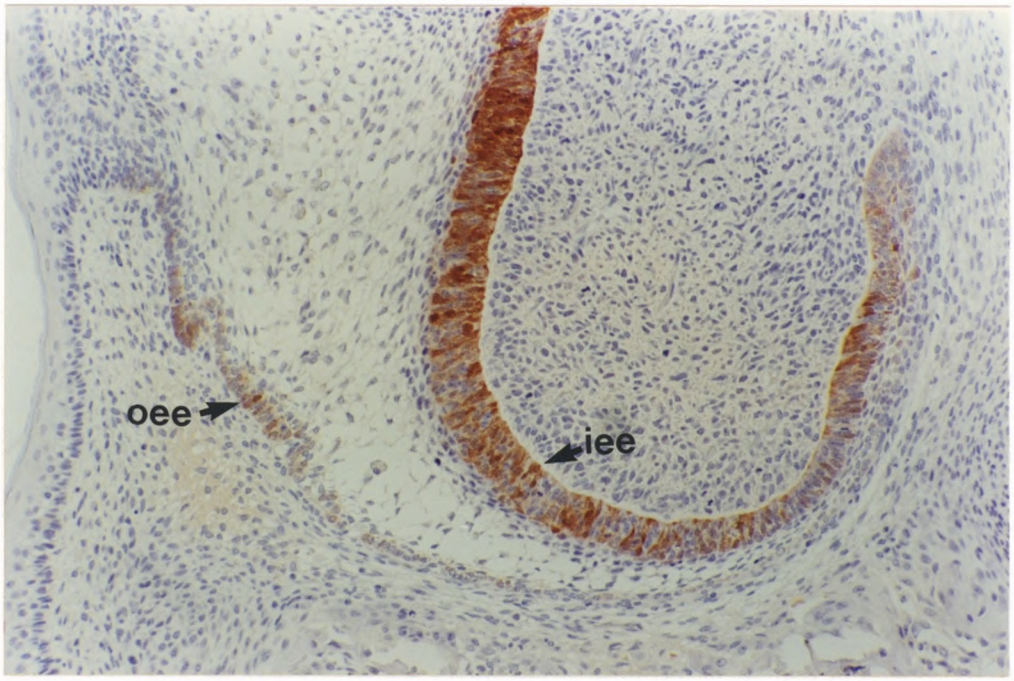


Figure 24: A section through a molar tooth germ showing positive calretinin immunostaining of inner enamel epithelium and outer enamel epithelium. Calretinin x 256. Counterstained with Meyer's Haematoxylin.

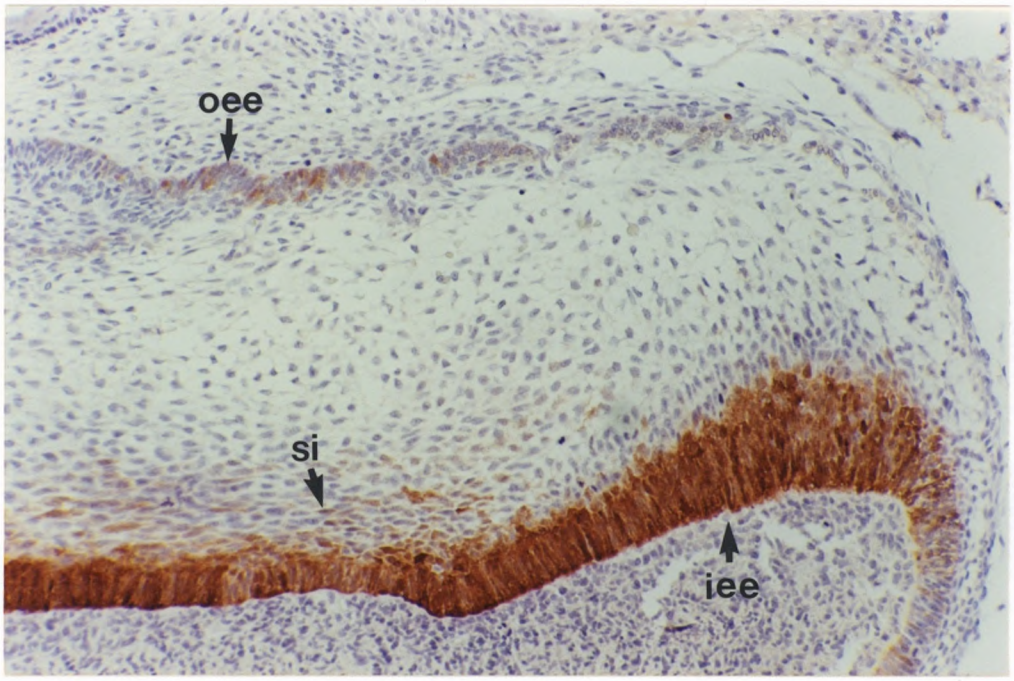


Figure 25: A section through a molar tooth bud of a P0 day old specimen showing positive calretinin immunostaining of the inner enamel epithelium (pre-ameloblasts), outer enamel epithelium, stratum intermedium (arrowed) and a few cells of the stellate reticulum. Calretinin x 256. Counterstained with Meyer's Haematoxylin.

5.6 Stellate reticulum (SR)

In the cap (E18) and late cap (E20) stages of tooth development approximately two-thirds of the specimens of stellate reticulum (SR) stained positively for calretinin, while this number increased to more than 90% for specimens in the early bell (P0) and late bell (P7) stages of tooth development (Figure 26). Staining varied from weak to moderate in the late cap (E20) and moderate to intense in the early bell (P0) and late bell (P7) stages of tooth development. In some specimens individual positive cells were seen, while in others the distribution was more focal in nature. Staining was never diffuse. In some cells the cytoplasmic extensions stained intensely. In the more advanced stages of tooth development the intensity of staining and frequency of distribution increased (Figures 22, 25 and 27).

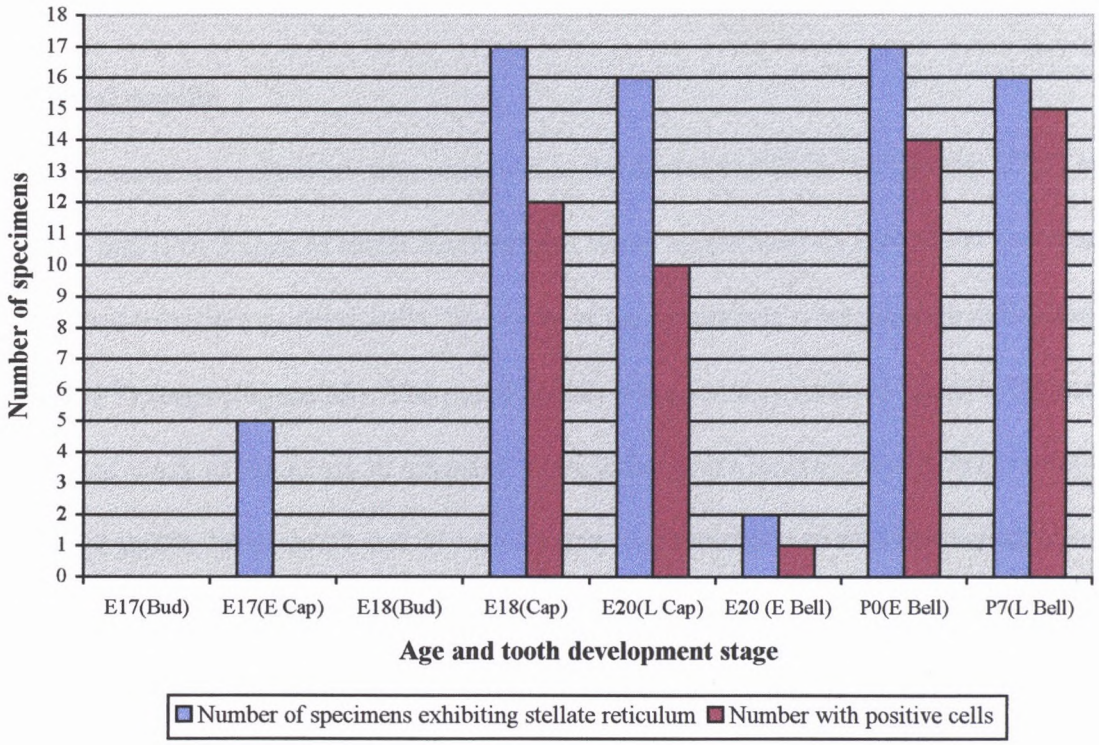


Figure 26: Histogram showing the frequency of stellate reticulum immunopositivity.

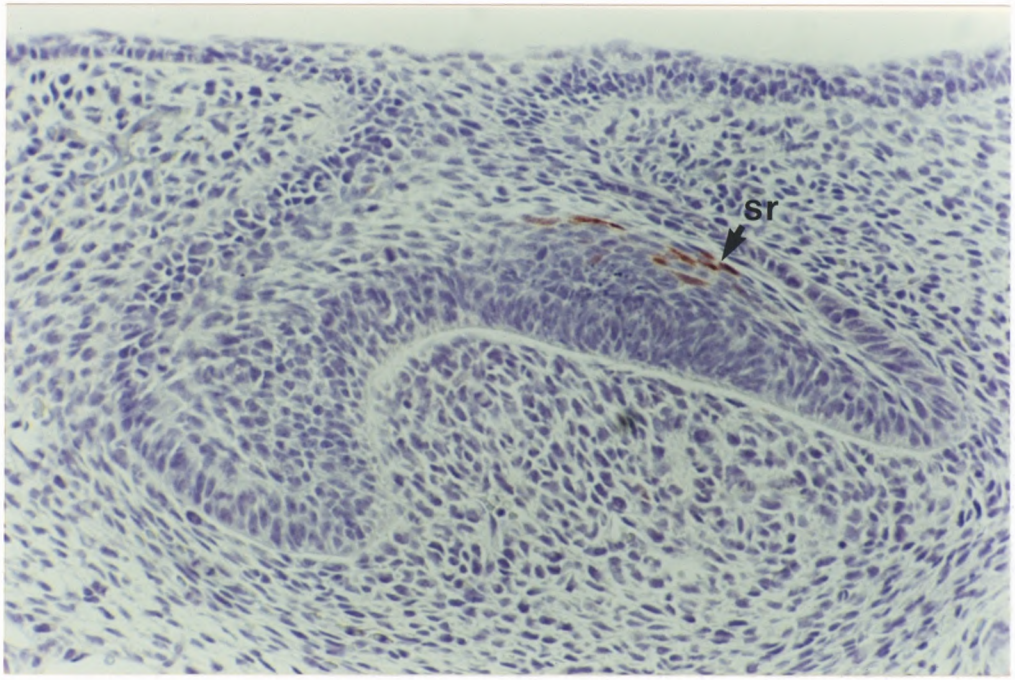


Figure 27: A tooth germ in the cap stage of tooth development showing positive calretinin immunostaining of stellate reticulum. Calretinin x 256. Counterstained with Meyer's Haematoxylin.

5.7 Dental papilla

Immunostained cells were first observed in the dental papilla in the late cap (E20) and early bell (P0) stages of tooth development when approximately half of the specimens showed calretinin positive cells. Very few of the specimens were positive in the late bell (P7) stage (Figure 28). The positive cells were focal or isolated (Figure 29) and stained weak to moderate in intensity. They either were located superficially in the dental papilla (Figures 23 and 31) where they were interpreted as representing pre-odontoblasts or deeper where they were thought to be neural cells (Figure 29). The stromal cells of the dental papilla did not express calretinin at any stage of tooth development.

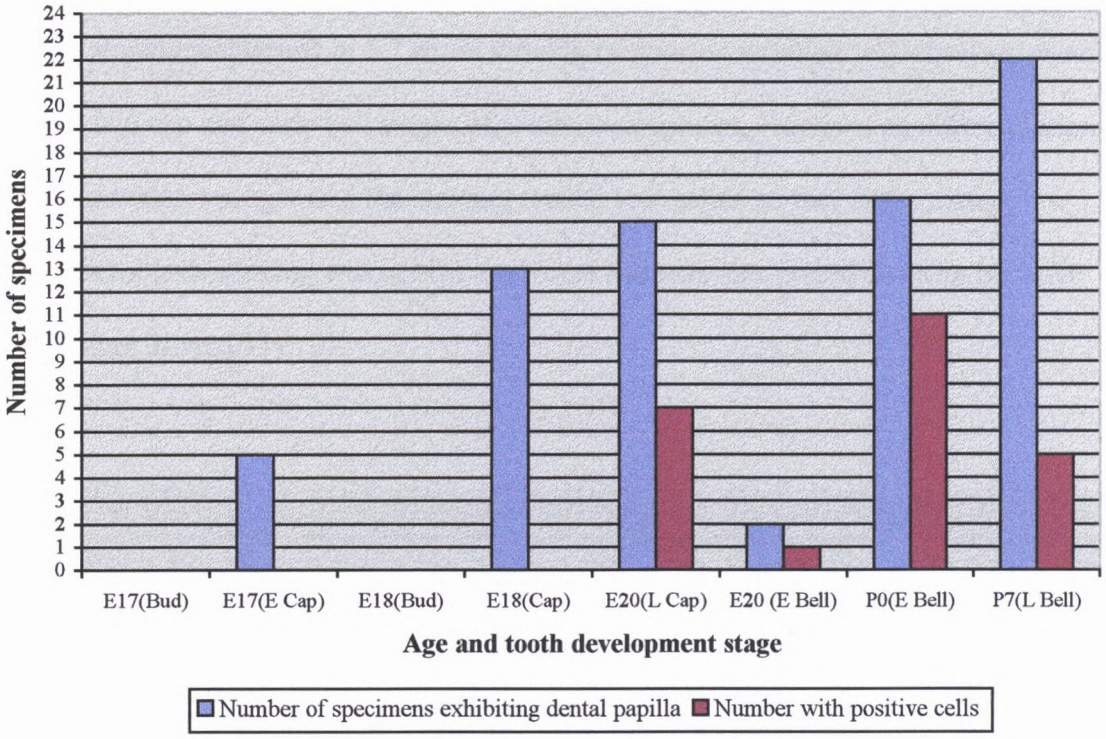


Figure 28: Histogram showing the frequency of dental papilla immunopositivity.

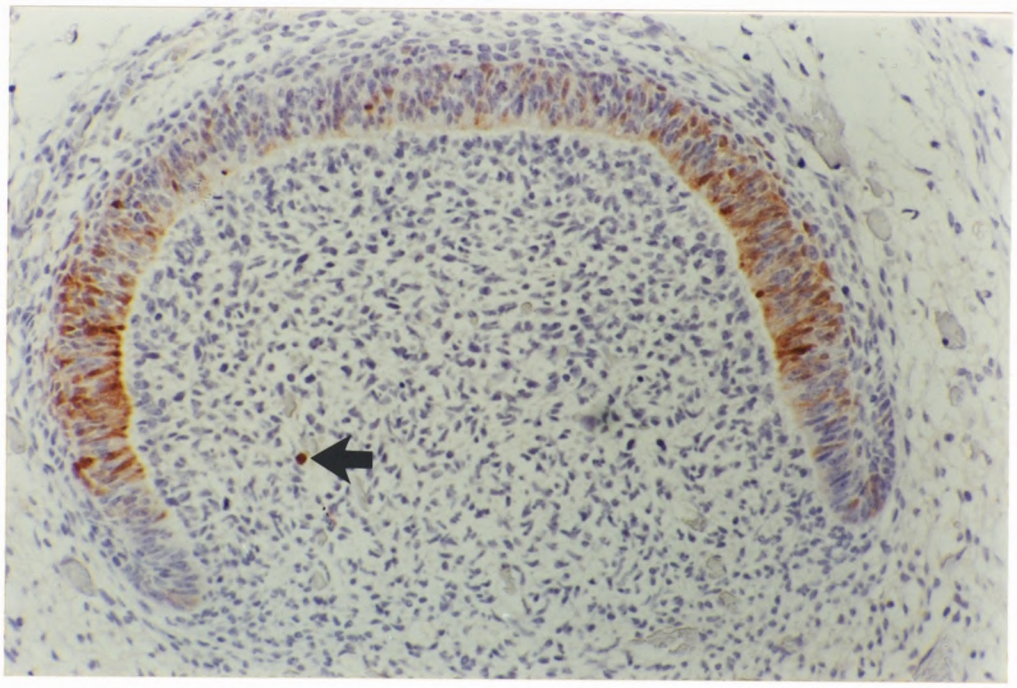


Figure 29: A coronal section through an incisor tooth germ showing positive calretinin immunostaining of the inner enamel epithelium and an isolated positive dental papilla cell (arrow). Calretinin x 160. Counterstained with Meyer's Haematoxylin.

5.8 Odontoblasts

As already mentioned in 5.7 some staining of pre-odontoblasts was observed mainly in the late cap and early bell stages of tooth development. However as differentiation of odontoblasts progressed the staining tended to weaken so that in the mature odontoblasts staining was at best equivocal and was interpreted as being negative (Figure 30 and 31).

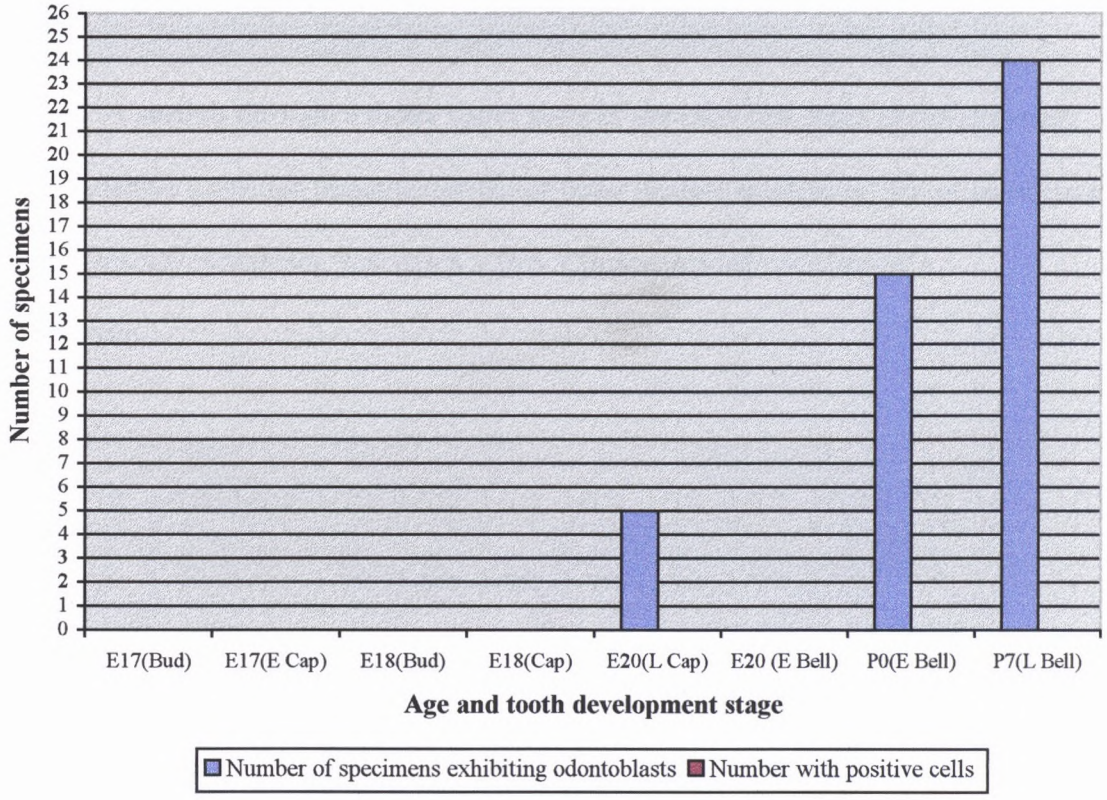


Figure 30: Histogram showing the frequency of odontoblast immunopositivity.

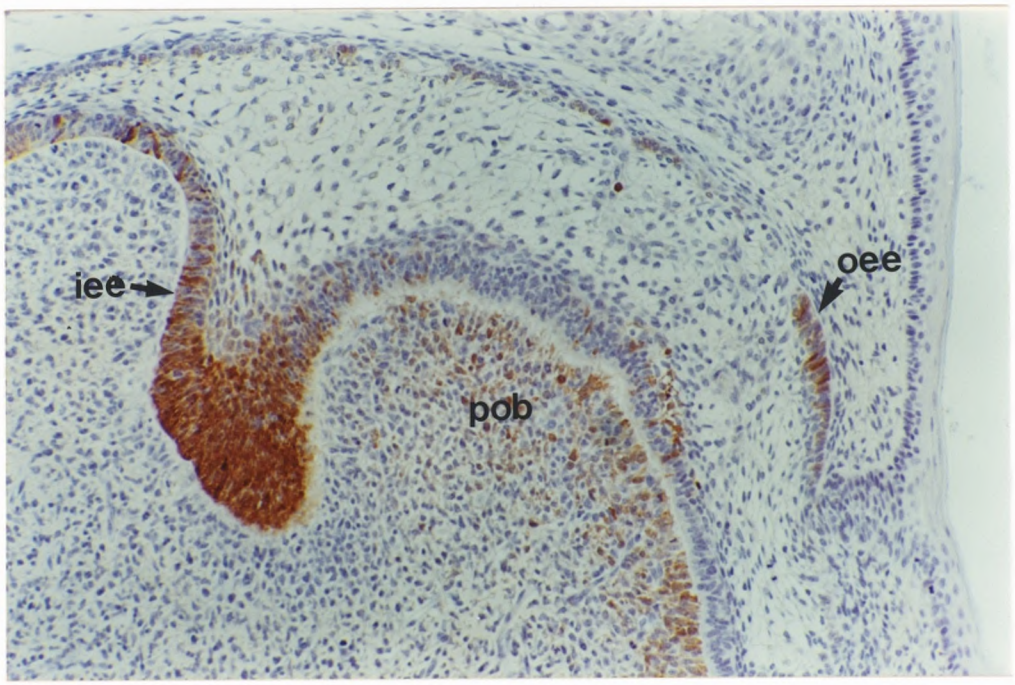


Figure 31: A section through a molar tooth germ in the early bell stage of development showing positive calretinin immunostaining of outer enamel epithelium, pre-odontoblasts and inner enamel epithelium. Calretinin x 256. Counterstained with Meyer's Haematoxylin.

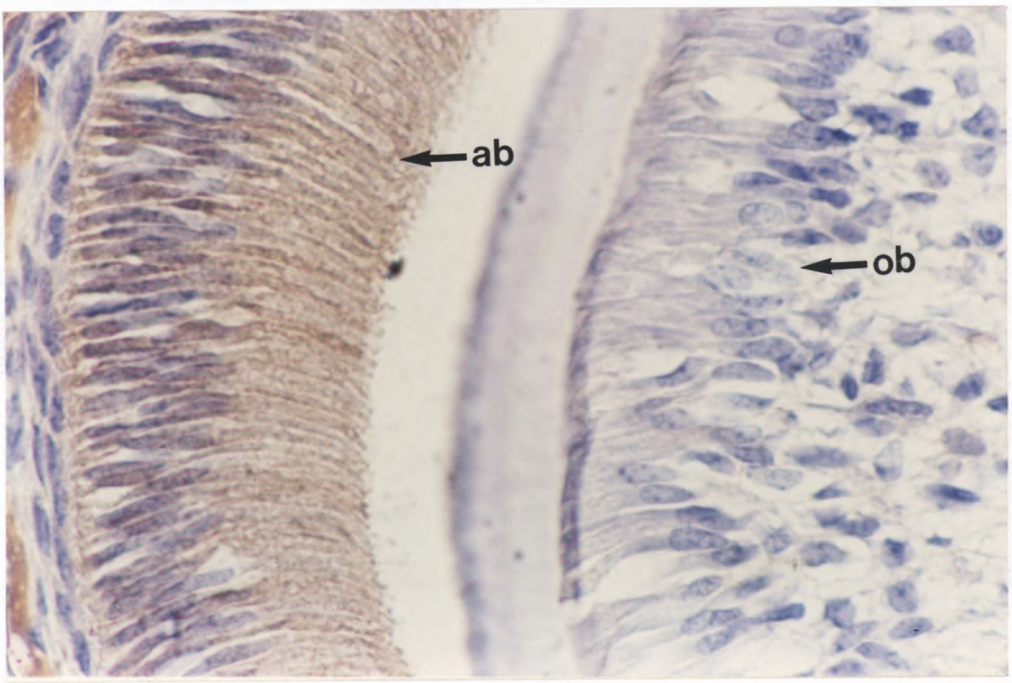


Figure 32: Tooth germ of a P7 day old specimen in late bell stage of development showing calretinin immunostaining of ameloblasts. Calretinin x 400. Counterstained with Meyer's Haematoxylin.

5.9 Ameloblasts

Most of the specimens in the early bell (P0) and all of the specimens in the late bell (P7) stages of tooth development showed positive staining. (Figure 33). Staining was at its most intense prior to enamel production, i.e. in pre-ameloblasts. Once enamel formation could be seen the staining intensity decreased so that in the mature functioning layer of ameloblasts staining was diffuse in nature, weak to moderate in intensity and located only in the cytoplasm including the Tome's processes (Figures 31, 32, 34, 35 and 36). It was interesting to note that the majority of the specimens examined showed intense staining of the ameloblasts in the pits and fissure regions, but the ameloblasts over the cusp tips remained negative (Figure 37). This correlates well with the absence of enamel over the cusp tips in the mature teeth.

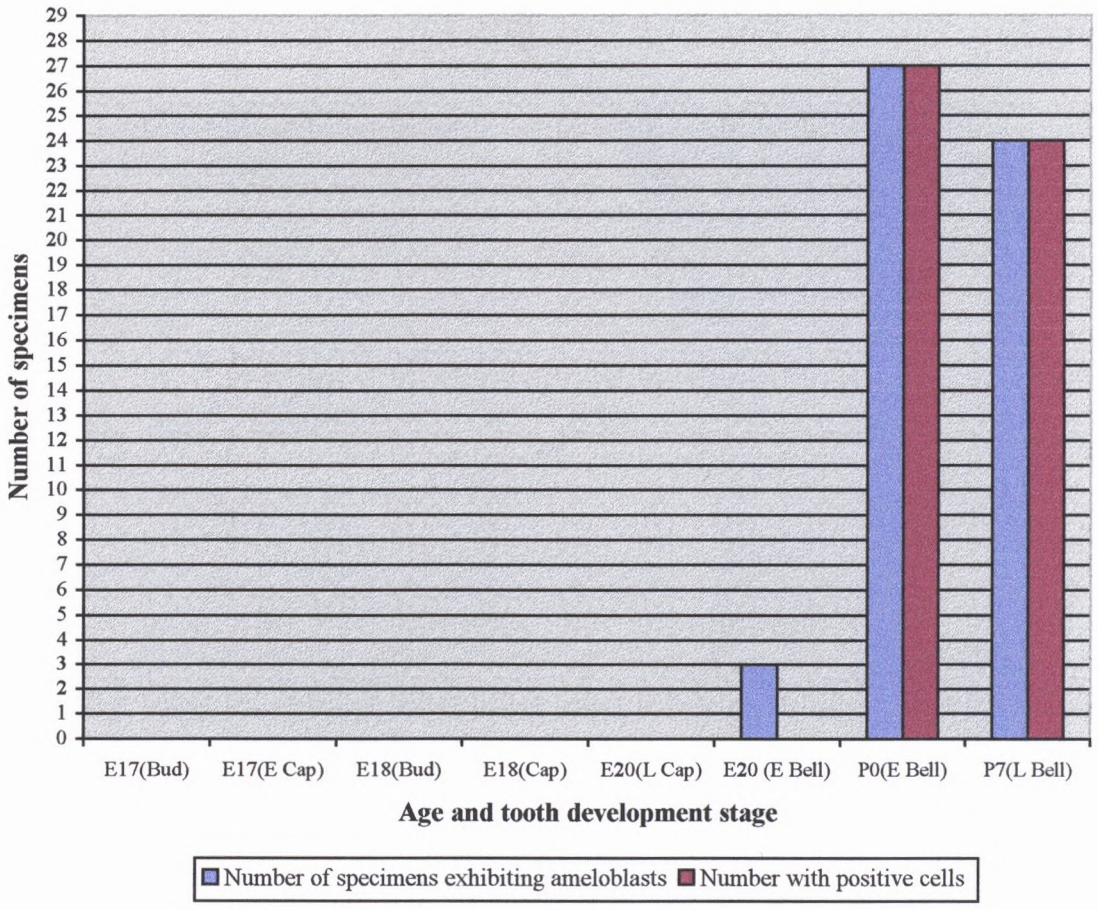


Figure 33: Histogram showing the frequency of ameloblast immunopositivity.

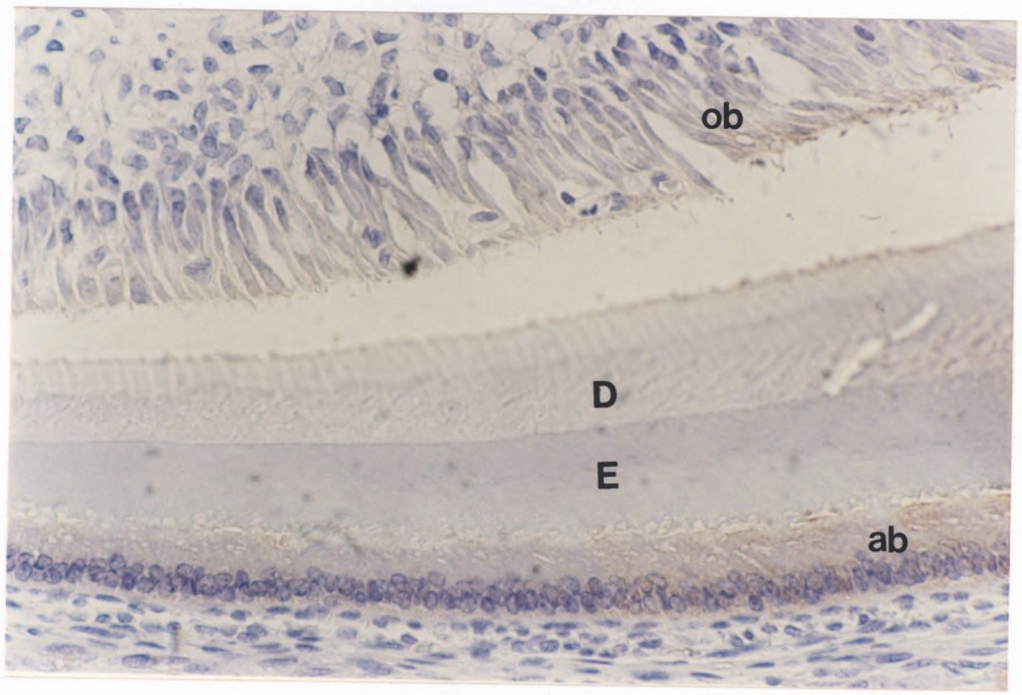


Figure 34: Tooth germ section of a P0 day old specimen showing negative calretinin immunostaining of mature ameloblasts. Calretinin x 400. Counterstained with Meyer's Haematoxylin.

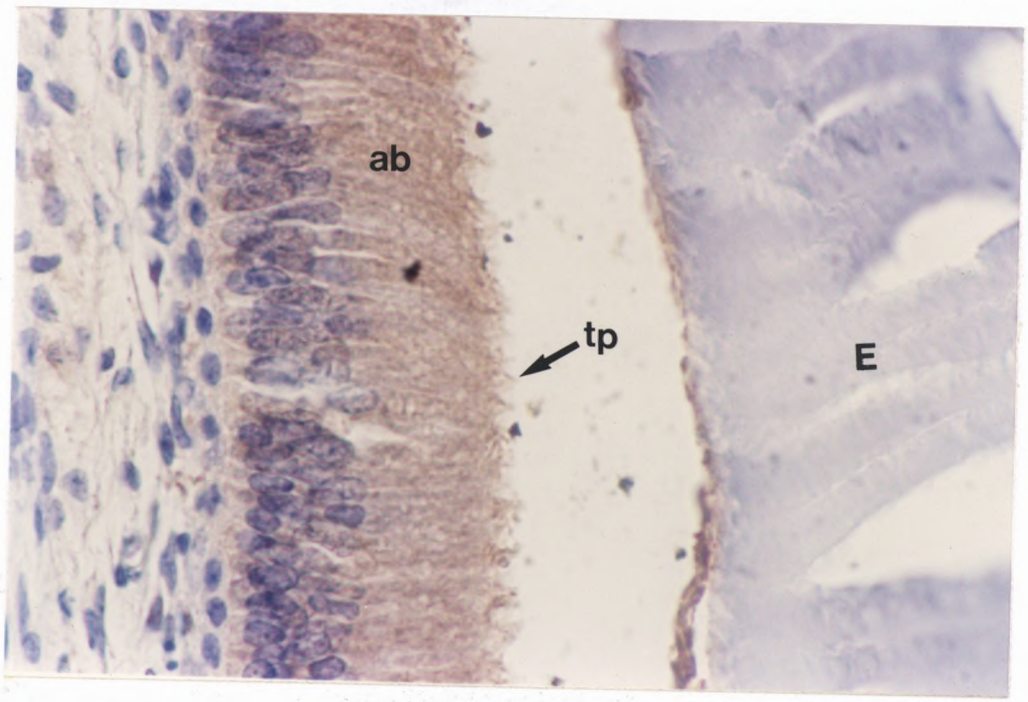


Figure 35: P0 day old specimen in the early bell stage of tooth development showing moderately intense calretinin immunostaining of ameloblasts and their associated Tome's processes. Calretinin x 400. Counterstained with Meyer's Haematoxylin.

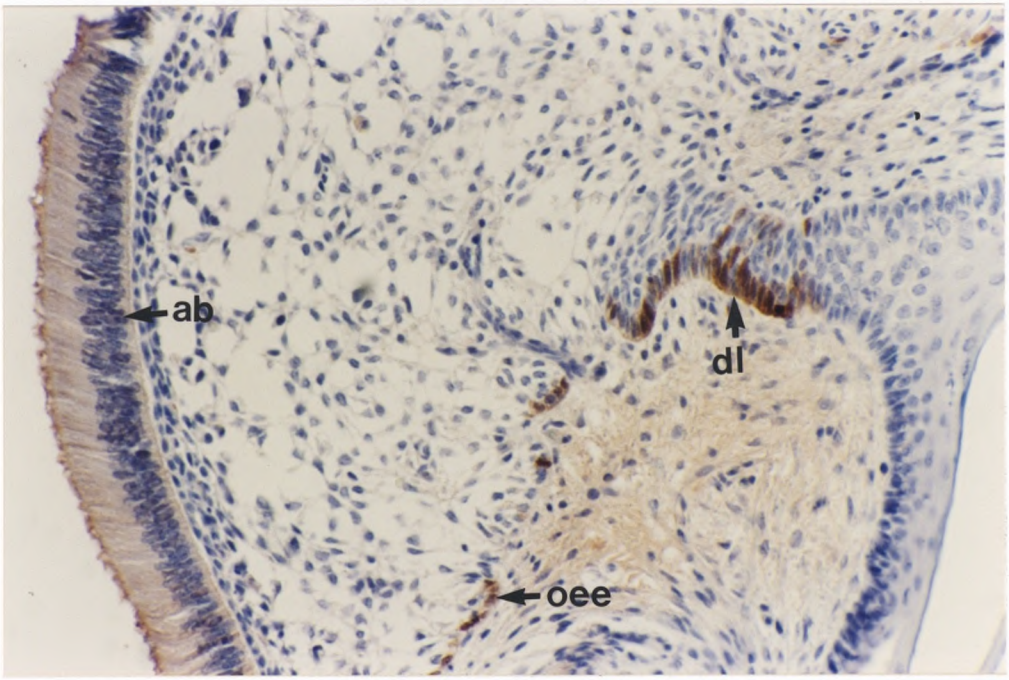


Figure 36: A section through a molar tooth germ showing moderate calretinin immunostaining of ameloblasts, and intense staining of the outer enamel epithelium and dental lamina. Calretinin x 160. Counterstained with Meyer's Haematoxylin.

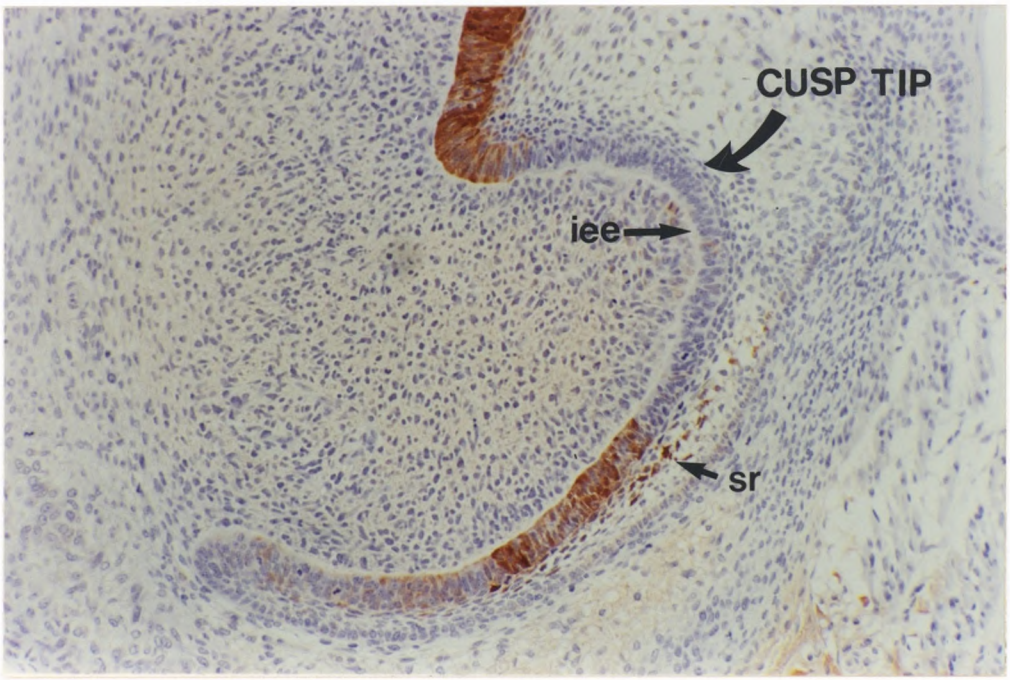


Figure 37: A section through a molar tooth bud of a P0 day old specimen showing positive calretinin immunostaining of the inner enamel epithelium, stellate reticulum, and no staining of the inner enamel epithelium in the region of the cusp tips since no enamel forms in this region. Calretinin x 160. Counterstained with Meyer's Haematoxylin.

5.10 Stratum intermedium

When stratum intermedium was present it stained weak to moderate in a few specimens and was focally distributed (Figure 25).

5.11 Reduced enamel epithelium

This epithelium was not represented in the developmental stages studied.

CHAPTER 6

6.0 DISCUSSION

6.1 Statement of the principle findings.

The results of this study show that calretinin is mainly expressed in oral ectoderm derived tissues during odontogenesis in the developing enamel organ in the rat. It is consistently expressed in the dental lamina (DL), outer enamel epithelium (OEE), stellate reticulum (SR) and stratum intermedium (SI) throughout all the stages of tooth development studied. In the inner enamel epithelium (IEE) and ameloblasts (AB) calretinin expression shows both a temporal and spatial distribution depending upon the degree of differentiation of the cells. In the IEE staining intensity and distribution increases with advancing differentiation reaching a maximum when pre-ameloblasts are formed. Thereafter staining decreases so that in mature ameloblasts it is restricted to the cytoplasm and is only graded as weak to moderate in intensity. In addition some staining of pre-odontoblasts occurred in the early developmental sections, but this disappeared in the maturing odontoblasts which were negative.

Calretinin is not expressed in dental papilla, dental sac and ectomesenchyme which showed no staining throughout the period of tooth development studied.

6.2 Comparison of results with other studies.

As already mentioned there are no previous published reports on calretinin expression in odontogenic tissues during tooth development. We are however aware of some unpublished work by Gotzos and co-workers at the University of Friborg (*personal communication*) in which such expression was demonstrated and indeed have had an

opportunity of examining some photomicrographs illustrating the results of this study. From this limited information it appears as if their results were very similar to ours in terms of immunostaining of the various odontogenic epithelia with the exception that the mature ameloblasts in their study stained very intensely in contrast to our study in which the ameloblasts stained only weakly to moderately and showed a decreasing intensity with increasing differentiation. Since we have no details of the experimental procedure they used we cannot comment on possible reasons for this variation but they may include differences in antisera, staining protocol or in antigen retrieval system used. In addition it remains open to question whether the staining pattern and intensity demonstrated in their photomicrographs was an isolated finding or was consistently reproduced throughout their experiment on a large series of animals.

There are however several published papers reporting the expression of a closely related calcium-binding protein (CaBP), namely calbindin in odontogenic tissues during tooth development in the rat (13, 14, 15). Although there is a high degree of homology between calretinin and calbindin with cross-reactivity having been demonstrated (36) we are not sure whether a direct comparison can be made between their results and our own. The anti-calretinin that we have used does not cross-react with calbindin.* In their studies the same experimental animal was used, namely the Sprague-Dawley rat, but they studied only mandibular teeth, namely incisors (13, 14) and third molars (15), decalcified their specimens, used the peroxidase anti-peroxidase (PAP) staining method and concentrated mainly on ameloblasts while commenting only very briefly on the other odontogenic tissues (13). They do not state whether the staining patterns and intensity reported were consistent or whether variability occurred

* Zymed Laboratories Inc., San Francisco, CA 94080 Lot Number: 81044621. States that "this antibody reacts with human calretinin. Reactivity has also been seen with mouse and rat calretinin. No calbindin reactivity was seen on immunoblots".

between specimens. Nevertheless the results they have reported are of considerable interest.

In their study Taylor, Gleason and Lankford (13) investigated the expression of calbindin in the developing rat incisor. They found the presence of calbindin in the cytoplasm of the ameloblasts in the zone of maturation. The calbindin was present in a relatively uniform distribution within the cytoplasm, but was much lower in concentration or absent from nuclei. The apparent concentration of calbindin in ameloblasts appeared to vary, since the intensity staining varied from cell to cell. However, cells throughout the length of the zone of maturation contained calbindin. Smooth-ended ameloblasts, ruffle-ended ameloblasts, and those ameloblasts apparently in transition between the two forms all contained cytoplasmic calbindin. A dark line of calbindin staining at the basal end of the ameloblasts was routinely noted with smooth-ended ameloblasts, while it was almost always absent at that location in the ruffle-ended ameloblasts. Calbindin persisted in the cytoplasm of ameloblasts, as the cells became shorter in the region of reduced ameloblasts near the gingival margin. However, no calbindin was observed in ameloblasts in developmental stages earlier than the zone of maturation, i.e. zone of secretion and pre-secretion. Also, no calbindin was observed in other cells involved in tooth formation, i.e. pulpal cells, odontoblasts, cells of the stratum intermedium, stellate reticulum, papillary layer, and the outer enamel epithelium.

In a later paper Taylor (14) commented as follows on the results achieved by his group. "The continuously erupting rat incisor provides a useful model for the study of amelogenesis, with ameloblasts existing in all developmental stages from immature undifferentiated cells through various stages of maturity and secretory/absorptive

activity to inactive protective cells in the continuously erupting incisor. The ameloblast was the major type of cell seen to contain calbindin in this study. The binding protein first appeared in the cytoplasm of scattered young ameloblasts that were facing newly formed dentine. It was consistently observed that random ameloblasts at this developmental stage clearly contained calbindin at a time before enamel matrix could be detected with routine histologic stains. At this point in ameloblast development the nuclei have not yet attained their basal location. Very shortly thereafter, when the ameloblasts had further elongated, the nuclei had shifted to the basal aspect of these cells and the first thin line of enamel matrix could be seen, the ameloblasts responsible for the formation of this enamel all uniformly contained calbindin. When the ameloblasts matured to the point of developing Tome's processes, and were actively secreting enamel, the DAB reaction for calbindin was much darker, and completely filled the cytoplasm; including the Tome's processes. Later, as the ameloblasts shortened and assumed either a smooth or ruffled apical end in the zone of maturation, the apparent calbindin content remained the same."

Calbindin expression in developing rat third molars was investigated by Elms and Taylor (15). They reported that "calbindin was not detected in any cells of the enamel organ, dental papilla, or dental sac during early odontogenesis from the dental lamina stage through the advanced bell stage. The protein first appeared in secretory ameloblasts that were situated opposite odontoblasts with newly secreted dentine. Calbindin was present in the cytoplasm of more mature ameloblasts, but not in less mature ameloblasts. Some stratum intermedium cells subjacent to well developed secretory and maturation zone ameloblasts also contained calbindin. The protein was not detected in odontoblasts, pulp cells, or other cells associated with the developing molars. In was also absent from the demineralised enamel and dentine matrix. In

developing rat molars, the time-course of appearance of calbindin, a protein dependent for its synthesis on the vitamin D endocrine system in other organ systems, suggests a potential role of this hormonal system in enamel mineralisation."

A comparison between these results and our own show that calbindin appears much later than calretinin, is much more restricted in its distribution and is expressed more intensely in the ameloblasts without a temporal variation with a decreasing intensity as enamel formation progressed. Why this should be the case remains unknown, but clearly different roles for these two calcium-binding proteins in odontogenesis must exist.

6.3 Strengths and weaknesses of this study.

A possible weakness of our study was that the staining patterns were inconsistent with some specimens staining diffusely and intensely while in others the staining was weak or moderate in intensity and only focal in distribution or no staining occurred at all. In fact even in the same section variation occurred between the various enamel organs that were present. A possible explanation for this is that not all specimens were stained at the same time but consecutive batches were stained on different occasions. It is doubtful whether true variation between individual animals exists.

Decalcification of the tissues also proved to be a problem. In the pilot study regardless of the decalcification system used an increase in background staining and a decrease in staining intensity occurred. As a result no tissue decalcification was used in the experimental sections. Without decalcification considerable tearing and lifting of the tissues was seen especially in the 7 day old specimens which made it difficult to evaluate the staining in some of the developing teeth.

Older animals in which root formation and eruption of teeth was occurring were not studied and so a complete assessment of calretinin expressivity throughout the period of tooth development and eruption could not be obtained. It remains to be determined whether tissues such as root sheath of Hertwig, cementoblasts and reduced enamel epithelium show expression of calretinin.

Nevertheless the results of our study have shown that calretinin is present and may play an important role in tooth formation. It was expressed in all the tissues derived from odontogenic epithelium and only pre-odontoblasts derived from odontogenic mesenchyme. A possible explanation for the peculiar distribution of calretinin in dental lamina, outer enamel epithelium, stellate reticulum, stratum intermedium and inner enamel epithelium is that the protein may be present, as suggested by Elms and Taylor (15), in cells which reside directly in the path of calcium in transition on its way to the enamel matrix. The results suggest that calretinin may play a vital role in the early stages of amelogenesis. It was strongly expressed in pre-ameloblasts and was not expressed at all in those parts of the inner enamel epithelium and in those ameloblasts over the cusp tips where no enamel formation occurs. The lack of calretinin expression in the mature odontoblasts would seem to indicate that it plays a minor role, if any, in dentine formation.

6.4 Possible roles of calcium-binding proteins in odontogenesis.

The exact role of calretinin in odontogenesis remains unknown. As already mentioned in the literature review the physiologic function of calcium binding proteins is achieved via calcium-dependent interactions with other proteins thereby regulating their activity. Their major role is assumed to be buffering, transport of Ca^{2+}, and regulation of various enzyme systems. Also with respect to the distribution pattern of

this protein in the nervous system a role in electrical transmission would be consistent. Because there is no general agreement on the biochemical role of the calcium-binding proteins (CaBP's) in the other systems where it has been more extensively studied, its role in mineralisation of enamel also remains speculative. "The suggestion that calcium-binding proteins may function as a cytoplasm calcium ferry is certainly compatible with its spatial and temporal localisation in odontogenesis."(15).

6.5 Directions for future research.

As mentioned above the favoured suggested role for calretinin is as a calcium ferry. Further research will be required to confirm this function and to determine whether other roles exist during odontogenesis. Another question to be addressed in future studies is the possible influence of diet and nutritional status on the expression of calretinin and its role in tooth development.

Elms and Taylor (15) raised interesting questions on the possible role of vitamin D dependence of CaBP's in tooth formation. In previous studies (37) they showed that when the hormonal form of vitamin D, i.e. calcitriol, was reduced in concentration in the plasma, the synthesis of calbindin-9kD in the intestine of the rat was reduced, but this had no influence on the CaBP content of cells associated with tooth development. These somewhat surprising findings suggested the possibility that receptors for calcitriol in ameloblasts were more sensitive to lower circulating levels of the hormone than were receptors in intestinal absorptive cells. Since animals in their current study were raised on a normal diet no conclusion with respect to the possible effect of vitamin D on calbindin-28kD expression could be made (15).

6.6 Calretinin in tumour pathology.

The use of calretinin in the nervous system by neuro-anatomist to study and identify specific subsets of neurones is well established. Recently however, calretinin has emerged as an immunohistochemical marker with a high discrimination potential in specific areas of pathology. For example, calretinin represents by far the most specific and sensitive marker of both benign and malignant mesothelial cells (7, 8). In addition, calretinin is now established as a marker of neuronal differentiation in central nervous system tumours (38). Calretinin may also have a role to play in the differential diagnosis of cutaneous inflammatory infiltrates since it is consistently expressed in mast cells and in cutaneous mastocytosis (8). Its expression has also been investigated in many other human neoplasms but its potential role as a specific immunohistochemical marker of these tissues has yet to be elucidated (7). Only a partial correlation between staining of normal cells and their neoplastic counterparts has been observed (7).

Gotzos, *et al.* (30) have shown a correlation between the expression of calretinin and the differentiation of tumours. Well-differentiated tumours expressed calretinin in only 5% of the cases, whereas this percentage was 20% for moderately differentiated tumours and 66.6% for poorly differentiated or undifferentiated tumours. They concluded that calretinin may be used as a tumour differentiation marker.

Recent work in our department has shown that calretinin is consistently expressed in the epithelium of both unicystic and solid and multicystic ameloblastomas, but is not expressed in other odontogenic epithelia which line odontogenic keratocysts, dentigerous cysts and residual cysts (39, 40). Based on these results it was suggested that calretinin may be used as a specific immunohistochemical marker of ameloblastic

epithelium and may have an important role to play in the differential diagnosis of odontogenic tumours and cysts.

CHAPTER 7

7.0 CONCLUSION

The present study was stimulated by the ongoing work in our department on calretinin expression in odontogenic tumours and cysts. Analysis and correlation of our results will lead to a better understanding of the potential role of calretinin as a specific immunohistochemical marker of ameloblastic epithelium. In addition this study has shown that calretinin possibly plays an important role in odontogenesis. What this role is remains to be determined.

It is well known that complex epithelial-mesenchymal interactions occur during odontogenesis. In our opinion the spatial and temporal appearance of calretinin has more to do with the variation of maturity and secretory/absorptive activity of the cells than initiating events involved in the process of induction.

CHAPTER 8

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APPENDIX A: Animal Ethics Screening Committee clearance certificates

This appendix contains the approved Animal Ethics Screening Committee's (AESC) clearance certificates and AESC Modification and Extension clearance certificates for this research project.

AESC 3

STRICTLY CONFIDENTIAL

UNIVERSITY OF THE WITWATERSRAND. JOHANNESBURG

ANIMAL ETHICS SCREENING COMMITTEE

CLEARANCE CERTIFICATE NO:

99	6	1

APPLICANT: Dr D Mistry

DEPARTMENT: Anatomical Pathology, Division of Oral Pathology

PROJECT TITLE: Calretinin-immunoreactivity during odontogenesis in the rat

Species	Number	Expiry Date
Rats	1 (for pilot study)	27 January 2001
Rats	34	27 January 2001

i) Approval is hereby given for the experiment described in the above application.

The use of these animals is subject to AESC Guidelines for the use and care of animals, is limited to the procedures specified in the application form, and to:

the researcher discussing the method of euthanasia with the CAS Veterinarian.

SIGNED _____ DATE: 27 January 1999
(Chairman: Animal Ethics Screening Committee)

ii) I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23(1)(c) of the Veterinary and Para-veterinary Professions Act (19 of 1982)

SIGNED _____ DATE:
(Registered Veterinarian)

NOTE:

First-time users of the CAS should contact the Director of the CAS in order to familiarise themselves with the facilities available, and the procedures required by the CAS for the carrying out of experiments.

Please note that only typewritten applications will be accepted. Should additional space be required for section "i" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Dr Dharmesh Mistry

b. Department: Division of Oral Pathology, Department of Anatomical Pathology

c. Experiment to be modified / extended

AESC NO:		
99	6	1

d. Project Title: Calretinin immunoreactivity during odontogenesis in the Rat.

e. Number and species of animals originally approved:

35	Rattus Norvegicus

f. Number of additional animals allocated on M&Es:

g. Total number of animals **allocated** to the experiment to date:

0	

h. Number of animals **used** to date:

0	

i. Specific modification / extension requested:

To change the ages of the animals to be used in this study. By changing the age categories the number of animals used in the study will be reduced to 24.

Modified age categories:

Species	Strain	Sex	Age/Body Mass	Total number of animals required
Rattus	Norvegicus	Female	Pregnant Mature in day 17 of gestation	1
Rattus	Norvegicus	Female	Pregnant Mature in day 18 - 19 of gestation	1
Rattus	Norvegicus	Female	Pregnant Mature in day 20 - 21 of gestation	1
Rattus	Norvegicus	Both	0 days old	1 for pilot study 10 for full study
Rattus	Norvegicus	Both	7 days old	10

j. Motivation for modification / extension:

To be able to more accurately identify the calretinin calcium-binding protein during odontogenesis (tooth development). The original ages that were to be used in the study did not coincide with the development of teeth in the rat.

Date: 17/2/99 Signature: D Mistry

RECOMMENDATIONS:

Reduction of numbers from 35 to 24. (see approval of 27.1.99)

Date: 17.2.99 Signature: GJ Cliffe

Chairman, AESC

November 1998

AESC 4/99 This form is also available on disk (Word Perfect 6 or MSWord 97)

Please note that only typewritten applications will be accepted. Should additional space be required for section "i" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Dr Dharmesh Mistry

b. Department: Division of Oral Pathology, Department of Anatomical Pathology

c. Experiment to be modified / extended

AESC NO:		
99	6	1

d. Project Title: Calretinin immunoreactivity during odontogenesis in the Rat.

e. Number and species of animals originally approved:

35	Rattus Norvegicus

f. Number of additional animals allocated on M&Es:

g. Total number of animals allocated to the experiment to date:

24	Rattus Norvegicus

h. Number of animals used to date:

24	Rattus Norvegicus

i. Specific modification / extension requested:

To obtain approximately 25 - 30 ml of Rat serum required for histological staining purposes, i.e. Immunohistochemistry.

j. Motivation for modification / extension:

By using the Rat serum cross reactivity will be eliminated.

Date: 21/5/99 Signature: DMistry

RECOMMENDATIONS:

Approved: use of serum from rats kiled for other purposes
Details to be arranged with Mr Dawson, CAS Biochem.

Date: 25.5.99 Signature: .....
Chairman, AESC

November 1998

Please note that only typewritten applications will be accepted. Should additional space be required for section "i" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Dr Dharmesh Mistry

b. Department: Division of Oral Pathology, Department of Anatomical Pathology

c. Experiment to be modified / extended

AESC NO:		
99	6	1

d. Project Title: Calretinin immunoreactivity during odontogenesis in the Rat.

e. Number and species of animals originally approved:

35	Rattus Norvegicus

f. Number of additional animals allocated on M&Hs:

g. Total number of animals **allocated** to the experiment to date:

24	Rattus Norvegicus

h. Number of animals **used** to date:

24	Rattus Norvegicus

i. Specific modification / extension requested:

Require further 10 0 day and 10 7 day old rats for further histological preparation and staining.

j. Motivation for modification / extension:

During histological preparation encountered large amounts of hard tissue which causes the histological section to tear, and hence need to decalcify specimens prior to histological preaparation.

Date: 25/5/99 Signature: .....

RECOMMENDATIONS:

Approved: ten newborn and ten seven-day old rats.

Date: 26.5.99 Signature: .....

Chairman, AESC

November 1998

APPENDIX B: Raw data

This appendix contains the raw data.

KEY

DL	Dental lamina
EM	Ectomesenchyme
OEE	Outer enamel epithelium
IEE	Inner enamel epithelium
DP	Dental papilla
SR	Stellate reticulum
O/B	Odontoblasts
O/B differentiating	Odontoblasts differentiating
A/B	Ameloblasts
A/B (D present)	Ameloblasts - dentine present
A/B (D & E)	Ameloblasts - enamel and dentine present
A/B (TP)	Ameloblasts - Tome's processes
S/I	Startum intermedium
Cervical loop	Cervical loop

INCISORS

Specimen No	Age	Development Stage	DL	EM	OEE	IEE	DP	SR	O/B	A/B	A/B (D present)	A/B (no dentine)	A/B (D &E)	A/B (TP)	S/I	Cervial Loop
A10/99	E17															
A11/99	E17	Bud	Negative	Negative												
A12/99	E17	Bud	Negative	Negative												
A13/99	E17	Bud	Negative	Negative												
A14/99	E17	Bud	Negative	Negative												
A15/99	E17															
A16/99	E17	Bud	Negative	Negative												
A17/99	E17	Early cap	Negative	Negative	Negative	Negative	Negative	Negative								
A18/99	E17															
A19/99	E17															
A70/99	E17															
A71/99	E17	Early cap	Negative	Negative	Negative	Negative	Negative									

INCISORS

Specimen No	Age	Development Stage	DL	EM	OEE	IEE	DP	SR	O/B	A/B	A/B (D present)	A/B (no dentine)	A/B (D &E)	A/B (TP)	S/I	Cervial Loop
A20/99	E18	Cap		Negative	Negative	Negative		Focal weak single cells								
A21/99	E18	Cap		Negative	Negative	Negative		Focal weak single cells								
A22/99	E18	Cap		Negative	Negative	Negative	Negative	Focal weak single cells								
A23/99	E18	Cap	Negative	Negative	Negative	Negative	Negative	Focal weak								
A24/99	E18															
A25/99	E18	Cap	Negative	Negative	Negative	Negative	Negative	Negative								
A26/99	E18	Cap	Negative		Negative	Negative	Negative	Negative								
A27/99	E18															
A28/99	E18															
A29/99	E18	Bud	Negative	Negative	Negative	Negative	Negative	Negative								
A65/99	E18	Cap		Negative	Negative	Negative		Negative								
A66/99	E18	Cap	Negative	Negative	Negative	Negative	Negative									
A67/99	E18															
A67/99	E18															
A68/99	E18															
A69/99	E18															
A69/99	E18															

INCISORS

Specimen No	Age	Development Stage	DL	EM	OEE	IEE	DP	SR	O/B	A/B	A/B (D present)	A/B (no dentine)	A/B (D &E)	A/B (TP)	S/I	Cervial Loop
A30/99	E20	Cap	Negative	Negative	Negative	Negative	Negative	Negative								
A31/99	E20	Cap			Focal intense	Focal intense		Focal intense	Focal weak							
A32/99	E20	Cap	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative						
A33/99	E20	Cap	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative						
A34/99	E20	Cap	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative						
A35/99	E20															
A36/99	E20															
A37/99	E20															
A38/99	E20															
A39/99	E20															
A40/99	E20															
A41/99	E20															
A64/99	E20	Cap			Focal weak	Negative	Negative	Focal weak	Negative							

INCISORS

Specimen No	Age	Development Stage	DL	EM	OEE	IEE	DP	SR	O/B	A/B	A/B (D present)	A/B (no dentine)	A/B (D &E)	A/B (TP)	S/I	Cervial Loop
A42/99 A&B	0	Early bell			Focal intense	Focal intense	Focal weak scattered +ve cells	Focal weak	Negative	Focal weak		Focal weak			Occassional focal weak	
A43/99 A&B	0	Early bell														
A44/99 A&B	0	Early bell			Negative	Negative	Negative		Negative			Focal weak	Focal moderate	Focal moderate	Focal weak +ve cells	
A45/99 A&B	0	Early bell			Focal moderate	Focal moderate	Isolated +ve cells	Focal weak	Negative	Diffuse intense						
A46/99 A&B	0	Early bell			Focal moderate	Focal moderate	Focal moderate isolated cells	Scanty		Diffuse weak						
A47/99 A&B	0	Early bell			Focal moderate	Focal moderate		Negative	Negative	Diffuse weak-moderate						
A48/99 A&B	0	Early bell			Scanty		Isolated +ve cells		Negative	Diffuse weak-moderate						
A49/99 A&B	0	Early bell			Focal weak	Focal moderate	Isolated +ve cells	Focal weak	Negative	Diffuse intense (n+c)						
A50/99 A&B	0	Early bell			Scanty	Focal weak	Isolated +ve cells	Scanty		Diffuse weak						
A63/99 A&B	0	Early bell			Not seen	Not seen	Negative		Negative		Diffuse weak-moderate					

INCISORS

Specimen No	Age	Development Stage	DL	EM	OEE	IEE	DP	SR	O/B	A/B	A/B (D present)	A/B (no dentine)	A/B (D &E)	A/B (TP)	S/I	Cervial Loop
A51/99 A&B	7	Late bell			Not represented	Focal moderate	Negative		Focal weak	Focal moderate			Diffuse moderate (n+c)			
A52/99 A&B	7	Late bell				Focal moderate		Focal intense	Focal moderate			Focal intense				Focal moderate
A53/99 A&B	7	Late bell			Focal intense	Focal intense	Negative	Focal moderate	Diffuse moderate				Diffuse moderate (n+c)	Diffuse intense		
A54/99 A&B	7	Late bell			Not seen	Not seen	Negative	Not seen	Focal moderate	Diffuse moderate						
A55/99 A&B	7	Late bell			Focal intense	Focal intense	Negative	Not seen	Negative	Diffuse weak-moderate (n+c)				Diffuse moderate		
A56/99 A&B	7	Late bell			Focal moderate	Focal moderate	Negative	Focal moderate	Diffuse weak O/B processes	Diffuse moderate (n+c)				Diffuse moderate		
A57/99 A&B	7	Late bell			Focal intense	Focal intense	Isolated +ve cells	Not seen	Negative				Diffuse intense (n+c)	Diffuse intense		
A58/99 A&B	7	Late bell			Focal intense	Focal intense	Negative	Not seen	Negative				Diffuse moderate (n+c)			
A59/99 A&B	7	Late bell					Negative	Not seen	Negative		Diffuse weak (n+c)			Diffuse weak		
A60/99 A&B	7	Late bell					Negative	Not seen	Negative		Diffuse weak			Diffuse weak		
A61/99 A&B	7	Late bell			Focal weak	Negative	Isolated +ve cells	Negative	Focal weak		Diffuse weak			Diffuse moderate		
A62/99 A&B	7	Late bell					Negative	Focal weak	Negative				Diffuse weak			

MOLARS

Specimen No	Age	Development Stage	DL	EM	OEE	IEE	DP	SR	O/B	O/B (different lating)	A/B	A/B (D)	A/B (E & D)	A/B (TP)	S/I	Cervial Loop
A10/99	E17	Bud	Negative	Negative												
A11/99	E17	Early cap	Focal weak	Negative	Negative	Negative										
A12/99	E17	Early cap	Negative	Negative	Negative	Negative		Negative								
A13/99	E17	Bud	Negative	Negative												
A14/99	E17	Early cap	Negative	Negative	Negative	Negative	Negative									
A15/99	E17	Early cap		Negative	Negative	Negative	Negative	Negative								
A16/99	E17	Bud	Negative	Negative												
A17/99	E17															
A18/99	E17															
A19/99	E17	Early cap	Negative	Negative	Negative	Negative	Negative	Negative								
A70/99	E17	Early cap	Focal weak	Negative	Focal weak			Negative								
A71/99	E17															

MOLARS

Specimen No	Age	Development Stage	DL	EM	OEE	IEE	DP	SR	O/B	O/B (different lating)	A/B	A/B (D)	A/B (E & D)	A/B (TP)	S/I	Cervial Loop
A20/99	E18	Bud	Negative	Negative												
A21/99	E18	Cap		Negative	Negative	Negative		Focal weak single cells								
A22/99	E18	Bud	Negative	Negative												
A23/99	E18															
A24/99	E18	Cap	Negative		Negative	Negative	Negative	Focal moderate								
A25/99	E18	Cap	Negative		Negative	Negative	Negative	Focal weak								
A26/99	E18															
A27/99	E18	Bud	Negative	Negative												
A28/99	E18	Cap		Negative	Negative	Negative	Negative	Negative								
A29/99	E18	Cap		Negative	Negative	Negative	Negative	Focal moderate								
A65/99	E18	Cap		Negative	Focal weak	Negative		Focal weak								
A66/99	E18	Cap	Focal moderate		Focal weak	Negative	Negative	Focal intense								
A67/99	E18	Cap	Focal moderate		Negative	Negative	Negative	Focal moderate-intense								
A67/99	E18	Bud	Focal moderate	Negative												
A68/99	E18	Cap	Focal moderate		Focal moderate	Negative	Negative	Focal intense								
A69/99	E18	Bud	Focal moderate	Negative												
A69/99	E18	Cap	Focal moderate	Negative	Focal intense	Negative	Negative	Focal intense								

MOLARS

Specimen No	Age	Development Stage	DL	EM	OEE	IEE	DP	SR	O/B	O/B (different lating)	A/B	A/B (D)	A/B (E & D)	A/B (TP)	S/I	Cervial Loop
A30/99	E20	Early Bell			Focal weak	Focal weak	Negative	Negative								
A31/99	E20	Cap	Focal weak		Focal intense	Focal intense	Negative	Focal intense								
A32/99	E20	Cap			Focal weak moderate	Focal weak moderate	Negative	Focal moderate								
A33/99	E20	Cap			Focal weak	Focal weak @ tips	Negative	Focal weak								
A34/99	E20	Cap			Focal moderate	Focal moderate	Focal moderate single cells	Focal weal								
A35/99	E20	Cap	Focal moderate		Focal moderate	Focal moderate	Focal moderate	Focal moderate								
A36/99	E20	Cap			Focal weak	Focal weak	Focal moderate single cells	Negative								
A37/99	E20	Cap			Focal weak	Focal weak	Focal moderate single cells	Negative								
A38/99	E20	Cap	Focal weak		Focal weak	Focal weak	Focal moderate isolated cells	Focal weak cytoplasmic extensions								
A39/99	E20	Cap			Focal moderate	Focal moderate	Focal moderate single cells	Focal weak								
A40/99	E20	Cap	Focal moderate		Focal moderate-intense	Focal moderate-intense	Focal moderate with isolated +ve cells	Focal weak								
A41/99	E20	Early Bell			Focal moderate	Focal moderate	Focal moderate isolated +ve cells	focal moderate-intense								
A64/99	E20															

MOLARS

Specimen No	Age	Development Stage	DL	EM	OEE	IEE	DP	SR	O/B	O/B (differentiating)	A/B	A/B (D)	A/B (E & D)	A/B (TP)	S/I	Cervial Loop
A42/99 A&B	0	Early Bell			Focal moderate	Focal weak	Scattered +ve cells	Focal weak							Focal weak-occasional	
A43/99 A&B	0	Early Bell	Focal moderate		Focal moderate	Focal moderate	Negative	Focal moderate	Focal weak							
A44/99 A&B	0	Early Bell			Focal intense			Focal intense		Diffuse intense		Diffuse intense				
A45/99 A&B	0	Early Bell			Focal moderate	Focal moderate	Negative	Focal weak	Focal weak				Diffuse intense			
A46/99 A&B	0	Early Bell			Focal moderate	Focal moderate	Focal weak @ differentiating O/B	Focal moderate-intense	Focal weak		Diffuse intense (n+c)					
A47/99 A&B	0	Early Bell			Focal moderate	Focal moderate	Isolated +ve cells	Focal moderate	Focal moderate just @ dentine production		Diffuse intense (n+c)					
A48/99 A&B	0	Early Bell			Focal weak	Focal weak		Focal moderate in SI area	Focal weak in differentiating areas		Diffuse intense (n+c) only in pits and fissures					
A49/99 A&B	0	Early Bell			Focal moderate	Focal moderate	Focal weak	Focal moderate-intense			Diffuse intense (n+c)					
A50/99 A&B	0	Early Bell			Focal intense	Focal moderate		Focal weak against A/B	Focal weak before dentine production		Diffuse intense (lateral vallies)					
A63/99 A&B	0	Early Bell	Focal moderate		Focal moderate	Focal weak	Negative	Focal intense	Focal weak moderate		Diffuse intense					

MOLARS

Specimen No	Age	Development Stage	DL	EM	OEE	IEE	DP	SR	O/B	O/B (different lating)	A/B	A/B (D)	A/B (E & D)	A/B (TP)	S/I	Cervial Loop
A51/99 A&B	7	Late Bell			Focal moderate		Negative		Negative				Focal moderate	Diffuse moderate (Questionable)		
A52/99 A&B	7	Late Bell	Focal intense		Focal intense	Focal moderate	Isolated +ve cells	Focal intense in SI area	Focal moderate		Diffuse moderate		Diffuse moderate	Diffuse intense		
A53/99 A&B	7	Late Bell	Focal intense		Focal intense	Negative	Negative	Focal moderate in SI area	Diffuse moderate in SI area				Diffuse moderate	Diffuse moderate		
A54/99 A&B	7	Late Bell			Focal intense	Focal weak	Negative	Focal weak	Diffuse moderate in SI area (O/B processes)				Diffuse moderate	Diffuse moderate		
A55/99 A&B	7	Late Bell	Focal intense		Focal intense		Negative	Focal moderate	Negative				Diffuse weak	Diffuse moderate		
A56/99 A&B	7	Late Bell	Focal intense		Focal intense	Focal weak	Negative	Focal weak in SI area	Diffuse weak (O/B process)		Diffuse moderate-intense (n+c)			Diffuse moderate		
A57/99 A&B	7	Late Bell	Focal intense		Focal intense	Not seen	Negative	Focal moderate in SI area	Focal weak and O/B processes diffuse moderate-weak		Diffuse intense (n+c)					
A58/99 A&B	7	Late Bell	Focal intense		Focal intense	Focal moderate	Negative	Focal intense	Focal weak in crest of pulp		Diffuse moderate (n+c)			Diffuse moderate		
A59/99 A&B	7	Late Bell	Focal intense		Focal intense		Negative	Granular +ve	Focal weak				Diffuse moderate (n+c)	Diffuse intense		
A60/99 A&B	7	Late Bell	Focal intense		Focal intense		Focal weak	Granular +ve cells in SI area	Focal weak O/B processes staining				Diffuse moderate-intense (n+c)	Diffuse intense		
A61/99 A&B	7	Late Bell	Focal intense		Focal intense			Focal moderate	Diffuse weak in crest of pulp				Diffuse moderate (n+c)	Diffuse moderate		
A62/99 A&B	7	Late Bell			Focal moderate @ tips	Focal moderate @ tips	Focal weak +ve cells	Focal intense granularity of SI	Focal weak @ O/B processes				Diffuse moderate	Diffuse moderate		

APPENDIX C: Frequency tables

This appendix contains the tables that show the frequency of immunostaining of calretinin in incisors and molars at various stages of tooth development in different ages. Since each odontogenic tissue was not necessarily always present at each developmental stage the figure in brackets denotes the number of tooth germs in which this tissue was represented. In the tables below an empty value indicates that this odontogenic tissue is not represented at this stage of odontogenesis. A value of zero (0) indicates no staining. N = Number of tooth germs examined. n = Number of tooth germs examined in specific developmental stage.

E17 (Bud and early cap stages of odontogenesis)

Number of animals = 12

Odontogenic tissue examined	Incisors N = 7		Molars N = 9	
	Bud n = 5	Early Cap n = 2	Bud n = 3	Early Cap n = 6
Dental lamina	0(5)	0(2)	0(3)	2(5)
Ectomesenchyme	0(5)	0(2)	0(3)	0(6)
Outer enamel epithelium		0(2)		1(6)
Inner enamel epithelium		0(2)		0(5)
Dental papilla		0(2)		0(3)
Stellate reticulum		0(1)		0(4)
Odontoblasts				
Ameloblasts				
Cervical loop				
Stratum intermedium				

Table 2: Frequency of calretinin immunoreactivity during odontogenesis in foetal rats, embryonic day 17 (E17).

E18 (Bud, late cap and early bell stages of odontogenesis)

Number of animals = 15

Odontogenic tissue examined	Incisors N = 9		Molars N = 15	
	Bud n = 1	Cap n = 8	Bud n = 5	Cap n = 10
Dental lamina	0(1)	0(4)	2(5)	4(6)
Ectomesenchyme	0(1)	0(7)	0(5)	0(5)
Outer enamel epithelium		0(8)		4(10)
Inner enamel epithelium		0(8)		0(10)
Dental papilla		0(5)		0(8)
Stellate reticulum		4(7)		8(10)
Odontoblasts				
Ameloblasts				
Cervical loop				
Stratum intermedium				

Table 3: Frequency of calretinin immunoreactivity during odontogenesis in foetal rats, embryonic day 18 (E18).

E20 (Late cap and early bell stages of odontogenesis)

Number of animals = 13

Odontogenic tissue examined	Incisors N = 6	Molars N = 12	
	Late Cap n = 6	Late Cap n = 10	Early bell n = 2
Dental lamina	0(4)	4(4)	0(2)
Ectomesenchyme	0(4)		
Outer enamel epithelium	2(6)	10(10)	2(2)
Inner enamel epithelium	1(6)	10(10)	2(2)
Dental papilla	0(5)	7(10)	1(2)
Stellate reticulum	2(6)	8(10)	1(2)
Odontoblasts	1(5)		
Ameloblasts	0(3)		
Cervical loop			
Stratum intermedium			

Table 4: Frequency of calretinin immunoreactivity during odontogenesis in foetal rats, embryonic day 20 (E20).

Postnatal day 0 (P0) - (Early bell stage of odontogenesis)

Number of animals = 10

Odontogenic tissue examined	Incisors N = 10	Molars N = 10
	Early bell n = 10	Early bell n = 10
Dental lamina		2(2)
Ectomesenchyme		
Outer enamel epithelium	6(9)	10(10)
Inner enamel epithelium	7(8)	9(9)
Dental papilla	7(9)	4(7)
Stellate reticulum	4(7)	10(10)
Odontoblasts	0(7)	7(7)
Odontoblasts (differentiating)		1(1)
Ameloblasts	9(9)	8(8)
Cervical loop		
Stratum intermedium	2(2)	1(1)

Table 5: Frequency of calretinin immunoreactivity during odontogenesis in postnatal day 0 (P0) old rats.

Postnatal day 7 (P7) - (Late bell stage of odontogenesis)

Number of animals = 12

Odontogenic tissue examined	Incisors N = 12	Molars N =12
	Late bell n = 12	Late bell n = 12
Dental lamina		9(9)
Ectomesenchyme		
Outer enamel epithelium	6(6)	12(12)
Inner enamel epithelium	7(8)	5(6)
Dental papilla	2(11)	3(11)
Stellate reticulum	4(5)	11(11)
Odontoblasts	6(12)	10(12)
Ameloblasts	12(12)	12(12)
Cervical loop	1(1)	
Stratum intermedium		

Table 6: Frequency of calretinin immunoreactivity during odontogenesis in postnatal day 7 (P7) old rats.