THE PASSAGE OF CALCITONIN THROUGH THE DENTAL ROOT.

In vitro studies of diffusion kinetics and parameters affecting diffusion.

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LIST OF ABBREVIATIONS

Å	angstrom
ATPase	adenosine triphosphatase
°C	degrees Celcius
BSA	bovine serum albumin
cAMP	cyclic adenosine monophospate
CDJ	cemento dentinal junction
CEJ	cemento enamel junction
Ci	curie
cm^2	square centimetre(s)
cpm	counts per minute
CT	Calcitonin
1,25 Vit D_3	1,25 dihydoxy cholecalciferol (Vitamin D)
DEJ	dentino enamel junction
DMEM	Dulbecco's modification of Eagle's medium
EDTA	ethylene-diamine-tetra-acetic acid
EDTAC	ethylene-diamine-tetra-acetic acid with cetyltrimethylammomium bromide
GAG	glycosaminoglycan(s)
h	hour(s)
IU	International Units of penicillin
l	litre(s)

mg	milligramme(s)
min	minutes(s)
ml	millilitre(s)
mmHg	millimetre(s) of mercury
mol wt	molecular weight
mm^2	square millimetre(s)
MRC units	British Medical Research Council units
nm	nanometre(s)
PBS	phosphate buffered saline
PDM	periodontal membrane
pg	picogramme(s)
pmol	picomole(s)
PTH	Parathyroid Hormone
rpm	revolutions per minute
SD	standard deviation
sec	second(s)
SEM	scanning electron microscopy
TEM	transmission electron microscopy
[I^{125}]-CT	iodine^{125}-labelled calcitonin
μCi	microcurie(s)
μg	microgramme(s)
μl	microlitre(s)
μm	micrometer(s)
wk	week(s)

SUMMARY

Calcitonin, a polypeptide hormone, inhibits osteoclastic activity both *in vivo* and *in vitro*. This hormone has been used in the treatment of Paget's disease, hypercalcaemia and osteoporosis. Moreover, it has been shown to both prevent experimentally-induced external inflammatory root resorption in monkey teeth (Pierce et al 1988a), and reduce pulpal inflammation (Cullum and Kline, 1985). The current study examines the notion that intracanal placement of the hormone will result in a protracted delivery to resorption sites at external root surfaces. To validate such a proposed route of therapeutic delivery, some of the features which might regulate the kinetics of calcitonin diffusion from the root canal to the periodontal membrane were examined.

In an attempt to identify the contribution that mineralized matrix makes to the diffusion kinetics in dentinal tubules, the conditions have been limited to tooth material which has been freed of soft tissue by alkaline hydrolysis. Thus, to determine the status of the dentinal tubules before and after this maceration, scanning electron-microscopic (SEM) studies were undertaken.

Further SEM investigations were performed to determine the effect, if any, of:

i) two commonly used tooth storage media,

ii) the time of storage in these media, and

iii) endodontic root-canal preparation

on the dentinal tubule contents in extracted teeth. These investigations were carried out in order to describe and compare the effects of these various parameters on diffusion within dentinal tubules pathways. These studies have been discussed in respect of future

studies of putative therapeutic diffusants in non-macerated teeth, a situation more closely resembling that seen *in vivo*.

Superior preservation of loose organic material in the dentinal tubules of extracted teeth was achieved: a) by storing the teeth, at 4°C, in phosphate buffered saline as compared with Dulbecco's modification of Eagle's medium, and b) by chemo-mechanical preparation of the root canals. The degree of preservation of loose organic material in the dentinal tubules of extracted teeth stored in either of the above-mentioned media at 4°C observed after 24 h was maintained for up to 3 wk. The proportion of dentinal tubules containing loose organic contents was significantly greater in the pulpal third of the root canal under all conditions.

One feature which may regulate the diffusion of calcitonin through the endodontically-prepared root canal to the external surface is the tightness of calcitonin binding to mineralized matrix. Relevant exchanges on the binding sites will provide further control over the movement of calcitonin through the dental root. This study has specifically focused on the diffusion and binding characteristics of calcitonin to the mineralized matrix of the dental root.

Three experimental *in vitro* approaches were undertaken: (1) to establish whether calcitonin will diffuse through dentinal tubules of intact teeth from the root canal to the external environment; (2) to establish to what extent the binding coefficient of calcitonin for mineralized tooth matrix contributes to the kinetics of this process and; (3) to establish whether non-specific protein binding will exchange, inhibit or otherwise affect these diffusion kinetics.

To address the first approach, intact, single-rooted premolar teeth extracted for orthodontic reasons from healthy adolescents were macerated and apically sealed. The

kinetics of diffusion from the root canal to the external root surface of calcitonin was then established. Controlled standard cross-sectional diffusion exits on the tooth-roots were established by exposing windows of dentine or cementum on root surfaces which had been coated with sealant. Nail varnish, waxes, unfilled resin materials, composite resins, and poly-vinyl acetate resulted in unacceptable leakage of indicator dyes and radiolabelled calcitonin. Acrylic paint provided a satisfactory root surface seal for in excess of 2 wk.

In the experimental teeth, the standard windows were either cut in the sealant so as to expose the cementum, or the cementum was cut back further to expose dentine. SEM investigation of these window preparations confirmed that the desired ultrastructural status was achieved. Teeth without windows served as controls. [I^{125}]-calcitonin (0.04 ml) was pipetted into the root canal and the access cavity was sealed. Diffusion into standard 2 ml aliquots of PBS was monitored, as a function of time, with a gamma counter.

The calcitonin appeared on the distal side of the windows by 2 h. Since material continued to appear in the PBS over the nine days of the study, it is proposed that tooth matrix might provide an intrinsic slow-release mechanism for calcitonin delivery.

To establish the role of calcitonin binding in the diffusion kinetics within dentinal tubules, radioactive calcitonin was bound to ground tooth-root matrix. Both values for the saturation of binding sites and modified Scatchard plots were prepared. Macerated root matrix was also pretreated with bovine serum albumin (BSA) or non-labelled calcitonin prior to the addition of radiolabelled calcitonin. These experiments were designed to establish what other levels of binding constraints may be relevant in this binding regulated diffusion. Briefly, binding of [I^{125}]-labelled calcitonin to mineral could be reduced by approximately 600% by preloading the system with non-radiolabelled

calcitonin and by about 100% where BSA was used as a competitor. The data provided us with straight lines on modified Scatchard plots, which suggest simple non-interactive binding. BSA was less competitive than non-radioactive calcitonin. It was concluded that this binding capacity contributes to diffusion kinetics which in turn will affect the levels of calcitonin detected outside the experimental window.

These *in vitro* data suggest that an integral mechanism exists for the delivery of a slow release, localized therapeutic dose of calcitonin to external root resorption sites. Based on the data from our BSA pre-treated experiments, such a slow-release mechanism may be augmented in the presence of tissue proteins, but is nonetheless likely to be dominated by the diffusion characteristics *in vivo* of the dentinal tubules and cementum themselves.

DECLARATION

This research report is submitted as a partial requirement for the Degree of Master of Dental Surgery in Endodontics at the University of Adelaide. Further requirements for the Degree were completed during 1988 and 1989.

This research report contains no material previously accepted for the award of any other degree or diploma in any University. To the best of my knowledge, this report contains no material previously published or written by another person, except where due reference is made in the text of this report.

I consent to this research report to be photocopied and loaned if it is accepted for the Degree.

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INTRODUCTION

External inflammatory root resorption is a process frequently encountered in endodontic practice as a post-traumatic sequel. It is thought to be maintained by an inflammation in the periodontal membrane caused by bacteria in the pulp, root canal and dentinal tubules (Andreasen 1980a, Hammarström et al 1986a). The insertion of various medicaments, such as an antibiotic/corticosteroid preparation (Ledermix paste[1]), into root canals following chemo-mechanical preparation is a therapy aimed at treating this condition (Pierce & Lindskog 1987). By passing through the dentinal tubules to the external root surface, a medicament may act directly on the cells of the periodontal membrane (PDM), particularly in areas where cementum has been removed (Pierce et al 1988b). Hence the resorption process may be inhibited by reduction of inflammation and its associated release of osteoclast-activating cytokines, or via a direct inhibitory action on the dentine-resorbing cells themselves. Although intracanal medication with Ledermix paste is effective in the treatment of most cases of external inflammatory root resorption, teeth showing severe resorption may not respond to this therapy. Furthermore, the use of this medicament may be precluded in external-resorption cases where there are medical contraindications to its corticosteroid or tetracycline components. Therefore, an alternative therapy to treat external-inflammatory root resorption is desirable.

Calcitonin (CT), a polypeptide hormone, has recently been shown to be successful in eliminating experimentally-induced, inflammatory root resortion in monkey teeth when the hormone is applied topically into the root canals (Pierce et al 1988a). This hormone

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[1]Ledermix paste - Lederle Pharmaceuticals, Wolfratshausen, West Germany

may act to both directly inhibit dentinoclasts, via specific cell surface receptors, and suppress inflammation. Regulation of these therapeutic events will depend upon, in part, the rate of arrival of the hormone at the site of activity and the biological status of the hormone at the site.

The notion that the placement of CT within root canals will result in a protracted delivery to the external root surface has been considered. Studies on the kinetics and factors affecting simple diffusion of proteins across the dental root are lacking. The following discussion examines current concepts of both external root resorption and the structure of the dental root. It also reviews previous dentine diffusion studies as well as aspects of CT as a medicament.

1. EXTERNAL ROOT RESORPTION

Hard-tissue resorption in the dento-alveolar complex may be either a physiologic or a pathologic process and can result in loss of enamel, dentine, cementum and alveolar bone. External root resorption is initiated in the periodontium and affects the cementum and/or the underlying dentine.

The causes of external resorption include:

1) Periapical and periodontal inflammation induced by bacteria and bacterial toxins from the root canal (Sundqvist 1976).

2) Prolonged and excessive mechanical forces usually produced by poorly designed orthodontic appliances (Barber & Sims 1981, Langford & Sims 1982, Malmgren et al 1982).

3) Dental traumatic injuries, especially luxation and avulsion injuries. The degree of external progressive root resorption is directly related to the degree of injury to the PDM (Andreasen 1970, Skieller 1980, Hedegard & Stalhane 1973).

4) Tumours, cysts and other fibro-osseous lesions (Cohen & Burns 1987).

5) Impacted and erupting teeth (Azaz & Shtayer 1978, Holcomb et al 1983, Nitzan et al 1963).

6) Radiation therapy (Cohen & Burns 1987).

7) Galvanic corrosion of non-precious posts (Cohen & Burns 1987).

8) Systemic diseases including hypoparathyroidism, Gaucher's disease, hyperparathyroidism, Turner's syndrome and Paget's disease (Cohen & Burns 1987).

1.1 Forms of external root resorption

There are four clinically-distinct forms of external root resorption, the most rapidly destructive being external inflammatory root resorption.

1.1.1 Surface resorption

Surface resorption is characterized by small areas of superficial resorption lacunae in cementum which may extend into dentine. The condition is the result of local injury and is usually self-limiting, that is, spontaneous lacuna repair by reparative cementum can occur without treatment (Andreasen & Hjörting-Hansen 1966a).

1.1.2 Inflammatory resorption

Root resorption sustained by infection is commonly referred to as inflammatory resorption. The inflammation and resorption are apparently related to the presence of infected necrotic pulp tissue in the pulp canal. This is a common sequela to luxation or avulsion injuries where damaged areas on the dental root denuded of cementum, or areas of surface resorption, allow access for bacteria and their toxins from dentinal tubules to the PDM (Andreasen 1980a, 1981b, Lindskog et al 1987).

A number of different methods have been used in the treatment of external inflammatory root resorption, both clinically and experimentally. The aim has been to either

1) eliminate bacteria by the systemic and/or intracanal use of antibiotics or intracanal placement of calcium hydroxide,

2) reduce inflammation within the PDM by systemic or topical corticosteroid therapy,

3) both remove bacteria and reduce inflammation by using an intracanal corticosteroid-antibiotic preparation (e.g. Ledermix paste) or,

4) directly inhibit dentine-resorbing cells.

Long-term calcium hydroxide intracanal dressings have been the most frequently employed therapy for treatment of inflammatory root resorption (Tronstad 1988, Heithersay 1975, Stock 1985). However, intracanal placement of calcium hydroxide can damage or kill cells within the PDM due to its high pH (Hammarström et al 1986b). It is the death of these cells which results in a higher incidence of ankylotic repair (fusion between alveolar bone and the dental root) at the expense of healed PDM tissues (Hammarström et al 1986b). Ankylotic repair, although not ideal, is a preferable clinical result to continued inflammatory root resorption. Nevertheless, the necrotizing effect of calcium hydroxide on PDM cells and its inability to totally eliminate inflammatory resorption indicates that this is not an ideal material for the treatment of inflammatory root resorption (Hammarstöm et al 1986b, Lindskog et al 1990).

Hammarström et al (1986a) demonstrated that intracanal application of antibiotics alone in monkey teeth with experimentally-induced inflammatory resorption resulted in 10% of root surfaces showing signs of resorption after 5 wk of therapy. These authors concluded that there may be additional factors involved in maintaining the inflammation

and resorption in the PDM.

When an antibiotic/corticosteroid preparation (Ledermix paste) is used in a similar experimental model, no evidence of inflammatory root resorption was observed after 5 wk (Pierce & Lindskog 1987). Although it is unlikely that the corticosteroid component eliminated the cause of the inflammation, the authors suggested that its action as an inflammatory suppressant was effective in allowing the PDM to heal over and cover the open dentinal tubules with reparative cementum.

CT, a polypeptide hormone, reduced residual inflammation in the PDM to less than 1% when placed topically in monkey teeth with experimentally-induced inflammatory root resorption (Pierce et al 1988a). Its mode of action is thought to relate to the specific inhibition of dentine-resorbing cells (dentinoclasts) and suppression of inflammation (Abdullahi et al 1977, Pierce & Lindskog 1989) rather than acting on bacteria, as do the previous forms of therapy.

Ledermix paste therapy is commonly used in Australia for the treatment of inflammatory root resorption. However, in rare cases where there are medical contraindications to its corticosteroid or antibiotic component or where these components contravene existing drug laws in some countries other strategies such as CT-mediated therapy may be required.

1.1.3 Replacement resorption and ankylosis

Replacement resorption involves the gradual resorption of dental hard tissue and its replacement by bone. Ankylosis refers to a physical union between bone and the dental root. Replacement resorption of replanted teeth usually occurs subsequent to extensive necrosis of the PDM cells on the root surface, usually the result of either a prolonged dry storage extra-oral period (Andreasen 1980b), an unsuitable storage

medium (Blomlöf et al 1983), or partial or complete removal of the PDM (Andreasen & Kristersson 1981, Lindskog et al 1987).

The pathogenesis of replacement resorption is poorly understood. However, the vitality of the PDM cells is of critical importance to its prevention (Blomlöf et al 1983, Lindskog et al 1983, Lindskog et al 1988). It has been suggested that replacement resorption is initiated by endosteal osteoblasts and is thus a hormonally-regulated process (Hammarström et al 1989).

Currently, no definitive treatment for this condition is available. However, prompt emergency measures to maintain the vitality of PDM cells together with intracanal placement of Ledermix paste have been shown to reduce the incidence of replacement resorption (Blomlöf 1981, Blomlöf et al 1983, Pierce & Lindskog 1987).

1.1.4 Invasive cervical resorption

Invasive cervical resorption occurs in the coronal third of the dental root near the cemento-enamel junction (CEJ). Although its aetiology is unknown, predisposing factors include orthodontic treatment, trauma or surgery involving damage to the cervical attachment apparatus, and bleaching procedures on teeth. Current treatment of these conditions do not primarily involve placement of intracanal medicaments.

1.2 The resorbing cells

Osteoclasts are the cells responsible for bone resorption. These cells are large, multinucleated and motile, and located in close proximity to bone. They are formed from the fusion of mononuclear precursor cells of haemopoietic origin, thus differing from osteoblasts and osteocytes which are derived from local mesenchymal osteoprogenitor cells (Marks 1983). Osteoclasts and other mineralized tissue-resorbing cells have been

named according to the substrate tissues they resorb, such as odontoclasts (teeth) and dentinoclasts (dentine). Dentinoclasts are morphologically similar, if not identical, to osteoclasts (Furseth 1968, Nilsen 1977, Lucht 1980, Ten Cate & Anderson 1986, Jones & Boyde 1988). Both cell types share similar enzymatic properties, and intense tartrate-resistant acid phosphatase activity, characteristic of osteoclasts, has also been demonstrated in dentinoclasts (Nilsen & Magnusson, 1979, 1981). Clastic activity and mineralized-tissue formation are processes necessary for skeletal development and maintenance. However, in pathological states such as Paget's disease, giant cell tumours (Chambers 1985) and external inflammatory root resorption (Andreasen 1981b), clastic activity is elevated to a level where excessive mineralized-tissue destruction can occur. This discussion considers the morphology, mechanism of attachment to mineralized surfaces and the mechanism of action of clastic cells.

The inactive clastic cell is ovoid in shape and detached from the adjacent hard-tissue surface. These cells lack specialized structures in the cell membrane that are required for a) attachment to the hard-tissue surface and b) hard-tissue degradation. In contrast, the actively-resorbing cell assumes a flatter, "scrambled egg" profile as it extends over the adjacent hard-tissue surface. The resorbing cells are usually larger than inactive cells, are highly vacuolated, contain specialized microfilaments which aid in cellular movement as well as cell attachment, and possess a well developed organelle and lysosomal system compared with their inactive counterparts indicating a higher metabolic activity (Jones & Boyde 1977). Although these active cells are usually large and multinucleated, they can exist as mononucleated cells (Boyde & Jones 1979). The active cells possess two cytoplasmic membrane specializations on the surface adjacent to the hard tissue which are unique to resorbing cells: (1) the "clear zone" - which is an annular region of organelle-free cytoplasm responsible for cell attachment to the hard tissue

surface (Lucht 1980), and (2) the "ruffled border", a series of membranous folds located within the clear zone which is thought to be the site where hard-tissue resorption occurs.

The active osteoclast moves along the mineralized-tissue surface during and in between episodes of resorption indicating that this cell is highly motile (Hancox & Boothroyd 1961). The life span of osteoclasts *in vivo* is in excess of 7 wk with a half-life of approximately 6 to 10 days (Jaworski et al 1981, Loutit & Townsend 1982). Thus a sustained suppression of dentinoclasts by intracanal medicaments such as CT may be required to inhibit external inflammatory resorption.

The mechanisms involved in the breakdown of both the organic and mineralized components of hard tissue are not yet fully understood. However, there appears to be three distinct phases of resorption:

(1) the initial removal of osteoid (the unmineralized organic matrix on the mineralized-tissue surface) by latent collagenase released by osteoblasts (Rodan & Martin 1981, Chambers & Fuller 1985). Spreading of osteoclasts on mineralized surfaces is not possible in the presence of osteoid (Chambers et al 1984), but may proceed after its removal (Blair et al 1986). Chambers & Fuller (1985) have hypothesized that osteoblast-induced degradation of non-mineralized collagen shielding mineralized surfaces is necessary to allow osteoclasts to resorb bone.

(2) the degradation of collagen and other organic materials embedded within the mineralized matrix. This process is thought to occur extracellularly and is caused by enzymes released at the ruffled border of the osteoclast. These enzymes are optimally active at an acid pH, and recently, an ATPase proton pump has been identified within the ruffled border cell membrane (Baron et al 1985). The protons for transport are thought to be produced by the conversion of carbon dioxide and water to bicarbonate and hydrogen ions and the reaction is catalysed by carbonic

anhydrase isoenzyme 11 (Marie & Hott 1987).

(3) the degradation of the mineralized component of bone. The mineral component of bone is initially partly degraded extracellularly by acid dissolution in the ruffled border region (Baron et al 1988). The remaining partially degraded mineral crystals are then thought to be internalized into lysosomes within the osteoclast possibly aided by receptor-mediated endocytosis (Pierce 1988). Hence, final degradation of the bone is thought to occur within the lysosomes (Pierce et al 1990).

The regulatory mechanisms responsible for activation and deactivation of the resorbing cells are complex and closely related to those operating on bone-forming osteoblasts, which results in a coupling of bone deposition to resorption. (For a detailed review of these regulatory mechanisms, see Vaes 1988). One such regulatory mechanism involves the inhibitory action of CT on the osteoclast. A number of authors had suggested that CT may act directly on osteoclasts via specialized membrane receptors (Chambers & Magnus 1982, Warshawski et al 1990). These receptors have recently been shown to exist (Nicholson et al 1987). The mechanism by which CT inhibits bone resorption may be through inhibition of cytoplasmic motility, presumably essential for enzyme exocytosis and endocytosis. The motility of osteoclasts is abolished by picomolar concentrations of CT and reduced within the physiological range (Chambers & Magnus 1982). Cyclic adenosine monophosphate (cAMP) has been implicated as a secondary messenger in CT responsiveness (Ransjö 1988). Analogues of cAMP inhibit both motility and hard-tissue resorption in isolated osteoclasts (Chamber & Moore 1983). Furthermore, agents which reduce cAMP degradation enhance CT responsiveness, and the hormone is known to increase cAMP concentations in bone (Marx et al 1972).

The potential use of CT as an intracanal therapy to inhibit external inflammatory root resorption depends on the ability of the hormone to diffuse through the dental root

and be available to its target cell, the dentinoclast, in a biologically-active form for a protracted period. This study focused upon the diffusion kinetics of CT through the dental root. The biological activity of CT after it has diffused through the dental root was not considered.

2. STRUCTURE OF THE DENTAL ROOT

The most characteristic feature of dentine is the presence of dentinal tubules which extend peripherally from the odontoblast-predentine junction throughout the thickness of dentine to the cementum. Dentinal tubules also contain lateral extensions, or canaliculi, which branch from the main tubules. Permeability within the dentine is a direct consequence of the presence of the tubules. The dentinal tubules contain the cell processes of odontoblasts, the main body of which reside at the pulpal-predentine interface.

Dentine is highly mineralized with over half its volume taken up by an extracellular organic matrix material (Figure 1). This matrix consists of a tightly woven network of collagen fibres and various extracellular macromolecules such as phosphophorins, glycolipids, glycoproteins and proteoglycans.

2.1 Contents of dentinal tubules

The structural interrelationships within dentinal tubules are best visualized in cross section (Figure 2).

2.1.2 Peritubular dentine

The walls of the tubules and their lateral branches are lined by peritubular dentine. This tissue is highly mineralized and contains a light organic matrix.

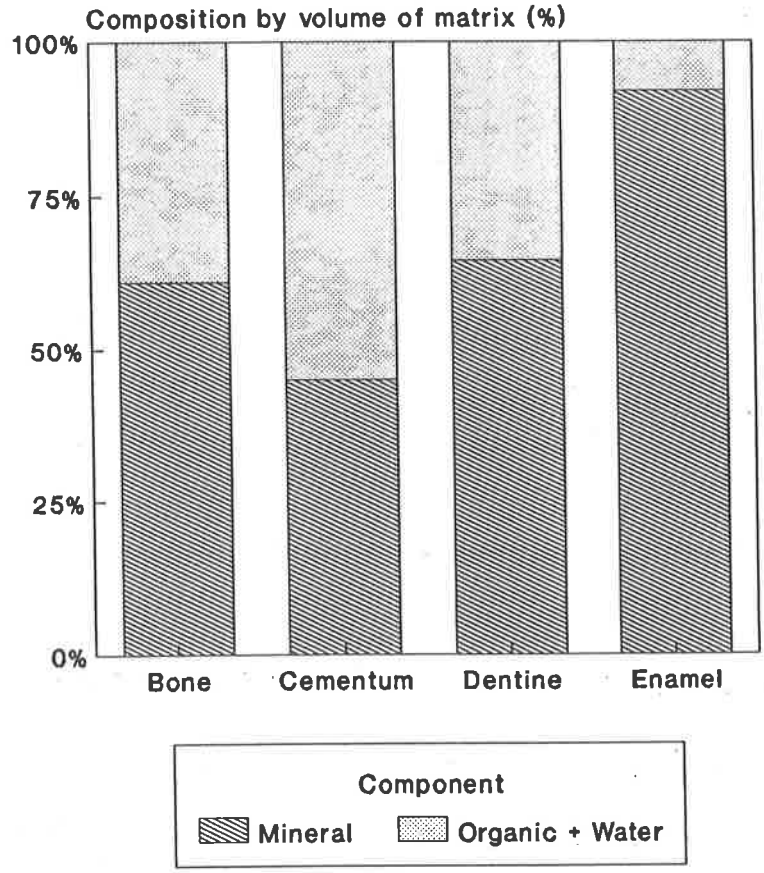
Peritubular dentine is usually lost in demineralized sections due to difficulties in specimen preparation.

The presence of peritubular dentine at the predentine-dentine junction has been reported (Takuma 1967), but it is usually present in lesser amounts in this region, reaching maximum thickness in mid- and peripheral dentine (Atkinson & Harcourt 1961). The observation that a progressive narrowing of dentinal tubules occurred with increasing age was interpreted from light microscopy to be due to the growth of peritubular dentine (Bradford 1958).

A clearly demarcated interface between peritubular and intertubular dentine is evident when non-decalcified specimens of dentinal tubules, in cross section, are examined. This interface has been described as a sheath-like separating medium, the "Sheath of Neumann" (Orban 1976). Electron microscopic examination of similar dentine specimens, however, suggests that such a sheath does not exist. Rather, the organic fibrils of the peritubular dentine appear to intermix with the fibrils of the intertubular dentine (Orban 1976).

Figure 1.

Composition of Hard Tissues



(Adapted from Orban 1976)

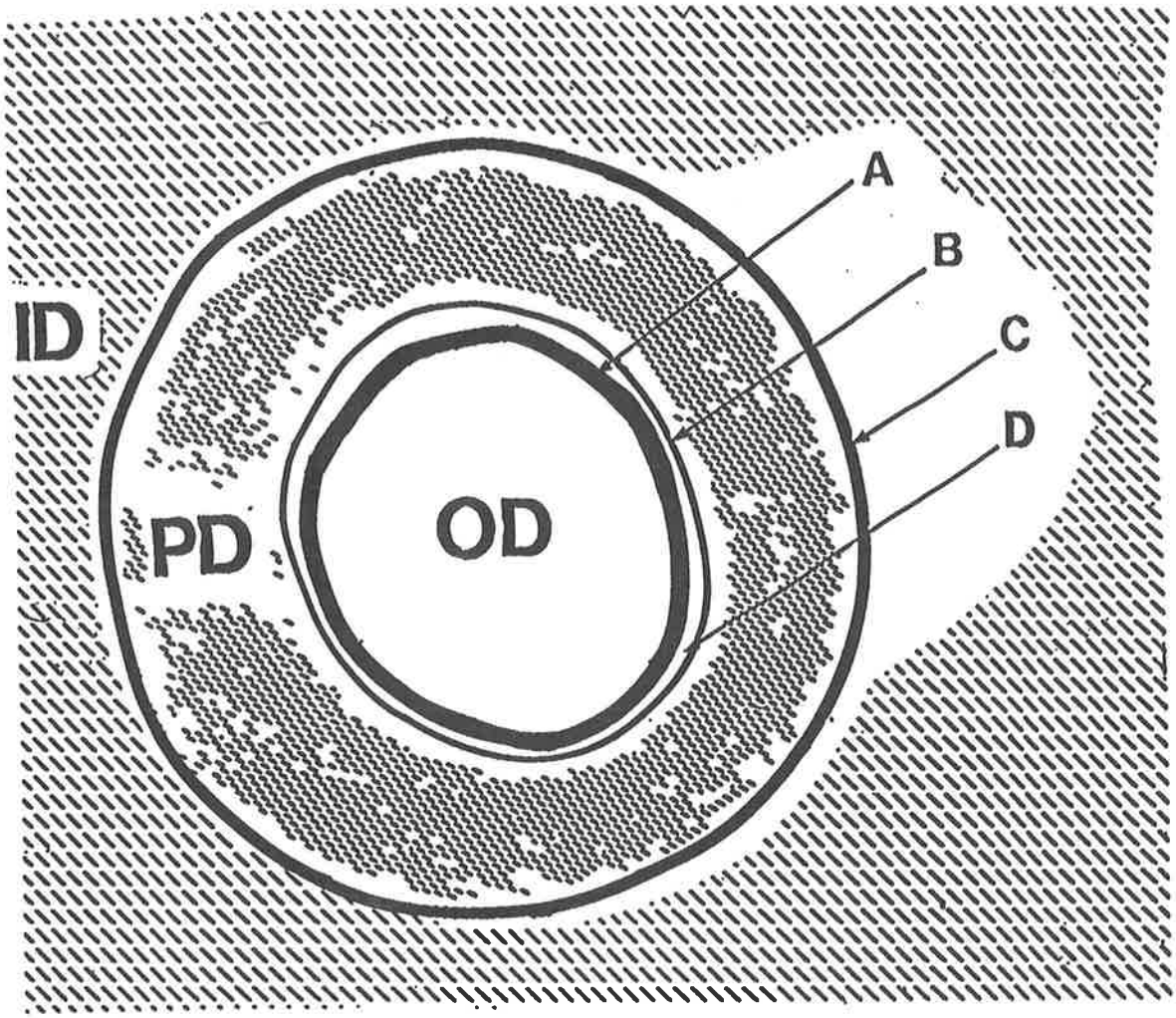


Figure 2. Diagram of a dentinal tubule in cross section. **ID** = intertubular dentine, **PD** = peritubular dentine, **OD** = odontoblastic process,
A = odontoblastic process cell membrane (trilaminar)
B = Lamina limitans (seen as an electron dense lining in decalcified sections using TEM)
C = Sheath of Neumann (seen in undecalcified SEM or ground sections)
D = Peri-odontoblastic space

2.1.2 Intertubular dentine

The main body of the dentine is composed of intertubular dentine (Figure 2). This tissue is also highly mineralized but less so than peritubular dentine, with over one half of its volume being taken up by the organic matrix. Peripheral dentine adjacent to both enamel and cementum has a different microscopic appearance as from the main body of dentine. This difference is attributed to the coarse peripheral fibre bundles which are arranged at right angles to the external dentinal surface.

2.1.3 Odontoblastic processes

Odontoblastic processes are the cytoplasmic tubular extensions of the odontoblast cell bodies which line the pulpal surface of dentine. Each process traverses the predentine and occupies a canaliculus in the dentine, predominantly filling the lumen of the dentinal tubule. The processes contain only a few cytoplasmic organelles. Numerous coated vesicles are present and those close to the secretion front in predentine are pinocytotic, enabling the odontoblasts to ingest material from the predentine (Garant et al 1968, Reith 1968). Many vesicles are contiguous with the cell membrane in the region of the predentine. Dense secretory granules and lysosome-like bodies are also present and numerous filaments are oriented parallel to the cell membrane. According to Reith (1968), these fine filaments are the most characteristic feature of the odontoblastic process and its branches. Microtubules are the next most prevalent cytoplasmic component. Seltzer and Bender (1984) reported that microtubules are seen in virtually every portion of the process and are oriented longitudinally to its long axis. Groups of microvesicles, similar to those seen in the Golgi apparatus, are situated near the plasma membrane.

The odontoblast process can completely fill the dentinal tubule at the dentine-predentine junction (Thomas 1979). However, further into dentine a distinct separation appears between the process and the tubule wall. This separation, termed the "periodontoblastic space" (Frank 1966), contains collagen fibrils and unidentified granular material (Frank 1966, Thomas 1979) and is a consistent feature in both mineralized and demineralized specimens. The effects of these components in the periodontoblastic space on the permeability of dentine are unknown. The presence of odontoblasts and their processes, however, has been reported to have an effect on dentine permeability (Pashley et al 1978b). The resistance to fluid movement through dentine in extracted human molars is greater in freshly extracted teeth, where odontoblasts and odontoblast processes are present, than in teeth stored for one week in buffer, where degeneration of odontoblasts and their processes have presumably occurred.

The odontoblastic process has long been assumed to extend throughout the entire thickness of dentine. The reason for this assumption stems from an observation made by Tomes (1856), in which fibrils (termed "Tomes' fibres") of soft tissue were described throughout the length of dentinal tubules.

Using the electron microscope, Frank (1959) noticed that in the middle and outer portion of undecalcified dentine, the odontoblast process assumed a tubular form; he found the part near the predentine to be a tube of cytoplasm, which was sometimes absent. In a further study Frank (1966) concluded that the dentinal tubule contained the cellular process of the odontoblast. In the predentine area he also found structures in the tubules resembling myelinated nerve fibrils.

Much debate exists in the current literature as to the true extent of the process. Many investigators using scanning electron microscopy (SEM) and transmission electron microsopy (TEM) of mineralized and demineralized tissue have described the process as

being limited to the pulpal third of the tissue in a variety of animal species of different ages (Brannström & Garberoglio 1972, Tsatsas & Frank 1972, Garant 1972, Thomas 1983).

Recently, immunological techniques have been used to determine the extent of the odontoblast process. Sigal et al (1984a) used an immunofluorescent technique in rat molar dentine at the light microscopic level. Antibodies were used against tubulin and actin, proteins known to be components of the cytoskeleton of the odontoblastic process. The study demonstrated intense staining in the inner third of the dentine. The same authors (Sigal et al 1984b) later used a combined SEM and immunofluorescent study on human molars and confirmed their previous results. In addition, the authors consistently stained and observed tubulin containing structures at the dentino-enamel junction (DEJ). This study supports recent SEM observations (Manniatoupolis & Smith 1983, Yamada et al 1983), suggesting that the odontoblastic process extends to the DEJ.

Weber and Zaki (1986) queried whether the immunofluorescent staining was intratubular, or a result of non-specific staining of the tubule wall. They also suggested that the specimens were too thick, thus casting doubt on the results of the studies by Sigal et al (1984a, 1984b).

La Fleche et al (1985) offered an apparent TEM confirmation of processes near the DEJ. The observations strongly suggest the presence of odontoblastic processes in the most peripheral dentine when the teeth had been frozen in liquid nitrogen prior to fixation. When specimens were not frozen, the processes were only observed in the inner third of dentine. This led them to hypothesize that the odontoblastic processes contract during processing prior to fixation. The odontoblastic process contains actin filaments and microtubules, a stretched system with the filaments arranged longitudinally throughout the cell body and its process. Freezing the contents of the tubules prior to

fixation, they suggested, prevents contraction of the odontoblastic processes. Weber and Zaki (1986), however, disputed these observations. They suggested that if the contraction hypothesis of La Fleche et al (1985) is correct, the investigators using immunofluorescence methods (Sigal et al 1984a) should not have demonstrated positive staining near the DEJ, because they did not employ liquid nitrogen freezing prior to fixation.

The degree of extension of the odontoblast process throughout dentine is a potentially important factor in the permeability of this tissue.

2.1.4 Other tubular contents

Type I collagen has been found arranged circumferentially, longitudinally and as interlacing networks within the lumen of dentinal tubules both in the periodontoblastic space, and in peripheral tubules (Frank 1966, Lester & Boyde 1968, Tronstad 1973, Thomas 1979, Takuma 1960, Frank 1959, Scott 1955). The thickness of the intratubular collagen fibres are 50 nm to 150 nm (Tronstad 1973) and the fibres are present throughout the entire course of the tubules (Tidmarsh 1981). These intratubular fibres may contribute to reducing dentine permeability (Thomas 1985). Conversely, the removal of intratubular collagen by endodontic chemo-mechanical debridement of root canals may increase dentine permeability to intracanal medicaments.

Nerve fibres are present within dentinal tubules but penetrate no further than 100-150 μm into the tubules (Lilja 1979). La Fleche et al (1985), however, using a freezing technique prior to fixation, demonstrated the occasional unmyelinated nerve fibril in peripheral coronal dentine.

It has been suggested that the fluid within dentinal tubules is a capillary transudate or filtrate. The sodium/potassium ion ratio in dentinal fluid has been shown by Coffey

et al (1970) to resemble that of interstitial fluid. Tanaka (1980) using lanthanum tracer studies in developing teeth confirmed that dentinal fluid originates from terminal capillaries in the pulp and diffuses to the DEJ along the periodontoblastic space. The presence of fluid within the tubules forms the basis of the hydrodynamic hypothesis of dentine sensitivity (Brannström 1963).

The diffusion of molecules through dentine is possible due to the presence of dentinal tubules. In addition, the presence of organic contents (such as odontoblastic processes) within these tubules may modify this diffusion.

2.2 Lining of dentinal tubule walls

The lining of the walls of dentinal tubules has been extensively studied, with several authors differing in their descriptions.

Johansen and Parks (1962) observed that, except in the predentine area, the lumen of the dentinal tubule was lined by a membranous structure containing collagenous elements closely associated with the highly calcified peritubular zone. In the predentine region, the only membranous structure present was the plasma membrane of the odontoblast process.

Symonds (1962) using a histochemical technique on transverse sections of dentinal tubules, described a strongly-staining boundary between the peritubular zone and the tubule contents. The author suggested that this lining might be a thin limit boundary of the peritubular matrix.

Ikosawa et al (1970) and Thomas and Carella (1983) described this lining as a sheet-like membranous structure. After acid demineralization, the structure can be more readily visualized as almost all of the peritubular dentine disappears, but an acid resistant lining remains in the innermost aspect of the peritubular dentine.

Brannström and Garberoglio (1972) also observed this lining in mineralized tissue which, according to Thomas (1985), was probably a result of having torn it away from peritubular dentine during specimen preparation. It has been termed the "lamina limitans", to conform with terminology used for a similar structure seen in bone canaliculi.

Szabo et al (1985), in an SEM study, showed that this lamina limitans consisted of a two-layered sheath separating the lumina of tubules from the mineralized dentinal tissue, and extending from the pulp-predentine border to the dentine-enamel junction. They observed that the inner layer of the sheath (nearest the peritubular dentine) is membrane-like and the outer layer is fibrous, and that the two layers can be separated by a fracturing process.

Thomas and Carella (1984), using both SEM and TEM investigations, suggested that the lamina limitans is not the odontoblast process because it does not possess a trilaminar structure and is too thick to represent a cell membrane. When both the lamina limitans and odontoblast process are seen together, they were clearly distinguishable.

The extracellular nature of the lamina limitans has been confirmed by demonstrating its susceptibility to digestion by hyaluronidase, indicating a high content of glycosaminoglycans (GAG) (Thomas 1985). Since GAGs have been suggested to play a role in the inhibition of mineralization (Fleisch et al 1975), the presence of the lamina limitans might provide a mechanism for maintaining the patency of the tubules, thereby maintaining the permeability of the tissue (Thomas 1985).

The interaction of the tubule surface with molecules passing through dentinal tubules may influence their diffusion characteristics. CT may bind to the hydroxyapatite mineral, collagen fibres or to a carrier protein. Although Pierce et al (1988) have shown biological activity of CT at resorption sites following intracanal application, interactions

of CT with tubular contents, especially degradative enzymes, may modify biological activity.

2.3 Dentinal tubule dimensions

In early studies, the number and diameter of the dentinal tubules were measured in decalcified human dentine using light microscopy and it was found that the tubule diameter can vary from 1.0μm near the enamel to 4 or 5μm near the pulp (Bradford 1955). There are indications that most of the peritubular dentine is dissolved during decalcification in acid (Ikosawa et al 1970, Brannström & Garberoglio 1972). The tubule diameter on decalcified sections thus gives an excessively high value.

Tronstad (1973) studied the incisal area of non-decalcified coronal dentine using SEM and TEM. The number of dentinal tubules observed varied from about 7,000 per mm^2 in peripheral dentine to 60,000 per mm^2 near the pulp. The diameter of the tubules was 2 to 3μm but near the enamel the diameter was usually less than 0.5μm (Tronstad 1973).

Garberoglio and Brannström (1976) used SEM to examine fractured coronal dentine of 30 intact human teeth in various age groups at various distances from the pulp. Close to the pulp they found approximately 45,000 tubules per mm^2 with a diameter of 2.5μm. In the middle of the dentine, there were 29,500 tubules per mm^2 with a diameter of 1.2μm. In the peripheral dentine, there were less dentinal tubules per surface area (20,000 tubules per mm^2) and the tubule diameters were narrower (0.9μm) when compared with the middle and pulpal regions of the dental root. No significant difference was found between young and old teeth. For a portion of their sample, the authors pretreated some longitudinally fractured teeth with an 8% solution of sodium hypochlorite for 24 h to remove pulp tissue and predentine. A somewhat larger diameter

of tubule was seen close to the pulp wall in teeth treated with sodium hypochlorite in contrast to the untreated ones. This is to be expected, considering that sodium hypochlorite removes organic material. To determine the difference in tubule diameter and number between decalcified and calcified dentine, some teeth were treated with nitric acid for 5 days. The authors found that the decalcified specimens shrank during dehydration *in vacuo* and thus gave a false value for the tubule diameter and number.

Garberoglio and Branström (1976) further calculated that the total tubular volume was 10% of the total coronal volume, a figure which differed from that obtained by Hoppe and Stuben (1965) using decalcified sections (21%). Garberoglio and Branström (1976) found no significant difference between young and old teeth. Both research groups, however, concurred on the 27% value for tubule volume near the pulp which suggests that in this region, the tubule diameter in undecalcified and decalcified dentine is almost the same; in other words, there is no peritubular dentine near the pulp.

The above studies indicate that there is an large surface area of dentinal tubule walls in the dental root. Therefore, the permeability of dentine to molecules that are likely to interact (for example, bind) to tubule walls will be modified. CT may be one such molecule.

2.4 Structure of cementum

Cementum is the mineralized dental tissue covering the anatomic roots of human teeth. It extends from the cervical portion of the tooth at the CEJ through to the apex. Cementum provides a medium for the attachment of collagen fibres that bind the tooth to surrounding structures. It is thinnest at the CEJ (20 to 50 μm) and thickest towards the apex (150 to 200 μm). A thin layer of unmineralized cementoid tissue lines the cementum surface. This, in turn, is lined by a layer of cementoblasts. Cementum can

be differentiated microscopically into two types, acellular and cellular. Acellular cementum covers the entire root surface. Cellular cementum is usually found at the apical third of a dental root. It is characterized by the presence of cementocytes in lacunae. There is no demarcation line between the acellular and cellular layers.

The interface between cementum and dentine is clearly visible in decalcified and stained sections using the light microscope. This interface has been termed the intermediate cementum layer of Hopewell-Smith. Lindskog (1982) suggested that this layer may be a type of enameloid, formed by the epithelial root sheath prior to its disintegration.

The continuous deposition of cementum is of considerable functional importance. In contrast to the physiological process of continuous bone remodelling, cementum is not resorbed under normal conditions. However, repeated incremental apposition of cemental layers does occur and represents the ageing of the tooth as an organ. Cementum serves as the major reparative tissue for root surfaces. Damage to roots such as fractures and resorptions can be repaired by the deposition of new cementum.

Cementum has also been shown to be a rate-limiting barrier to a number of substances including demeclocycline and triamcinolone when placed topically in root canals (Abbott 1985). However, further investigations are required to identify the parameters that may influence the diffusion of molecules through this tissue.

3. DIFFUSION OF MOLECULES ACROSS DENTINE

Dentine is penetrated by 30 000 tubules per mm^2 which represent pathways between the pulp and the PDM. This tissue can be regarded as a semi-permeable membrane, that is, readily permeable to solvent but differentially permeable to solute, and accordingly, there exists a potential for osmotic diffusion of solute through the dentinal tubules.

Unlike most biological membranes, dentine is unique in that the diffusional surface area, or the area of the dentine surface that is occupied by the lumina of the tubules, is not constant. Due to the convergence of the dentinal tubules, the diffusional surface occupies only about 1% of the total area near the dentino-enamel junction and approximately 22% near the pulp (Pashley 1985).

Total dentinal surface area of prepared cavities in dentine is difficult to quantitate and standardize from one specimen to another because of the variability of dentine thickness and the uneven contours of dentine.

Quantitative studies on dentine permeability involve measurements of either radioactive solute diffusion across dentine (Pashley & Livingstone 1978, Pashley et al 1981), or the movement of fluid across dentine (Reeder et al 1978, Pashley et al 1983).

3.1 Diffusion

Diffusion is the spontaneous tendency for molecules to distribute themselves evenly throughout the whole space available to them. That is, the molecules seek a state of minimum free energy or maximum entropy. For example, when a concentration gradient of a solute in solution exists between two different regions there will be a spontaneous net movement of solute from the region of higher to the region of lower concentration. When all concentration gradients have been dissipated, net solute movement between different regions of solutions will cease.

Most molecules diffuse through the dental root by simple diffusion (Pashley 1988). Initially, a diffusion gradient would be created between the root canal and the external dental-root surface when medicaments such as Ledermix paste or CT are placed into endodontically-prepared root canals: the dental root acting as a permeable membrane. The rate of solute diffusion (J) through this permeable membrane is equal to the amount of solute passing from the region of higher to that of lower solute concentration in unit time. The amount of consumption of the intracanal medicament in the PDM will also influence J. At constant temperature, J is given by Fick's Equation:

$$\mathbf{J = P.A.dc/dx}$$

where:

1) **J** = rate of solute diffusion

2) **P** = permeability coefficient of the solute through the dental root.

P is a measure of the inherent ability of solute to permeate through the dental root. Among several variables, it is proportional to temperature and inversely proportional to the molecular weight of solute. Furthermore, P is dependent on solubility of solute (i.e. the degree of dissociation in solvent). For example, bacterial endotoxins, large molecular weight molecules that poorly dissociate in solvent, do not readily diffuse through dentine. With large molecules, the greater extent and strength of their interactions with other molecules, can lead to a complex relationship existing between P and molecular weight.

Outhwaite et al (1976) reported that the size, charge, and water (or lipid) solubility of the diffusing molecules affected their diffusion through dentine.

3) **A** = cross-sectional area of the dentinal-tubule lumens, at their smallest diameter, available for diffusion of intracanal medicaments. As discussed previously, the tubule lumens are narrowest peripherally.

Using an *in vitro* model, Outhwaite et al (1976) examined the diffusion of I^{125} (in NaI^{125} form) through disks of coronal dentine cut from freshly-extracted human third molars. They reported that doubling the surface area available for diffusion caused a 95.5% increase in the rate of permeation through dentine by the isotope.

4) **dc** = concentration gradient of solutes between the regions

The greater the concentration of solute placed into root canals, the greater the rate of diffusion (Outhwaite et al 1976).

5) **dx** = thickness of the membrane between the two regions.

Extensive mechanical preparation of root canals will also reduce the thickness of the remaining dental root and lead to higher rates of diffusion.

The diffusion of intracanal medicaments (such as CT) through the endodontically-prepared dental root to the external root surface will be influenced by the parameters described in Fick's equation. Therefore, these parameters should be considered when analyzing the diffusion of currently used and potential medicaments through the dental root. However, it was not the intention of our study to investigate the influence of these parameters on CT diffusion through the dental root, but rather to investigate overall diffusion of CT from the root canal to the external dental-root surface. Therefore we have chosen to standardize the parameters that contribute to Fick's equation by using similarly prepared teeth.

3.2 Hydraulic conductance

The ability of fluid, under a positive hydrostatic pressure, to flow through dentinal tubules is termed the "hydraulic conductance".

The hydrostatic pressure in vital pulp tissue is 28 mmHg greater than that in the

adjacent PDM (Van Hassell 1971). This pressure gradient creates a net filtration, or bulk fluid flow, from the dental pulp to the external dental-root surface.

The use of hydrostatic pressure to examine the hydraulic conductance of solutes through cut dentine disks has been described (Merchant et al 1977). These studies intended to simulate the normal pulp-tissue environment.

When dental pulps are removed and the root canals endodontically prepared, the net outward filtration pressure is lost. Therefore, intracanal medicaments are most likely to reach the external dental-root surface by simple diffusion.

3.3 Radicular dentine permeability

Studies on the kinetics of diffusion, of intracanal medicaments through dental roots, are lacking. Most research on dentine permeability has focused on coronal dentine, especially under cavity preparations and dental caries. In addition to dentinal tubules, the tubule-free granular layer of Tomes and the cementum, may restrict the passage of molecules from the root canal, through the dentinal tubules, to the external dental-root surface.

Wasserman et al (1941) found that within 24 h, P^{32} readily diffused through the cemento-dentinal junction (CDJ) of pulpless dog teeth, *in vivo*, when systemically administered as $Na_3P^{32}O_4$.

Abbott (1985) examined the diffusion of demeclocycline (a tetracycline antibiotic) and triamcinolone (a corticosteroid), constituents of Ledermix paste, through intact dental roots *in vitro*. The rate of release of these molecules was highest after the first hour (490pmol/min and 95pmol/min respectively), and declined exponentially over 14 wk (0.3pmol/min and 0.6pmol/min). The major route of exit of these medicaments from the dental root was via the dentinal tubules and not via the apical foramen.

Fogel et al (1988) demonstrated that the hydraulic conductance of radicular dentine was much lower (up to 80%) than coronal dentine. This was presumed to be due to the smaller diameter and density of the dentinal tubules in radicular dentine.
In addition, radicular dentine was less permeable peripherally as compared to dentine adjacent to the pulp. This was due to the larger diameter of tubules (1.56μm) and greater tubule density (40,691/mm^2) of radicular dentine at the pulpal side as compared to peripheral dentine (1.07μm and 20,895/mm^2 respectively).

The passage of CT through the dental root will depend on its ability to diffuse through the radicular dentinal tubules (which are narrower and less dense than those in coronal dentine), through the granular layer of Tomes, and through the cementum. The results of Abbott (1985) suggest that the dental root does not act as an impermeable barrier to intracanal medicaments.

3.4 Effects of molecular size and shape

The diffusion of solute through the dental root may be influenced by its molecular weight and effective molecular size.

Pashley et al (1977) used an *in vitro* model to measure the rate at which small isotopically-labelled substances permeated through dentine in occlusal cavity preparations. The substances used were:

1) $H^3{}_2O$ (molecular radius 1.97Å, mol wt 22),
2) I^{131} (2.55Å, mol wt 131) available as NaI^{131} (carrier free),
3) Tc^{99m} (2.58Å, mol wt 163) available as $NaTc^{99m}O_4$ (carrier free) and,
4) C^{14}-urea (2.70Å, mol wt 62).

The relative rates of permeation were $H^3{}_2O > I^{131} > Tc^{99m} > C^{14}$-urea, a sequence that follows the molecular dimensions of these substances.

Pashley and Livingston (1978) examined the effect of increasing molecular radius of solute on its rate of permeation through dentine. An *in vitro* model in which disks of coronal dentine were placed between the halves of a split chamber device was used. Radioactive molecules were placed on the coronal side of the disks and allowed to diffuse through to the pulpal side. The osmotic pressure on both sides of the disks were then measured. The permeability coefficients of a wide range of solutes were then calculated using Fick's equation. A high correlation was obtained between molecular radius and permeability coefficient. Molecules that bound to dentine (such as F^{18} and C^{14}-chlorhexidine) were less permeable than their molecular weights may have indicated. In addition, Pashley et al (1979) examined the permeability of cut dentine disks to dextran molecules and proteins of different molecular radii, *in vitro.* Their results revealed a strong correlation between molecular radius and permeability within dentine. The dentine disks were less permeable to the proteins, haemoglobin and albumin, than to comparably-sized dextrans. This result could be due to their different molecular shapes. Proteins are usually in globular form whilst dextrans, are long-slender molecules; some of which are branched.

Other factors besides molecular weight and radius may have influenced the diffusion of the above isotopes through dentine. These include: ionic charge (I^{131} is available in solution as a negatively charged ion), solubility (Tc^{99m}, also negatively charged, may have been available as a colloid), and intermolecular interactions (C^{14}-urea may denature proteins increasing the osmotic pressure of the solution).

In summary, the diffusion of molecules, such as CT, through dentine will be influenced by their molecular weight, molecular radius, ionic charge, intermolecular interactions (such as binding), and solubility. If proteins such as albumin (molecular weight 69,000) can diffuse through dentine then, on the basis of molecular weight, smaller

molecules such as CT (molecular weight 3600) are likely to diffuse through dentine. However, limiting factors other than molecular weight may modify diffusion.

3.5 Effects of smear layer

Dentinal surfaces mechanically exposed by routine dental procedures, such as cavity preparation and endodontic root-canal debridement, are covered with a smear layer. This layer of microcrystalline cutting debris acts as a barrier to dentine diffusion. It cannot be rinsed or scrubbed off.

Pashley and Livingston (1978) investigated the permeability of cut dentine disks and disks that were acid etched (to remove smear layer) to I^{131}-albumin and H^3_2O. The permeability of acid-etched dentine to these diffusants was greater than that of cut dentine. This increase in permeability after acid etching was greater for I^{131}-albumin than H^3_2O. This suggests that the smear layer may have a greater effect in reducing the permeability for larger molecules than for smaller ones. In addition, the smear layer represents a surface area for molecular binding.

The smear layer can and should be removed by endodontic irrigants such as a 15% solution of ethylene-diamine-tetra-acetic acid, with or without cetrimide (EDTAC or EDTA respectively), to allow unrestricted access of intracanal medicaments, such as CT to the dentinal tubules.

3.6 Functional radii of dentinal tubules

The radius of dentinal tubules will influence the rate of diffusion of molecules across dentine. There are several possible mechanisms responsible for reducing the radii of dentinal tubules, thus decreasing the fluid permeability through dentinal tubules. They include:

1) the formation of mineralized deposits within exposed dentinal tubules within vital pulp tissue (Brannström & Garberoglio 1980),

2) the deposition of bacteria in the dentinal tubules (Michelich et al 1978), and

3) protein adsorption to the walls of tubules.

All of these mechanisms can reduce the permeability of dentinal tubules by reducing the residual functional radii of the dentinal tubules to the point where less fluid flow occurs (Pashley et al 1982).

3.7 Protein binding to hydroxyapatite

Molecular interactions, or binding, of intracanal medicaments to dentine will influence their rate of diffusion through the dental root.

Hydrogen ions have been reported to bind to the unprotonated trivalent phosphate ions in dentine matrix. This suggests that dentine may act a "solid buffer" (Chan & Jensen 1986, Wang & Hume 1988).

Unbound parathyroid hormone (PTH), a polypeptide, was found not to diffuse through dentinal tubules in physiological concentrations. The hormone did, however, diffuse across the tubules after it has been prebound to high concentrations of albumin (1 gm percent). The permeability coefficient of the PTH-albumin complex was similar to that for unbound albumin (Pashley 1988).

Protein purification procedures, *in vitro*, by use of hydroxyapatite columns is a widely-used biochemical technique. The protein-hydroxyapatite interaction is thought to occur between the protein carboxyl groups and the calcium ions of hydroxyapatite. In addition, phosphoproteins also bind to calcium ions via their phosphate groups.

The mineralized surface of dentinal tubule walls acts as an ion-exchange support matrix which allows for molecules diffusing through the tubules to interact with it. The

large surface area of dentinal-tubule surfaces available for this interaction has led to these tubules being described as "extremely efficient ion-exchange columns" (Pashley et al 1982).

A number of constituents in plasma and saliva have been reported to bind to the dental root in physiological concentrations. They include fibrinogen, albumin and salivary glycoproteins. Their binding to dental-root mineral reduces the functional radii of the dentinal tubules and therefore permeability.

CT, a polypeptide hormone, is likely to bind to dental-root mineral. When CT is placed topically into root canals, this binding may hinder its passage through dental roots. Therefore, strategies which may facilitate the hormone's availability to external dental-root surfaces include:

1) placement of high concentrations of CT into root canals,

2) pretreatment of the endodontically-prepared dental root with another protein such as albumin to occupy the binding sites on the dental roots prior to administering CT,

3) binding CT to a carrier protein such as albumin prior to insertion into root canals.

The binding characteristics of CT to dentinal-tubules surfaces are not known. If CT binds irreversibly to the dental-root surface, then the hormone will be of little therapeutic use for the treatment of external-inflammatory root resorption until all the dental-root binding sites are saturated. Conversely, a reversible binding to the dental root may allow a therapeutic slow-release of CT to the resorption sites on the external dental-root surface.

3.8 Sites of resistance to fluid movement

Pashley et al (1978a) described resistance to fluid movement through dentine in terms of three resistances placed in series:

1) surface resistance due to the presence of debris occluding dentinal tubules (i.e. smear layer). Surface resistance accounted for 86% of the total resistance.

2) an intratubular resistance due to mineralized irregularities within the tubules.

3) a pulpal resistance due to the presence of odontoblastic processes and cell bodies within the tubule.

Any odontoblast cell bodies remaining in the pulp chamber after removal of pulp would contribute to intratubular resistance. The authors observed a decreased intratubular resistance to fluid flow after the teeth had been stored in Kreb's Ringer phosphate buffer at 5°C for 1 wk. This reduction in resistance to fluid flow was probably due to odontoblastic processes disintegrating (Pashley et al 1978a).

Tubular resistances that may restrict the passage of CT through the endodontically-prepared dental root are likely to be reduced by chemo-mechanical debridement of the root canal. Such debridement, which usually involves the use of EDTAC and sodium hypochlorite, will remove the smear layer on root-canal surfaces and loose organic contents within root canals (Goldman et al 1981).

In conclusion, the above studies indicate that the rate of diffusion of molecules through dentine depends on a number of factors, including molecular size, the cross-sectional surface area available for diffusion, the patency of the dentinal tubules, the presence or absence of a smear layer and the nature of the dentinal tubule surface. In addition, the interaction of diffusants, such as CT, from the root canal reservoir with the dentinal-tubule surfaces may modify diffusion.

3.9 In vitro dentine diffusion models

Various *in vitro* experimental models have been developed to study the diffusion of molecules through dentine. These models have been used to study the diffusion of molecules; a) from the base of occlusal class 1 cavities to the pulp chamber (Hume & Kenney 1981, Hume 1984, Pashley et al 1977); b) through disks of coronal dentine (Outhwaite et al 1976, Merchant 1977) or c) through disks of root dentine (Fogel et al 1988).

Some of the studies examined the influence of hydrostatic pressure on molecular diffusion through dentine.

These experiments addressed parameters which may affect diffusion of molecules through dentinal tubules in general, and focused on diffusion towards the root canal in particular. However, research on (i) the diffusion of molecules through dentine and cementum following their placement into endodontically-prepared root canals, and (ii) the influence on simple diffusion characteristics of regional variability between coronal and radicular dentine was lacking.

Subsequently, Abbott et al (1988) studied the diffusion of some constituents of Ledermix paste through endodontically-prepared dental roots. This investigation addressed many of the parameters that may influence their diffusion. The surface area of the root-canal walls exposed to Ledermix paste components was estimated using a mathematical formula that assumes the dental root to be circular in cross section at the apex and elliptoid at the coronal end, with endodontically-prepared root canal having the shape of a "regular frustrum of an elliptiod cone". Furthermore, the surface area of the prepared root-canal surfaces was considered to be non-perforated, that is, devoid of dentinal tubules. The results from the studies of Abbott et al (1988) indicated that the cumulative diffusion of the Ledermix components demeclocycline and triamcinolone

through the dental root is related to the surface area of the root canal wall. In addition, the contribution of diffusion of these medicaments through the apical foramen to total diffusion was also investigated. This contribution was found to be negligible, that is, most of the diffusion of demeclocycline and triamcinolone through dental roots occurs through the dentinal tubules themselves. Abbott (1985) observed that cementum acts as a permeable, rate-limiting molecular barrier to the diffusion of the Ledermix components through the dental root. Abbott et al (1988) also examined the contribution of cementum to the diffusion of demeclocycline and triamcinolone through dental roots. The authors observed that cementum acts as a permeable, rate-limiting barrier to these substances.

In most cases of external inflammatory root resorption the dental root surfaces are only partially devoid of cementum. Therefore an *in vitro* dental-root model with a standardized surface area of exposed dentinal tubules on the dental-root surface may more closely mimic the *in vivo* situation.

4. CALCITONIN

4.1 History of calcitonin

It was originally thought that hypercalcaemia could be corrected by inhibiting PTH secretion. However, Copp (1962) established that hypercalcaemia causes the release of a short-acting second hormone "calcitonin" which actively lowers serum calcium. This was demonstrated by perfusing dog parathyroid and thyroid glands with hypercalcaemic blood which caused an immediate transitory hypocalcaemic effect in the systemic blood. This hypocalcaemic effect occured more rapidly than the hypocalcaemia observed in dogs which have had a total parathyroidectomy. These observations led Copp to conclude that the parathyroid glands secreted a factor or hormone in response to hypercalcaemia which reduced the elevated plasma calcium concentration (Copp 1969, Jenkins 1978). This factor was later isolated and purified and called CT. It was later noted that thyroid extracts also produced a hypocalcaemic agent termed thyrocalcitonin but the two agents were shown to be the same and the hormone originated from the thyroid. Nevertheless, CT is the name commonly used (Goodman & Gillman 1985).

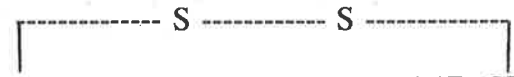
4.2 Origin of calcitonin

CT is produced and secreted by the parafollicular "c" cells of the thyroid gland. These cells are distinct from the thyroxine-producing follicular cells. The "c" cells are embryologically derived from the ultimobrachial body. In submammalian vertebrates, this body is a distinct structure arising from fifth brachial pouch. In mammals, however, the ultimobrachial body and the median portion of the fourth brachial pouch become incorporated into the lateral lobe of the thyroid gland (Klinck et al 1970).

4.3 Chemistry of calcitonin

CT exists as a single chain 32 amino-acid polypeptide of molecular weight 3600. The polypeptide has an N-terminal 7 membered disulphide ring and a C-terminal of proline-amide. The sequences of eight different forms of CT from five different species have been determined, and porcine, human and salmon CTs have been synthesized. The amino acid sequences of these different forms of CT are presented in a review by Wilson & Foster (1985).

The structure of human CT is as follows:



CYS-GLY-ASN-LEU-SER-THR-CYS-MET-LEU-GLY-THR-TYR-THR-GLN-ASP-PHE-ASN-LYS-PHE-HIS-THR-PHE-PRO-GLN-THR-ALA-ILE-GLY-VAL-GLY-ALA-PRO-NH_2

It may differ by as much as 19 residues between the human and the ovine forms. However, there are a number of features common to all the CT molecules, including:

1) cystine residues at positions 1 and 7 linked by a disulphide chain ,

2) N-terminal amino acid residue of proline-amide,

3) an acidic residue (aspartic or glutamic acid) is found uniformly at position 15, and the only other acid residue is found at position 30,

4) aromatic residues may exist at positions 12, 13, 16, 19, 22 or 27 but do not occur between positions 1 and 7 (Beesley et al 1968).

Fish and chicken CT are immunologically similar and are the most biologically active CTs. The biological potency of salmon CT is one hundred times greater than human CT.

This higher potency is due in part to salmon CT's greater resistance to degradation in peripheral tissues and also due to its greater affinity for receptors (Smith 1975).

Bioassays of CT preparations are performed by comparing the biological activity of the hormone in the preparations against a CT reference standard. The Division of Standards of the British Medical Research Council has set up the standard, the "Thyroid Calcitonin Research Standard". One Medical Research Council (MRC) unit is defined as the biologic activity of the CT present in 40 mg of a standard porcine thyroid extract. One MRC unit is equivalent to 4μg of pure porcine CT.

CT preparations, for medical purposes, are available in Australia in three forms;

1) A highly purified CT of porcine thyroid origin.[1]
2) A synthetic salmon CT[2]
3) A synthetic human CT[3].

4.4 Physiology of calcitonin

CT is a hormone involved in the maintenance of blood calcium homeostasis, and thus acts as an hypocalcaemic agent. The principal site of action of CT is in bone and, more specifically, on osteoclasts (see Section 1.2 above). The hormone also acts on the renal and the gastro-intestinal systems and may affect the host defence system and dentine calcification.

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[1] "**Calcitaire**" - USV Pharmaceuticals Pty. Ltd. Presentation; ten vials containing 160IU lyophilized calcitonin supplied with 10 vials of 2ml sterile gelatine diluent. Storage; 2-10°C for two years.

[2] "**Calsynar**" - USV Pharmaceuticals Pty. Ltd. Presentation; 2 ml vials containing 400IU Calsynar in saline acetate or 1ml ampoules containing 100IU calsynar in saline acetate. Storage; as for Calcitaire.

[3] "**Cibacalcin**" - Ciba Pharmaceuticals. Presentation; Five ampoules containing 0.5 mg of human CT supplied with 5 ampoules of 2 ml water for injections. Storage; below 30°C and protect from light.

CT, once secreted, has only a short half life of between two and fifteen minutes in the circulatory system (Harrison 1987). Normal blood CT concentration is approximately 100 picograms per millilitre. CT lowers serum calcium levels, in general, by decreasing the rate of bone resorption. This reduction results in an increase in net skeletal bone deposition. Hypophosphataemia is a secondary effect that can occur following a CT-induced decrease in bone resorption.

CT can act directly on renal tubules causing an increase in the clearance of calcium, phosphorous, magnesium, sodium, chloride and potassium ions (Murad et al 1970, Paillard et al 1972, MIMS Annual 1977). The gastric effects of CT include i) the inhibition of gastric-acid secretion, ii) the stimulation of the intestinal secretion of water and electrolytes, iii) the inhibition of pancreatic-enzyme production and motility, and iv) the modification of glucose-insulin relationships (Ganong 1983, MIMS Annual 1987).

CT may influence the immune system. Body et al (1988) have identified CT-specific receptors on unstimulated, circulating T lymphocytes with a positive cooperativity between the receptor binding sites under certain conditions. The authors have thus speculated that CT may activate lymphokine production. In addition, Adami et al (1988) have shown that salmon CT can suppress the delayed hypersensitivity response. Antibodies develop in 30% to 66% of patients given porcine or salmon CT, probably due to differences in the amino-acid sequence from human CT, but this is only rarely of clinical significance (Haddad & Caldwell 1972, Singer et al 1972, Hosking 1981, De Rose et al 1974, Martin et al 1977, Woodhouse et al 1977, Singer et al 1980). The presence of antibodies seems to reflect the patient's ability to mount an immune response (De Rose et al 1974, Singer et al 1972). Furthermore, the prevalence of such antibodies to CT correlates poorly with the dose of hormone administered (De Rose et al 1974, Singer et al 1972).

By noting that people with chronic CT deficiency (for example, those who have had a total thyroidectomy and only receive thyroxine supplementation) have wide predentine and interglobular dentine regions, Kline and Thomas (1977) suggested that CT plays a role in the normal calcification of dentine matrix. No other systemic effects due to CT deficiency have been reported. Furthermore, people with CT excess (for example, those with the CT-secreting tumour, medullary carcinoma of the thyroid) do not suffer from any abnormalities in bone metabolism and serum calcium concentrations, even with circulating levels of CT up to twenty thousand times normal serum hormone levels (Smith 1975, Harrison 1987).

Given the lack of systemic effects produced by CT excess, the use of the hormone as an intracanal medicament to treat external inflammatory root resorption is likely to be a safe form of therapy.

4.5 Calcitonin and inflammation

Repeated parenteral administration of CT can suppress inflammation both indirectly (Velo et al 1985) and directly (Strettle et al 1980).

Velo et al (1985) administered salmon CT parenterally twice daily for forty-eight hours to thirty patients suffering from rheumatoid arthritis. Eighteen of these patients showed an improvement in their clinical condition, seven patients showed no improvement and five patients reported a worsening of the disease. The therapeutic effect of the CT correlated with serum calcium concentrations, that is, patients that improved had reduced serum calcium levels whilst for the other patients the calcium levels were unaltered or increased. The authors suggested that a reduction in serum calcium concentration occuring after CT administration may lead to suppression of prostaglandin synthesis and this in turn leads to the reduction in inflammation.

Abdullahi et al (1977) investigated the effect of synthetic salmon CT on various models of experimental inflammatory processes in rats. These inflammatory models included, adjuvant arthritis, pertussis-vaccine oedema, tuberculin skin reaction, passive direct Arthus reaction and nystatin oedema. Their results indicated that repeated parenteral CT administration can delay the appearance of adjuvant arthritis, pertussis-vaccine oedema and tuberculin skin reactions whilst a single dose of the hormone can inhibit Arthus reaction and nystatin oedema.

Strettle et al (1980) suggested that, besides the indirect effect of serum calcium levels on inflammation via the suppression of prostaglandin synthesis, CT can also suppress inflammation directly. Strettle et al (1980) demonstrated that CT can reduce vascular permeability in histamine-induced inflammation in the mouse pinea by a mechanism that was independent of serum calcium levels.

The potential for successful treatment of external-inflammatory root resortion by the intracanal placement of CT is further enhanced by these inherent anti-inflammatory properties of the hormone.

4.6 Therapeutic uses of calcitonin

Medical interest in CT at present is centred principally upon its ability to reduce plasma calcium levels in Paget's disease and hypercalcaemia.

4.6.1 Paget's disease

Paget's disease is a condition characterized by increased bone turnover and defective bone remodelling which result from an abnormal increase in the size, number and activity, of multinucleated osteoclasts (Bijvöet et al 1978). The dose of CT used to treat patients with active Paget's disease is 50 to 100 MRC units daily, and administered

parenterally. Prolonged, systemic administration CT therapy produces short-term symptomatic relief and reduction in plasma alkaline phoshatase activity, urinary hydroxyproline and blood flow to bone. However, the use of CT to treat the disease after six to eight weeks is not recommended because:

1) even prolonged courses of high CT doses are unable to restore completely to normal, the bone turnover of very active disease (Bijvöet et al 1978, Woodhouse et al 1971),

2) in some patients the efficacy of long-term therapy is not maintained. This is probably due to the production of neutralizing antibodies (Haddad & Caldwell 1972, Singer et al 1972, Hosking 1981, De Rose et al 1974, Martin et al 1977, Woodhouse et al 1977, Singer et al 1972), and

3) once CT therapy is terminated, bone turnover increases rapidly and the speed with which this occurs is independent of the dose given, the duration of treatment and the degree of suppression achieved (Kanis et al 1974, Williams et al 1978).

Alternative medications are required to treat Paget's disease long term. These medications include glucocorticoids, non-steroidal anti-inflammatory agents, cytotoxic drugs and diphosphonates (Hosking 1981, Khairi et al 1974, Canfield et al 1977).

4.6.2 Hypercalcaemia

CT is effective in diminishing hypercalcaemia and decreasing concentrations of phosphate in plasma of patients with hyperparathyroidism, thyrotoxicosis, idiopathic calcaemia of infancy, 1,25 Vit D_3 intoxication, and hypercalcaemia due to malignancy and immobilization. The doses of CT used to treat hypercalcaemia are 25 to 50 MRC units parenterally every eight hours.

Although CT is effective in the initial treatment of hypercalcaemia, this form of therapy is to be regarded as an adjunct to more specific long-term measures. These alternative measures include the use of of diphosphonates and corticosteroids.

4.7 Pharmacology of calcitonin therapy

The pharmacology of current systemic CT therapy to treat Paget's disease and hypercalcaemia is discussed below in terms of route of administration, duration of action and excretion.

CT is administered parenterally to treat Paget's disease and hypercalcaemia by either intravascular, subcutaneous or intramuscular routes. The polypeptide cannot be administered orally as it is degraded by gastro-intestinal proteases and gastric acid. Although the half life of CT is reported to be in the range of two to fifteen minutes, once administered parenterally, the apparent biological activity of the hormone is considerably longer (8 h to 24 h). Information regarding the binding, if any, of CT to serum proteins is lacking. CT is thought to be rapidly metabolized in the liver, spleen, kidney, blood and peripheral tissues, presumably due to protease activity, and then excreted in the urine as unidentified inactive metabolites.

Systemic therapy with CT for the treatment of Paget's disease and hypercalcaemia focuses on osteoclast inhibition in general. With external inflammatory root resorption, however, CT is only required to inhibit those dentinoclasts resorbing the dental root. Therefore, the placement of CT into endodontically-prepared root canals is one potential therapeutic route of delivery to these cells. Information regarding the diffusion kinetics or availability of CT to the dentinoclasts on the external resorption sites, once the hormone is placed into such-prepared root canals, is lacking.

4.8 Contraindications for calcitonin use

Contraindications to the use of systemic CT therapy exist for pregnant and lactating patients, children and allergy-prone patients.

CT should not be administered to pregnant or lactating patients since safe usage in these patients has yet to be established. CT shows a variable lactation index and weight loss in offspring of dosed animals.

Long-term safety and efficacy has not yet been established in children, and therefore, CT is not recommended for long-term paediatric use. It has, despite this, been used successfully in Juvenile Paget's Disease but prolonged use may interfere with bone growth.

Being a polypeptide, CT has the potential for eliciting hypersensitivity reactions. Patients exhibiting such reactions should not be treated.

Although some contraindications to the use of systemic CT therapy exist, such therapy is generally safe for most patients if recommended doses are used.

4.9 Precautions for calcitonin use

Precautions to the use of CT can be discussed in terms of its immunological effects, long-term use and drug interactions.

4.9.1 Immunological effects

Patients exhibiting a predisposition to hypersensitivity-type reactions should have an intradermal skin test to determine sensitivity to CT before any systemic therapy is commenced.

4.9.2 Long-Term use

There are several problems related to long-term use of systemically-administered CT. First, some patients may exhibit a primary resistance to CT although this has been reported to be extremely rare (De Deuxchaisnes 1983).

Second, by increasing the dose of CT administered to patients with high alkaline-phoshatase levels, the therapeutic effect is increased up to a point whereby further increasing the CT levels will not reduce the levels of alkaline phoshatase below a certain level. This dose-limiting response is termed the "plateau phenomenon" (Bordier et al 1974). Nevertheless, there is continued suppression of symptoms and progressive amelioration of the histological findings in Paget's disease and hypercalcaemia, during the plateau phenomenon (Bordier et al 1974).

The third problem encountered with chronic CT usage is that of secondary resistance, the so called "rebound" or "escape" phenomenon, in which CT is no longer effective in inhibiting stimulated bone resorption *in vitro* after a certain period of time. Whether the rebound phenomenon reflects the presence of a more aggressive group of osteoclasts or whether calcitonn loses its effect on osteo-progenitor cell differentiation remains speculative (De Deuxchaisnes 1983).

The final problem is that of antibody-based resistance to CT. This is a characteristic of long-term administration of the salmon and porcine CT, but not their human equivalent (de Deuxchaisnes 1983). The significance of antibodies has discussed earlier.

Due to the above-mentioned problems relating to the efficacy of long-term systemic CT therapy, it has been suggested that patients placed on such therapy should have periodic measurements of urinary hydroxyproline concentrations; an indirect measure of bone turnover (MIMS Annual 1987).

4.9.3 Drug interactions

Subsequent to CT administration, plasma calcium levels may be transiently depressed to below normal levels. This should be taken into account if patients are receiving cardiac glycosides, as dosage adjustment of these drugs may be necessary.

4.10 Adverse reactions

Therapeutic doses of CT are generally well tolerated, but some minor adverse reactions have been reported by De Deuxchanaises (1983). These include:

1) Nausea and vomiting: This occurs in approximately 10% of patients and is usually a transient, dose related phenomenon. It can be overcome either by concomitant antiemetic therapy or by increasing the frequency of dose. Unusual taste and diarrhoea has also been reported.

2) Dermatological: Facial flushing, itching of the palms and skin rashes including maculo-papular eruptions. Local inflammatory reactions at the injection sites can occur.

3) Immunological: Urticaria. Fatal anaphylactic reactions to CT have not been reported.

4) Endocrine: Hypocalcaemia with associated parasthesiae has been reported, although it is extremely rare.

5) Fever and headaches.

6) Cramps in extremities.

7) Increased urination frequency.

Deuxchaisnes (1983) suggested that the side effects to CT is dose dependent and that the hormone is devoid of toxicity. Indeed, evidence for overdose from CT is not available.

In summary, systemic use of CT has been used safely and successfully to treat Paget's disease and hypercalcaemia. There are only a few minor side effects associated with its use (MIMS Annual 1987).

5. CALCITONIN AND EXTERNAL ROOT RESORPTION

The potential use of CT as a systemic or topical dental therapeutic agent has been considered in accelerating healing of tooth sockets (Foster & Kronmann 1974), capping exposed dental pulps (Smith & Soni 1982, Cullum & Kline 1985) and preventing external resorption in replanted monkey teeth (Barbakow et al 1981, Pierce et al 1988a).

Foster and Kronman (1974) investigated the effect of a single, sustained dose of CT on the healing of eight tooth extraction sites in four dogs. Collagen wound pads (1 cm^2 size; Avicon, Inc., Forth Worth, Texas, USA) were saturated with a solution containing i) porcine CT, ii) a gelatine solution and iii) an acetate buffer (pH 3.0). These pads were then sutured into dental-root sockets immediately after tooth extraction. Wound pads saturated with physiologic saline served as controls. The socket sites were examined histologically after sixty days. The experimentally treated sockets displayed an increased rate of bone formation, with a reduction in the maturity and density of the bone. The sites not treated with CT contained more osteoid and less trabeculation and less collagen which indicated a slower growth rate of new bone. Although the results were encouraging, the authors were guarded in making any clinical recommendations.

Smith and Soni (1982) investigated the effect of both a salmon CT in saline preparation and a calcium hydroxide in water preparation when each were placed topically on both direct and indirect pulp exposures in the molar teeth of twenty rats. The pulp-exposure sites were examined histologically for up to twenty-eight days after exposure to the preparations. Indirect pulp capping with either CT or calcium hydroxide allowed for the deposition of underlying secondary dentine. When CT was placed directly onto pulps, a fibrous scar was formed beneath the exposure sites by fourteen days. This scar tissue then progressively calcified to form a dentine bridge by twenty-eight days. In contrast, the placement of calcium hydroxide directly onto pulps produced an inflammatory reaction without the formation of a dentine bridge.

Cullum and Kline (1985) examined the potential use of CT as a direct pulp-capping agent. Synthetic salmon CT (1.1 μg) on a sterile absorbable surgical sponge was placed topically onto twelve direct pulp exposures in labial cavities in the teeth of two dogs and the cavities sealed with light-cured composite resin. Concomitant systemic antibiotics were administered for seven days. The placement of the CT carrier vehicle (2.75μl of 0.1% albumin in 1% sodium acetate on sterile, absorbable surgical sponge [name and manufacturer not supplied]) on separate exposure sites in the same dogs served as controls. After sixty-one and ninety days the exposure sites were histometrically examined. The use of CT did not increase the rate of healing in directly pulp-capped teeth, nor did it increase the amount of secondary dentine produced. However, CT did reduce the amount of inflammation in the dental pulps. The authors suggested that due to this anti-inflammatory effect, CT could be useful as an adjunct to the treatment of directly pulp-capped dental pulps. The results of the aforementioned studies are consistent with the anti-inflammatory capability of CT suggested by Abdullahi et al (1977) and Strettle et al (1980).

Barbakow et al (1981) investigated the healing of replanted monkey maxillary incisor teeth with systemic administration of CT. Ten teeth from five monkeys were endodontically treated, extracted for extraoral periods of between 30 min and 120 min, then replanted and splinted. Daily intramuscular injections of 20 MRC units of CT was administered to these monkeys for eight weeks. The dental roots were then histomorphometrically examined. No improvement in healing, reduction in inflammation nor reduction of resorption was observed where systemic CT was used as an adjunct to treatment when compared with replanted control teeth which did not have the CT therapy.

The potential use of CT as a new treatment modality for external root resorption stems from a study which demonstrated the potent intracanal effects of CT on inflammatory resorption in replanted monkey teeth (Pierce et al 1988a). The residual inflammation found within the periodontal membrane was reduced to less than 1% of the root surface by intracanal application of CT. This reduction was notably greater than that achieved in earlier studies using antibiotics alone (Hammarström et al 1986a) or calcium hydroxide (Hammarström et al 1986b), and was similar to that observed when Ledermix paste was used for the same purpose (Pierce and Lindskog 1987). It was suggested that CT may have directly inhibited the dentinoclasts in resorption lacunae thus allowing cementoblasts and/or osteoblasts to proliferate over and seal off the exposed dentinal tubules (Pierce and Lindskog 1987). Furthermore, CT may have suppressed inflammation (Velo et al 1985). Although the use of Ledermix paste to treat inflammatory root resorption is successful, some cases of resorption do not respond to this form of therapy. Furthermore, Ledermix paste may be preluded from use in cases where medical contraindications to corticosteroids or tetracyclines exist. Therefore, an alternative intracanal medication to treat external-inflammatory root resorption is

required. CT, a polypeptide hormone, may be one alternative.

If CT is to be considered as a topical therapeutic agent to treat external-inflammatory root resorption, then a protracted release of the hormone from the root canal to the external resorption sites is desirable. We have already suggested that binding of CT to dental-root mineral may provide one therapeutic slow-release mechanism. The use of a slow-release delivery vehicle with CT, such as those described by Smith and Soni (1982) and, Cullum and Kline (1985), may provide another slow-release mechanism. The present study focused on the CT binding to dental-root mineral as a potential slow-release mechanism. The use of a slow-release delivery vehicle for the topical application of CT into root canals was not considered.

The study of Pierce and Lindskog (1987) indicated that CT may be an effective agent in the treatment of inflammatory root resorption. Its inhibitory action on clastic cells and inflammation, combined with the fact that it is a biologic, endogenous hormone indicates that CT is a potentially attractive alternative to conventional therapies for the treatment of external resorption.

AIMS

The aims of this study were to:

1. Determine the status of dentinal tubule contents before and after maceration by alkaline hydrolysis in order to identify the contribution of mineralized matrix alone to the diffusion of CT through dental roots. The dentinal tubule contents were examimed by SEM.

Further SEM investigations were performed to determine the effect of:

(i) two commonly used tooth storage media,

(ii) the time of storage in these media, and

(iii) endodontic root-canal preparation

on the dentinal tubule contents of extracted teeth. It was hypothesised that the parameters described above will affect the preservation of dentinal tubule contents. These investigations were carried out in order to describe and compare the effects of such parameters on the permeability of non-macerated dental roots to putative therapeutic diffusants in future studies.

2. Examine the kinetics of CT diffusion through the endodontically-prepared dental root and parameters which may affect this diffusion. These parameters include; CT binding to dental-root mineral, competition for CT binding sites by other molecules, and the presence of cementum on the dental root.

The following hypotheses were considered:

(i) CT, placed topically into endodontically-prepared root canals, will diffuse from the root canal to the external root surface.

(ii) The presence of cementum on the tooth will delay, but not inhibit diffusion.

(iii) CT will reversibly bind to macerated, powdered dental-root mineral and,

(iv) this binding will have an influence on the kinetics of diffusion.

This study will be divided into three sections: **1.** A scanning electron microscopic investigation of dentinal tubule contents, **2.** Experiments examining CT diffusion through whole dental roots, and **3.** A series of experiments investigating binding characteristics of CT to ground dental-root mineral.

PART 1: A SEM INVESTIGATION OF DENTINAL TUBULE CONTENTS

1. MATERIALS

Twenty seven intact, single-rooted premolar teeth extracted for orthodontic reasons from young teenagers were obtained for SEM investigation. The teeth were stored immediately after extraction at 4°C either in phosphate buffered saline (PBS) or in Dulbecco's Modification of Eagle's Medium (DMEM) with antibiotics (see Appendix 4). The storage period was either 24 h or 3 wk prior to commencement of the SEM analyses. The teeth were sorted into into 9 experimental groups each of 3 teeth.

2. EXPERIMENTAL TECHNIQUES

1. Maceration - The teeth were boiled in water for 1 h and subsequently placed in an ultrasonic bath with a 4% solution of sodium hypochlorite for 30 min and washed copiously with running water. This technique is an adaptation of that used by Garberoglio and Brannström (1976).

2. Fixation - Teeth were placed in a solution of 2.5% glutaraldehyde in PBS for 4 h, after which they were dehydrated through a sequential series of graded alcohols to acetone. The sequence of solutions used was:

i) 50% ethanol in distilled water for 30 min.

ii) 70% ethanol in distilled water for 30 min.

iii) 90% ethanol in distilled water for 30 min.

iv) 95% ethanol in distilled water for 30 min.

v) 100% ethanol for 15 min.

vi) 100% ethanol for 15 min.

vii) 100% acetone for 60 min.

3. Splitting - Teeth were split longitudinally by placing a longitudinal slot on the mid-buccal and mid-lingual surfaces of the crowns with a bur and compressing them in the jaws of a vice.

4. Endodontic preparation - Access to pulp chambers was obtained using a 169L tapered fissure tungsten carbide high speed bur. After location of canals, irrigation was performed with a 1% solution of sodium hypochlorite. The root canals were filed to the apical foramen until a size 35 file was reached. A 15% solution of ethylene diamine tetra-acetic acid with cetyltrimethyl ammonium bromide (EDTAC) was used for irrigation. Sendoline "S" files (Sjödings, Stockholm, Sweden) were used in sequential order from a size 10 and the canals recapitulated to their full length after every change of file size, and copiously irrigated.

5. SEM Investigation - All of the specimens were allowed to dry naturally overnight and then mounted on SEM mounting stubs with a silver adhesive. Specimens were subsequently coated with a gold-palladium alloy using a vacuum evaporation method. SEM examination was carried out using a Siemen's ETEC Autoscan electron microscope (ETEC Corp., USA) at tilt angles of 25° to 35°. The area of tooth-root examined was between the mid-cervical third to the mid-root level. This region corresponds to the location of standardized windows in the next part of the study.

For each specimen examined, the following micrographs were taken:

1) One low magnification (30x) photograph.

2) Three medium magnification (400x) photographs. One adjacent to the pulpal border, one adjacent to the cemento-dentinal junction, and one approximately halfway between the two.

3) Seven higher magnification (4000x) photographs in each of the three medium magnification areas.

The higher magnification micrographs were used to examine the contents of the tubules.

In order to examine the effects of storage medium, time, and endodontic preparation on dentinal tubule contents, the nine experimental groups, each of three teeth, were treated as follows (Table I):

Group 1. Teeth were macerated irrespective of how long they had been stored, and split longitudinally. They were allowed to dry naturally overnight and prepared for SEM investigation. These teeth were used as negative controls.

Group 2. Teeth stored in PBS for 24 h at 4°C were fixed in 2.5% glutaraldehyde for 30 min, split, refixed for a minimum of 4 h, dehydrated and prepared for SEM investigation.

Group 3. As for Group 2 except that teeth were stored in DMEM for 24 h.

Group 4. As for Group 2 except that teeth were stored in PBS for 3 wk.

Group 5. As for Group 2 except that teeth were stored in DMEM for 3 wk.

Group 6. Teeth stored in PBS for 24 h were endodontically prepared with the roots still bathed in the same storage medium to keep them from drying. The rest of the preparation proceeded as for Group 2.

Group 7. As for group 6 except that the teeth were stored in DMEM for 24 h.

Group 8. As for group 6 except that the teeth were stored in PBS for 3 wk.

Group 9. As for group 6 except that the teeth were stored in DMEM for 3 wk.

TABLE I. Treatment Groups for SEM Analysis

Group	Storage Time	Storage Medium	Root-Canal Debridement
1	macerated (negative controls)		
2	24 h	PBS	No
3	24 h	DMEM	No
4	3 wk	PBS	No
5	3 wk	DMEM	No
6	24 h	PBS	Yes
7	24 h	DMEM	Yes
8	3 wk	PBS	Yes
9	3 wk	DMEM	Yes

The high magnification (4000x) micrographs were scrutinized blind and scored according to the following criteria:

"0" Tubules devoid of organic contents. These specimens displayed a smooth inner surface outlined by mineralized bundles of fibres. Canaliculi were commonly discernable.

"+" Tubules contained loose organic contents. These specimens displayed interlacing arrangements of individual fibres on the inner surface without the presence of remnants of odontoblast processes. No canaliculi were visible.

"++" Odontoblast processes or their remnants were discernable.

The criteria used for identification of these structures were as for Garberoglio and Brannström (1976).

6. Statistical analysis - the results were collated and statistically analysed using the following null hypotheses:

1) Time spent in storage media did not affect dentinal tubule contents.

2) The type of storage media did not affect dentinal tubule contents.

3) Root-canal therapy did not affect dentinal tubule contents.

4) Dentinal tubule contents did not vary between the outer third, middle and inner areas of dentine.

5) Each of the above effects was non-interactive.

Hence the analysis comprised three experimental factors:

T (= time) at two levels (24 h, 3 wk),

M (= storage medium) at two levels (PBS, DMEM),

E (= endodontic preparation) at two levels (yes, no),

and one within-tooth factor:

R (= region of tooth) at three levels (inner, centre, & outer).

The variable used to assess the joint effect of the above factors is the proportion of the number of zero scores, to the total number present. The "+" and "++" scores were combined.

i.e. $y = N(0) / (N(0) + N(+) + N(++))$

The variable "y" was analysed on a logit scale, and the structure of the experiment required a repeated measures analysis of variance, with an unstructured covariance matrix. Programme 5V from the BMDP Statistical Software package (UCLA, Los Angeles, USA, ed. W. Dixon) was used for the calculations[1].

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[1]Statistical analysis was performed in consultation with Dr. Phillip Leppard, Statistical Unit, Department of Mathematics, The University of Adelaide, Australia.

3. RESULTS

Sample photographs relating to the scoring method are shown in Figures 3 - 6. The results of the SEM investigations are displayed in Table II.

It was found that only main effects terms for M,E and R were statistically significant at the 1% level, that is, there was no detectable time (T), effect or an interaction between the factors. The fitted model is shown in Table (III): for an "average" tooth the expected values of "y" are expressed as a percentage.

Macerated specimens did not exhibit any signs of loose organic tubular contents (Figure 7).

TABLE III. Expected Values of "y" for an Average Tooth

RCT	Yes			No		
Region	Inner	Centre	Outer	Inner	Centre	Outer
PBS	33	44	67	56	66	84
DMEM	54	64	82	75	82	92

Non-endodontically prepared teeth (that is, teeth with intact pulps) showed a significantly higher proportion of zero results (that is, tubules devoid of obvious organic contents) than endodontically prepared teeth ($P<0.01$).

Teeth stored in DMEM showed a significantly higher proportion of zero scores than those stored in PBS in both endodontically and non-endodontically prepared

TABLE II. SEM Investigation of Dentinal Tubular Contents

GROUP	TOOTH	TREATMENT			REGION								
					INNER			CENTRE			OUTER		
		A	B	C	0	+	++	0	+	++	0	+	++
1	1	-	-	-	12	0	0	16	0	0	7	0	0
1	2	-	-	-	15	0	0	10	0	0	7	0	0
1	3	-	-	-	20	0	0	11	0	0	9	0	0
2	4	1	1	2	5	10	0	10	3	0	8	0	0
2	5	1	1	2	0	24	0	0	7	2	7	0	0
2	6	1	1	2	3	11	0	7	1	0	7	0	0
3	7	1	2	2	15	0	0	10	1	1	8	0	0
3	8	1	2	2	7	5	0	7	2	0	9	0	0
3	9	1	2	2	9	4	0	12	0	0	9	0	0
4	10	2	1	2	11	4	4	7	3	1	6	2	0
4	11	2	1	2	6	14	0	13	0	0	7	1	0
4	12	2	1	2	3	11	0	12	0	0	8	0	0
5	13	2	2	2	23	0	0	9	0	0	7	2	1
5	14	2	2	2	20	0	0	17	0	0	7	1	0
5	15	2	2	2	6	2	0	5	2	0	9	0	0
6	16	1	1	1	9	2	0	1	4	4	2	5	1
6	17	1	1	1	8	9	0	0	14	2	3	5	1
6	18	1	1	1	0	13	1	7	3	1	10	0	0
7	19	1	2	1	3	11	0	10	3	0	3	4	0
7	20	1	2	1	9	6	2	5	2	0	7	0	0
7	21	1	2	1	6	8	0	6	2	0	9	1	0
8	22	2	1	1	7	4	0	7	3	0	6	2	0
8	23	2	1	1	9	4	0	4	4	0	6	2	0
8	24	2	1	1	14	6	0	6	2	0	6	1	0
9	25	2	2	1	5	2	0	5	3	0	5	1	0
9	26	2	2	1	5	5	0	6	2	0	9	0	0
9	27	2	2	1	9	1	0	6	2	0	3	4	0

TREATMENT:

A = Storage Time — A1 = 24h / A2 = 3 wk
B = Storage Medium — B1 = PBS / B2 = DMEM
C = Endodontic Debridement — C1 = YES / C2 = No

teeth ($P < 0.01$).

The pulpal (inner) third of the dental roots showed significantly lower zero scores (that is, more tubular contents) with respect to the middle third ($P < 0.01$). Correspondingly, the middle third showed significantly lower zero scores with respect to the outer third ($P < 0.01$). Results were significant for both DMEM or PBS storage and endodontically and non-endodontically prepared teeth ($P < 0.01$).

No significant differences in tubular contents were noted between teeth stored for 24 h as opposed to 3 wk ($P < 0.01$).

Some morphological observations noted in the specimens included:

(1) the presence of approximately 35 000 to 45 000 tubules per mm^2 adjacent to the pulpal surface. The diameter of these tubules ranged between approximately 1.8 μm and 2.5 μm. The corresponding values of tubule numbers and diameters in the middle third of the dental root were 23 000 to 27 000 tubules per mm^2 with an approximate diameter of 0.8 μm to 1.8 μm. In the outer third of the dental roots, there were 19 000 to 22 000 tubules per mm^2 and the approximate diameters of these tubules were 0.8 μm to 1.2 μm.

(2) few remnants of odontoblast processes were found in the dentinal tubules in all experimental groups.

(3) remnants of odontoblast processes were observed in the middle third (Groups 2, 3, 4, & 6) and outer third (Groups 5 & 6) of some dental roots (Figures 3 & 4).

(4) the dentinal tubule surfaces nearest the pulp appeared to be more corrugated in contrast to the smoother walls found in the middle and outer layers of the dental roots.

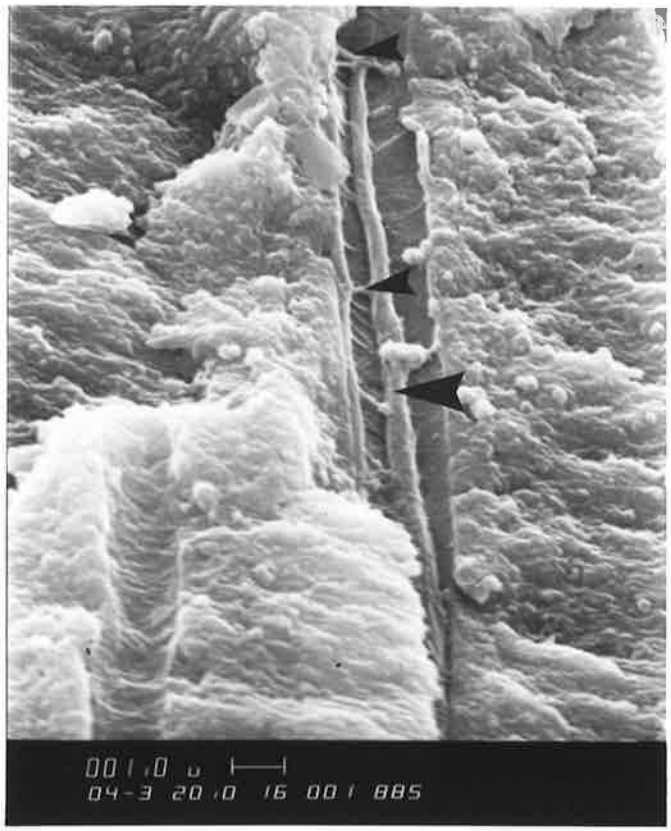

Figure 3. Odontoblast process located in the middle third of the tooth-root (large arrow). Note the small ramifications from the process extending into adjacent canaliculi (small arrows). This tubule was scored as "+". (Bar = 1μm)

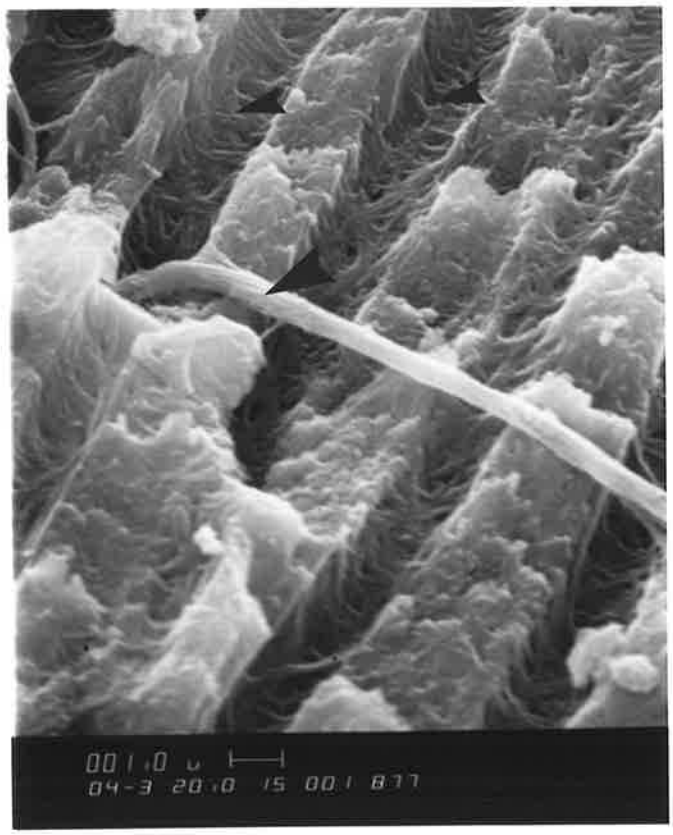

Figure 4. Odontoblast process (large arrow) in pulpal third of the dental root. Its tubular contents were scored as "+ +". Note the corrugated appearance of the walls of the adjacent tubules due to a lack of peritubular dentine (small arrows). These tubules were scored as "0". (Bar = 1μm)

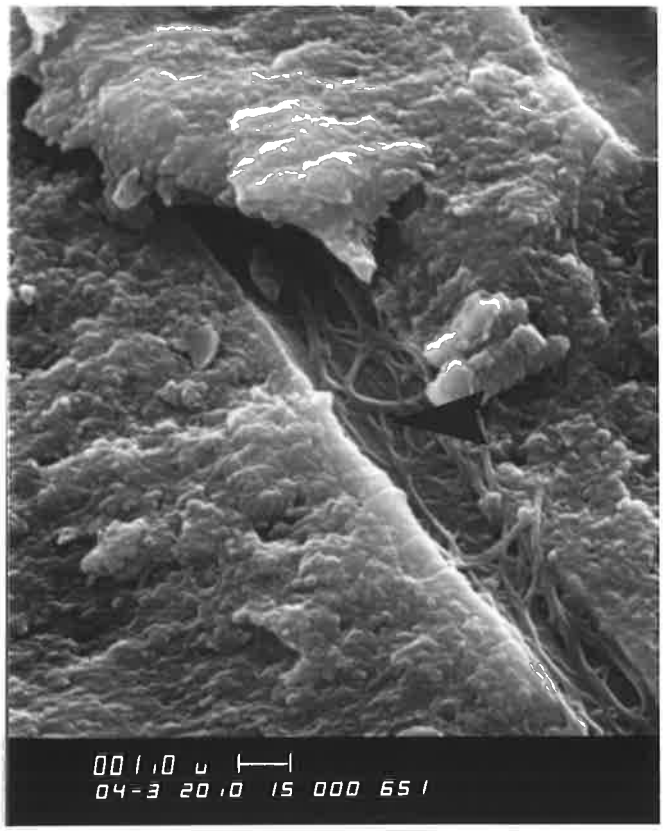

Figure 5. Interlacing network of collagen fibres in dentinal tubule (arrow). This tubule was scored as "+". (Bar = 1μm)

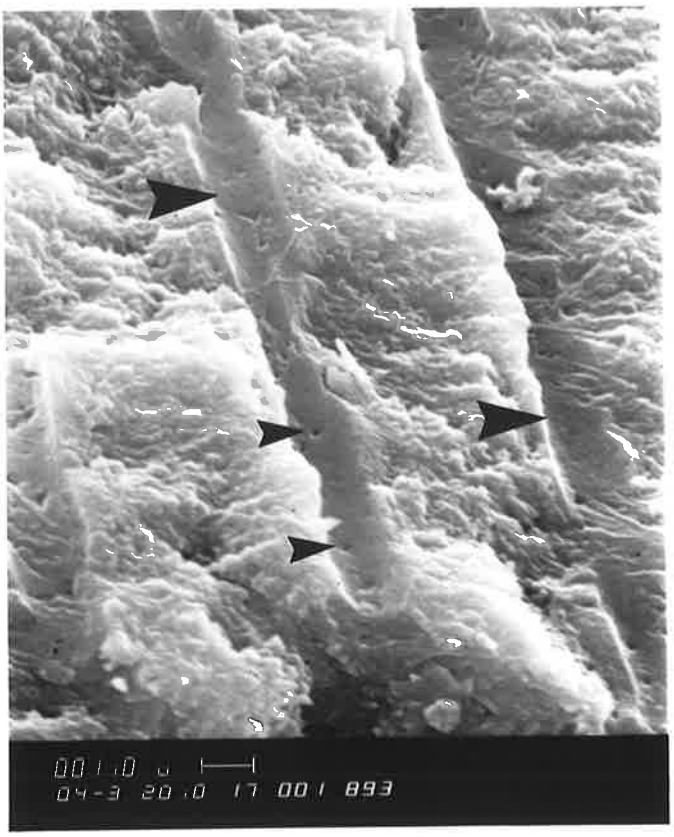

Figure 6. Dentinal tubules devoid of loose organic contents (large arrows). Note the smooth walls and presence of canaliculi (small arrows). The tubules were scored as "0". (Bar = 1μm)

Figure 7. Macerated dentinal tubule surfaces (arrows). Note corrugated and heterogeneous appearance with complete lack of loose organic contents. (Bar = 1μm)

4. DISCUSSION

Chemo-mechanical debridement of the root-canal systems using 1% sodium hypochlorite and 15% EDTAC prior to the placement of an intracanal medicament such as Ledermix paste or calcium hydroxide (or possibly CT) is the usual initial treatment for recently traumatized or avulsed permanent teeth exhibiting signs of inflammatory root resorption. This endodontic preparation will remove the odontoblasts and debris from the root canal surface leaving patent dentinal tubules (Cameron 1988). However, it cannot be assumed that all of the organic contents throughout the entire length of the tubules are concomitantly removed (Artener et al 1989). Indeed, information regarding the effect of endodontic chemo-mechanical debridement on the contents of dentinal tubules, throughout their course in dentine to the cemento-dentinal junction, is lacking. The diffusion of intracanal medicaments through dental roots may be affected by these dentinal tubule contents, particularly if the medicaments are able to bind to the contents as well as to mineral in dentine and cementum.

Although macerated teeth have been used for *in vitro* investigations on the diffusion of CT and other proteins through the tooth-root, current information on an optimal, readily available tooth storage medium and storage time necessary to preserve maximum tubular contents in extracted teeth is lacking. Therefore, we investigated the effect on dentinal tubule contents (in three areas) in extracted teeth of (i) two tooth storage media (DMEM and PBS), (ii) storage time (24 h and 3 wk) and (iii) endodontic preparation. The information thus collected will be of use in

future studies which examine diffusion through intact dental roots which are aimed to better mimic the *in vivo* situation than those teeth which employ macerated teeth.

Furthermore, in order to examine the contribution of mineralized dental root matrix to the kinetics of molecular diffusion through the dental root, an effective maceration technique was required to ensure complete removal of loose organic tubular contents. The maceration technique used on the teeth in Group 1 was effective in removing loose organic contents from the dentinal tubules. Hence, the SEM study confirmed that the diffusion kinetics of CT through macerated whole dental roots could be attributed to the mineralized matrix alone.

The more corrugated appearance of the dentinal tubule surfaces towards the pulp is due to the outline of mineralized coarse collagen-fibre bundles (Figure 4). The peripheral dentinal surfaces appeared smoother due to the greater amount of mineralized peritubular dentine (Figure 6). This surface appearance was taken into account when recording the loose organic contents because the corrugations may be easily confused with the unmineralized loose organic tubular contents.

The presence of the openings to canaliculi on the dentinal tubule walls was deemed to be a good marker for tubules that were completely devoid of loose organic contents. This assumption was made on the basis that the openings to these small transverse tubules are located on the mineralized surfaces of the tubules. Consequently, they are usually difficult to detect at the ultrastructural level as they are often masked by the adjacent loose organic contents. Therefore, these intertubular ramifications are usually only seen when the tubules are devoid of loose organic material. The canaliculi were more easily seen in the smoother, more peripheral tubule surfaces than in the more corrugated inner surfaces. This observation is similar to that reported by Boyde and Lester (1967).

Storing the extracted teeth in either PBS or DMEM at 4°C for 3 wk did not show any differences in preservation of loose-organic tubular contents compared with similarly stored teeth after 24 h. These results parallel those reported by Outhwaite et al (1976) which suggested that the permeability of dentine to NaI^{125}, after storage in PBS for 3 wk, is similar to that observed at 24 h.

The superior preservation of loose-organic tubular contents by PBS with respect to the nutrient-rich DMEM was also an unexpected but interesting finding. This could be due to a better buffering capacity in DMEM than PBS, thus enabling greater activity of degradative enzymes. This would, in turn, allow for prolonged proteolytic digestion of loose organic tubular contents. The contribution of bacterial enzyme degradation of loose organic tubular contents was unlikely as antibiotics were used with DMEM and bacteria were not evident in the SEM specimens. This was in contrast to a pilot study where marked bacterial and fungal growth was observed on SEM specimens of freshly extracted teeth stored in DMEM, without antibiotics, for up to 3 wk. Therefore, for collection of extracted teeth, PBS seems to be a reasonable storage medium which can be issued to general practitioners. Freeze drying of teeth may be an alternative method for storing teeth. However, this is impractical for the general dental practitioner, the major source of obtaining freshly-extracted teeth for research. In non-endodontically prepared teeth, the low proportion of dentinal tubules observed to contain odontoblast processes (20 out of 894 tubules examined, or 2.2%) may, in part, be due to the experimental techniques. In particular, the processes of splitting the teeth and fixation of tubule contents bear further discussion. Gotjamanos (1969) reported that odontoblasts in rat teeth were firmly adherent to the dental root and not removed from the root canal wall when the pulp was removed. Furthermore, Thomas (1983) demonstrated that adequate

preservation of tubule contents was achieved with glutaraldehyde fixation. In the present study, the teeth were placed whole in fixative for 30 min prior to being split longitudinally and refixing.

It is difficult to explain why endodontically-prepared teeth displayed more tubular contents than non-endodontically prepared teeth. One might postulate that the smear layer created during endodontic instrumentation may not be completely removed beneath the root-canal surface by the endodontic irrigants thus impeding the availability of these irrigants to the deeper extensions of the tubules. Indeed, Aktener et al (1989) demonstrated that chemo-mechanical debridement of root canals can force smear layer into the tubules. However, evidence of a subsurface smear layer was not observed in our study. Nevertheless, it is interesting to speculate that removal of the pulp does not necessarily alter tubule contents. Therefore, the efficacy of currents methods of endodontic chemo-mechanical debridement should be questioned.

Less organic tubular contents were observed in the middle third of the dental root with respect to the inner third and similarly, outer third with respect to the middle third. This supports the findings of Garberoglio & Branström (1976), Tsatsas & Frank (1972), Garant (1972) and Thomas (1983). Some remnants of odontoblast processes were evident in the middle and outer thirds of some dental roots. This supports the observations of Sigal et al (1984a, 1984b) and La Fleche et al (1985) who argued that odontoblast processes may extend throughout the entire length of the tubules. The observation of a greater number of dentinal tubules per surface area with a concomitant increase in tubular diameter towards the pulp (inner) surface was in accord with those of a previous investigation (Garberoglio & Branström 1976).

In summary, the maceration technique was effective in eliminating loose organic material from the dentinal tubules. Therefore, the contribution of mineralized dental-root matrix to diffusion through dentinal tubules can be studied in these teeth. Freshly extracted teeth can be stored in either DMEM or PBS at 4°C for at least 3 wk without further degradation of loose organic tubular contents. The odontoblastic processes in some of these stored teeth were observed in the outer third of the dental root. PBS proved to be a superior storage medium than DMEM in preserving loose organic tubular contents of extracted teeth at 4°C for in excess of 3 wk. Hence, PBS is recommended in preference to DMEM for the collection of extracted teeth for *in vitro* diffusion studies. Endodontic preparation of teeth actually contributed to the preservation of dentinal tubular contents in this study. This unexpected observation, although requiring further investigation, questions the efficacy of current methods of endodontic chemo-mechanical debridement of teeth.

PART 2: CALCITONIN DIFFUSION THROUGH THE DENTAL ROOT

In order to examine the kinetics of CT diffusion through dental roots, an *in vitro* model utilizing whole teeth was used. These teeth were endodontically prepared and apically sealed and their root surfaces coated with sealant. Standardized windows were cut in this sealant to expose the underlying dental-root surfaces. Radiolabelled CT was placed into the root canals of these teeth and the amount of label that appeared on the external dental-root surface was monitored over time. The materials and methods used in this experiment are described in detail below.

1. MATERIALS AND METHODS

Single-rooted premolar teeth extracted for orthodontic reasons from adolescents were used in this experiment. The teeth were treated in the following manner:

i) endodontic preparation: regular endodontic access cavities were prepared using a high-speed handpiece and a tapered fissure bur with water spray. The pulps were removed with a barbed broach and the canal filed to a size 25 endodontic file. A 1% sodium hypochlorite solution was used during instrumentation and a class I cavity was cut at the apical ends of the teeth with a high speed bur.

ii) maceration: the teeth were boiled in water for 1 h in a microwave oven. They were then placed in an ultrasonic bath for 30 min in a 4% sodium hypochlorite solution (Garberoglio & Brannström 1976). The teeth were then washed copiously with water and allowed to dry naturally overnight.

iii) apical seal: the apical class I cavities were filled using a glass ionomer/composite resin sandwich technique (Mclean et al. 1985) according to the following method: The surface was cleansed with a 20 sec application of a 25% polyacrylic acid conditioner (Ketac Conditioning Solution, ESPE, W.Germany), followed by a 60 sec wash with water. Cavities were air dried with compressed air, and a glass ionomer cement (Ketac Fil, ESPE, W.Germany) was placed within the cavities and allowed to dry for at least 30 min. The surface was recleansed with polyacrylic acid conditioner for 10 sec, washed for 60 sec and air dried. A dentine bonding agent (Visiobond, ESPE, W.Germany) was applied and light cured for 10 sec and composite resin (Visiodispers, ESPE, W.Germany) was placed over the glass ionomer cement and light cured for 60 sec. Dental floss was tied around the crowns of the teeth away from the CEJ in order to dip them into white, water-based acrylic paint (Weathershield Exterior Gloss Acrylic, Dulux, Aust.). This was determined to be most effective sealant available (see Appendix 1). The teeth were then suspended in air from the floss, allowed to dry for 24 h and the coating repeated. A minimum of 48 h was allowed before any experimental procedures were conducted. Figure 8 illustrates the diffusion model.

The teeth were divided into three groups of teeth.

Group 1 - dentine window: standardized rectangular windows were cut in these teeth by removing the paint with a scalpel blade and being careful not to damage the cementum surface. The exposed cementum was removed with an inverted cone bur to a depth of approximately 1 mm on a proximal surface in the coronal half of the dental root. The cut surface was cleansed of debris using EDTAC for 10 min and washed thoroughly with water. A pilot study indicated this procedure to be sufficient to expose dentinal tubules and remove the smear layer (see Appendix 2).

Group 2 - cementum window: standardized rectangular windows were cut out as in Group 1 except that the cementum was not removed. (see Appendix 3)

Group 3 - No windows were cut in the paint surface (controls).

The root canals of the teeth were thoroughly moistened with PBS from a syringe. The same syringe was used to withdraw excess PBS from the root canals in order to leave a moist root canal surface.

Forty microlitres of a 1μCi/ml solution of I^{125}-radiolabelled CT ([I^{125}]-CT) was then syringed into each of the root canals and blue periphery wax (Surgident Periphery Wax, Columbus Dental, St. Louis, Mo., USA) was used to seal the access cavities. The teeth were suspended above the CEJ (but not totally immersed) in plastic scintillation vial inserts containing 2 ml PBS. All teeth were sequentially transferred into other scintillation vials containing 2 ml of fresh PBS at 1, 2, 4, 6, 10, 14, 18, 24, 48 h, 5 days, and 9 days. The radioactivity, in counts per minute (cpm), in the scintillation vials was then measured. The data obtained from this experiment were plotted as both cumulative effluxion of CT and rates of release of CT.

The experiment was repeated using 11, 14 and 13 teeth in groups 1, 2 and 3 respectively. Ten microlitres of 2.7 Ci/μl I^{131} in PBS were added to 2 ml of 1μCi/ml of [I^{125}]-CT. Forty microlitres of this I^{131}/[I^{125}]-CT solution were syringed into the root canals of all teeth. The dental roots were transferred into vials of fresh PBS and the experiment proceeded as previously described at half-hourly intervals up to 10.5 h and thereafter at 12, 14, 18 and 31.5 h.

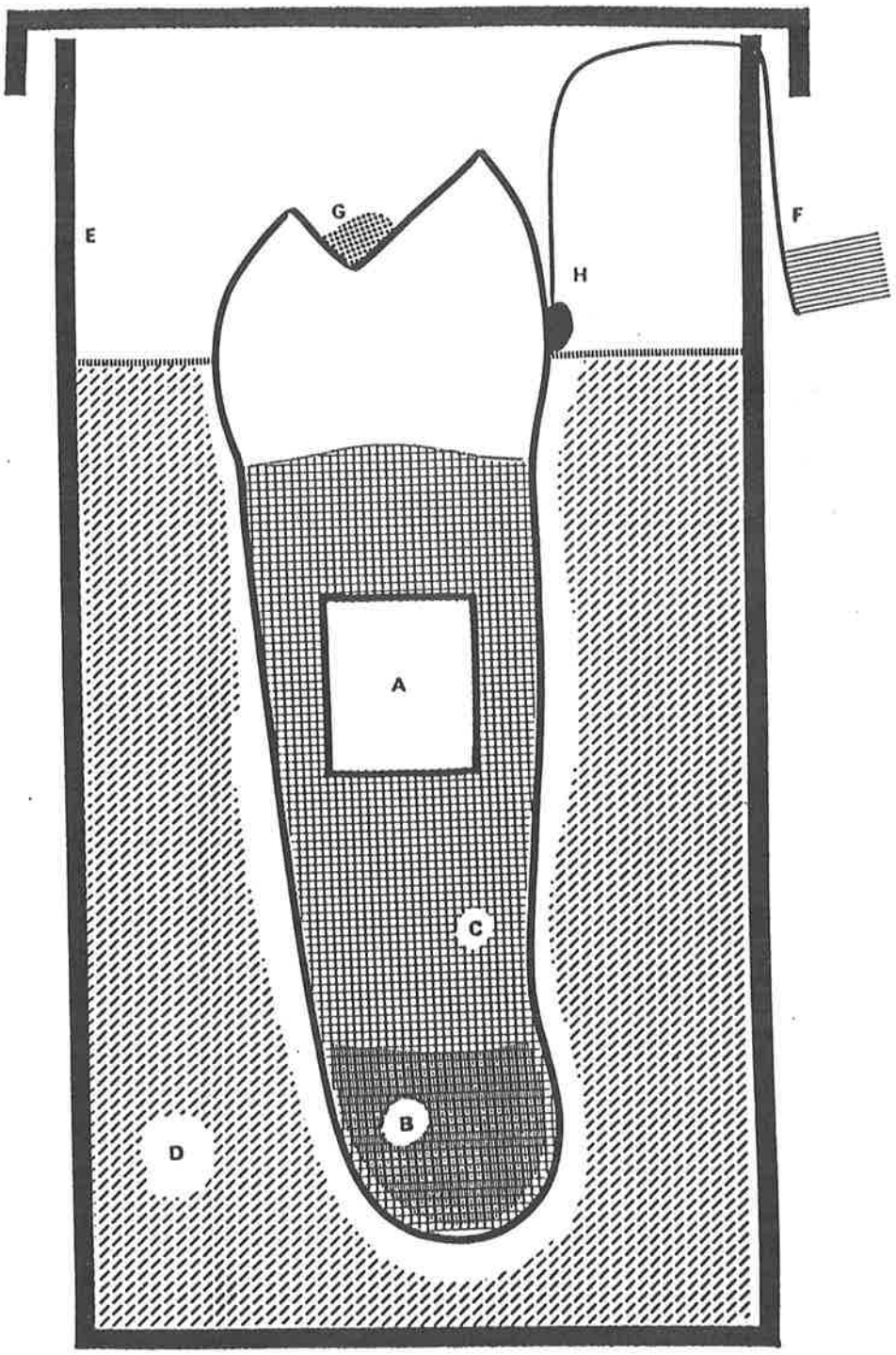

Figure 8. Diagram of tooth-root diffusion model used. **A** = standardized window of exposed dentine or cementum. **B** = apical seal (glass ionomer cement/composite resin). **C** = acrylic paint sealant covering tooth root. **D** = phosphate buffered saline. **E** = scintillation vial. **F** = dental floss to suspend tooth in vial. **G** = blue periphery wax to seal access cavity. **H** = blue periphery wax to stabilize dental floss.

2. RESULTS

The diffusion of [I^{125}]-CT from the root canal to the external dental-root surface is initially delayed but then proceeds for at least nine days (Figure 9, 10a). Once [I^{125}]-CT was detected on the external dental-root surface, its rate of effluxion was initially rapid during the first 10.5 h. This early, rapid rate of [I^{125}]-CT effluxion was followed by a slower, more prolonged effluxion. Given this biphasic mode of effluxion, we have chosen to describe the diffusion of [I^{125}]-CT through dental roots as either short term (0 to 10.5 h) or long term (10.5 h to 9 days). The data are presented as rates of effluxion and cumulative release of [I^{125}]-CT over time.

The results from the first series of experiments investigating the diffusion of [I^{125}]-CT through dental roots over nine days, are represented in Figures 9 and 10a. The subsequent series of experiments focusing on the diffusion of [I^{125}]-CT, and I^{131}, through dental roots during the first 10.5 h, are illustrated in Figures 10b, and 11 to 22.

2.1 Diffusion of [I^{125}]-CT through dental roots: short term

2.1.1 Initial delay period and rates of effluxion

An initial delay was evident prior to the detection of [I^{125}]-CT on the external dental-root surfaces. Following this the rates of effluxion of this hormone from the dental roots were high, when compared with the longer-term values (Figure 10a). The initial appearance of [I^{125}]-CT on the dental-root surfaces of teeth with exposed

dentine windows occurred at 2 h, with a maximal rate of effluxion at 5 h to 6 h. The delay prior to the detection of [I^{125}]-CT on the external dental-root surfaces of teeth with exposed cementum windows was 4 h. The maximal rate of effluxion for this group occurred at 6 h (Figure 10a).

A subsequent series of experiments focusing on short-term diffusion of [I^{125}]-CT, indicated that small cumulative amounts of CT could be detected on the external dental-root surface by 0.5 h in all groups. By 5 h about 20 to 30 percent less cumulative [I^{125}]-CT had diffused through teeth with cementum windows as compared with those teeth with exposed dentine windows (Figure 17). The retarded cumulative amount of label released from intact teeth, as compared with the amount released from teeth with cementum-free windows, was sustained after 10.5 h (Figure 18).

When the the rates of effluxion of [I^{125}]-CT from dental roots in this subsequent series of experiments were compared to the short-term rates of effluxion of [I^{125}]-CT from the dental roots in the initial series of experiments (Figure 10a), at similar time intervals, the biphasic trend in diffusion kinetics (as in the initial experiments) was also observed (Figure 10b). That is, the early, peak rates of [I^{125}]-CT effluxion from the dental roots (which occurred by 2 h to 3 h in teeth with cementum-free windows and 3 h in teeth with intact cementum) were followed by steadily declining rates of effluxion.

2.1.2 Cumulative release

Cumulative release of [I^{125}]-CT from dental roots during the first 10.5 h represented more than 20 (± 5)% of the total amount of hormone released over the nine-day experimental period (Figure 9). Some variability in the cumulative effluxion

of [I^{125}]-CT was observed between individual teeth in each experimental group (Figures 11-16).

Statistical analysis, using a repeated measures analysis of variance (Programme 5V, BMDP Statistical Software Package, UCLA, USA), of the cumulative release of [I^{125}]-CT from dental roots during the first 10.5 h demonstrated that there were no significant differences between the experimental groups ($P < 0.05$).

Leakage of control teeth was low during the first 10.5 h and represented less than 20 (± 5)% of total cumulative effluxion from teeth with cementum-free windows over the total nine day period.

2.2 Diffusion of [I^{125}]-CT through dental roots: longer term

2.2.1 Rates of effluxion

After attaining maximal levels in the rates of effluxion of [I^{125}]-CT through the dental roots at about 5 h to 6 h, the subsequent rates of effluxion steadily declined over nine days (Figure 10a). That is, a prolonged, slow release of [I^{125}]-CT from the dental root was evident.

Between 12 h and 15 h, small discrete plateaus were evident in the rates of effluxion of [I^{125}]-CT from both the teeth with exposed dentine windows and exposed cementum windows (Figure 10a).

2.2.2 Cumulative release

Approximately 80 (± 5)% of the total cumulative release of [I^{125}]-CT from dental roots, occurred after 10.5 h for both the teeth with exposed dentine windows

and exposed cementum windows (Figure 9). The cumulative release of [I^{125}]-CT from teeth with intact cementum was retarded by 30 (± 5)% when compared with the cumulative release from teeth with exposed dentine windows over the nine-day experimental period (Figure 9).

Differences in the cumulative release of [I^{125}]-CT from the teeth in all groups, over the nine-day experimental period, were not statistically significant ($P < 0.05$) (Repeated measures analysis of variance, Programme 5V, BMDP statistical software).

2.3 Diffusion of I^{131} through dental roots

The diffusion kinetics of [I^{131}]-iodide through dental roots were similar for all three experimental groups throughout the experimental period (0 to 31.5 h). A linear relationship was evident between cumulative effluxion of [I^{131}]-iodide and time during the first 10.5 h. Little variability was observed between teeth in each group. After 10.5 h, this cumulative effluxion of [I^{131}]-iodide through the dental root steadily declined until no further label was released at 31.5 h (Figures 19-22).

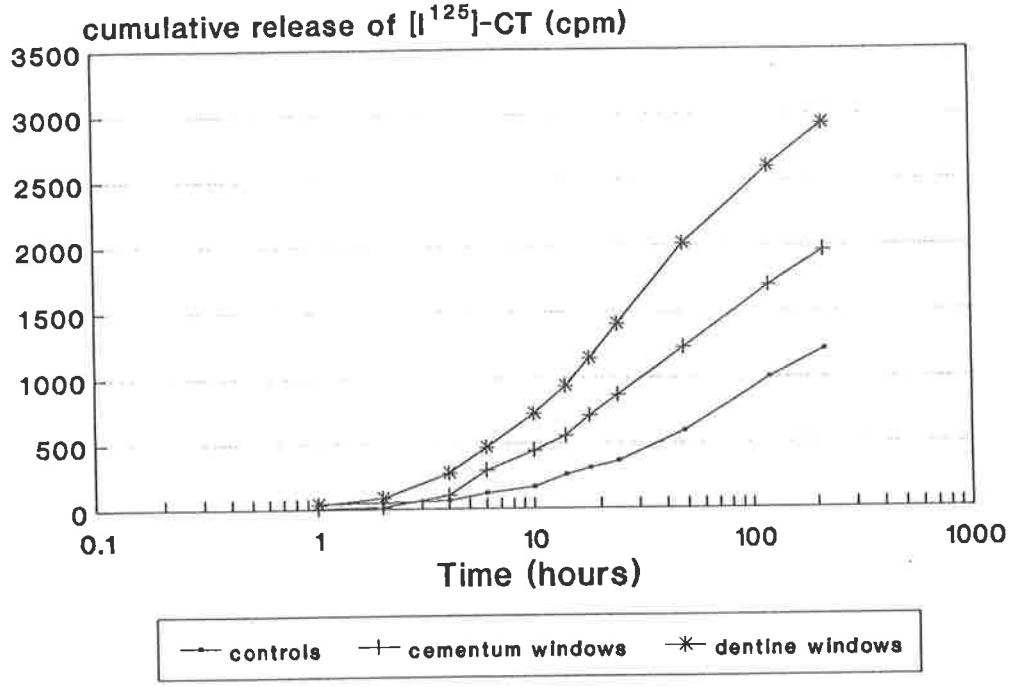

Figure 9. Cumulative effluxion of [I^{125}]-CT through dental roots for up to nine days. Low amounts of label were detected in all three groups during the first 10 h compared with the total released over nine days. After 10 h, intact cementum retarded the diffusion of [I^{125}]-CT from the dental roots.

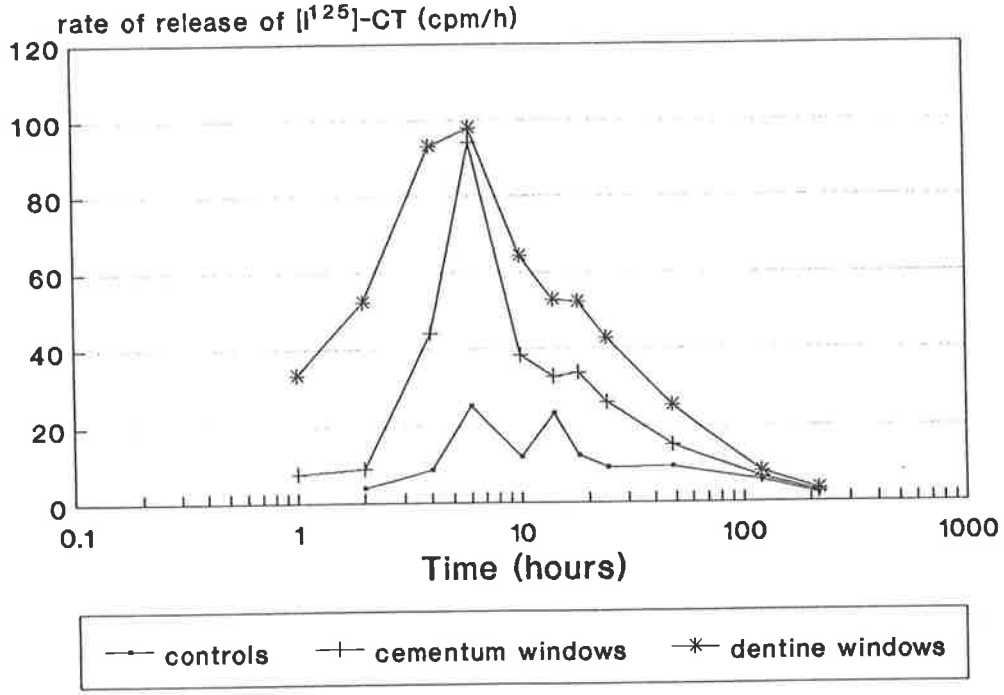

Figure 10a. This graph describes the same data as in Figure 9 but expressed as rates of effluxion of [I^{125}]-CT through dental roots. An initial 2 h delay prior to the detection of [I^{125}]-CT on the external dental-root surfaces is followed by an early, rapid release of label during the first 5 h to 6 h, in teeth with dentine windows. This is followed by a slower, prolonged rate of effluxion over the nine day experimental period. Intact cementum delayed the appearance of label by about 1 h. After the peak rate of release, at 6 h, of [I^{125}]-CT from teeth with intact cementum, the rate of release of label from this group is retarded as compared with teeth with cementum-free windows.

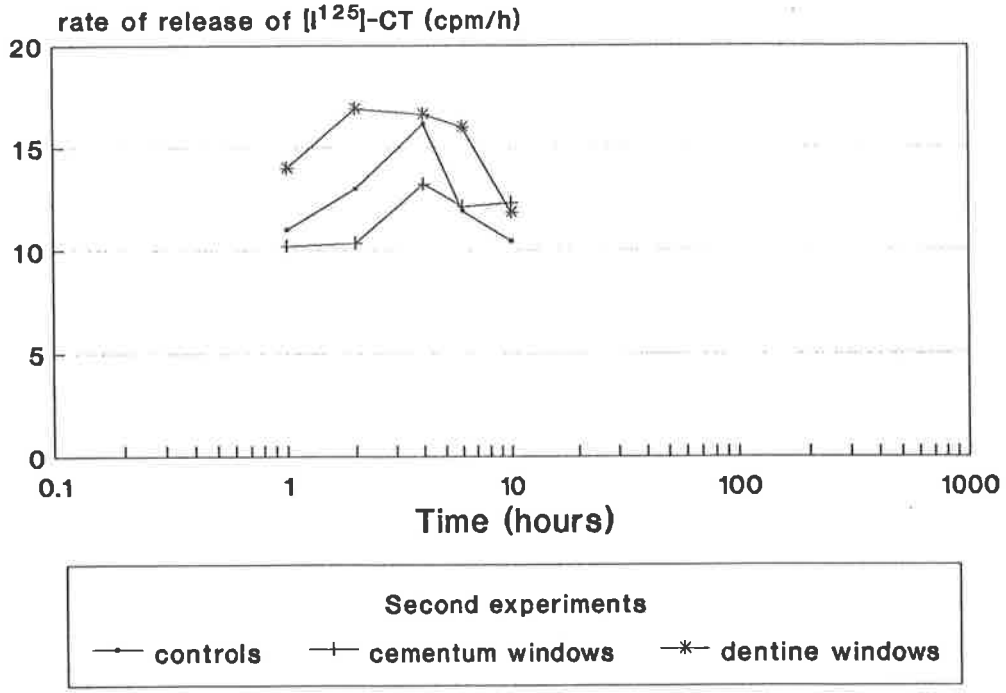

Figure 10b. Mean rates of effluxion of [I^{125}]-CT through dental roots in the subsequent series of experiments when plotted against the same time intervals as in the first experiment during the first 10.5 h.

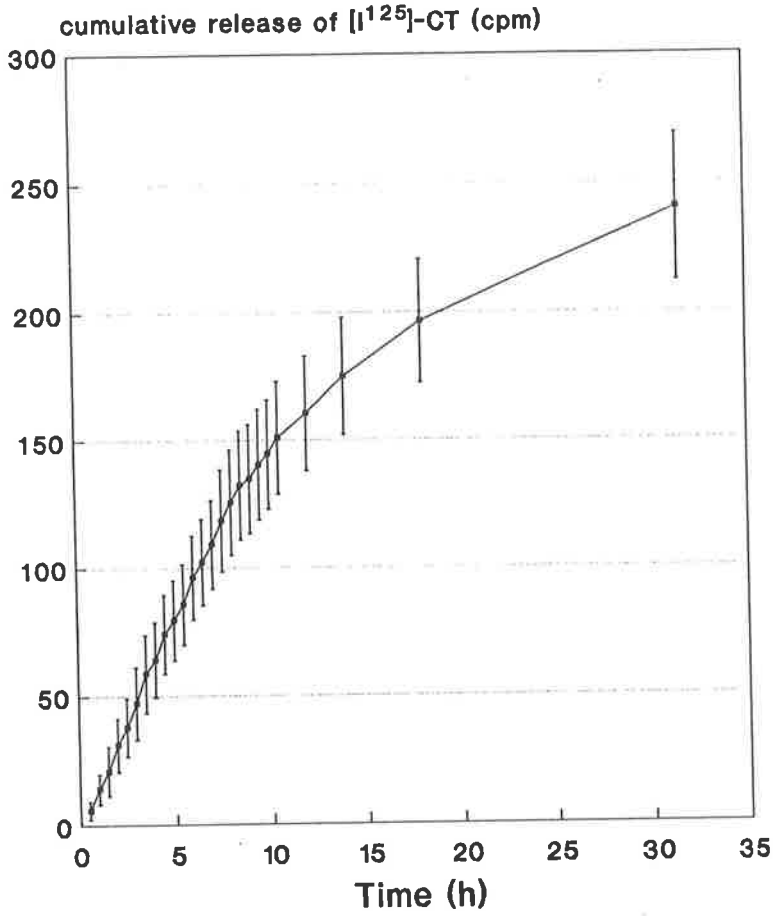

Figure 11. Mean cumulative effluxion of [I^{125}]-CT (± one standard deviation [SD]) through teeth with dentine windows (up to 31.5 h). Plotted as cumulative cpm of [I^{125}]-CT released versus time.

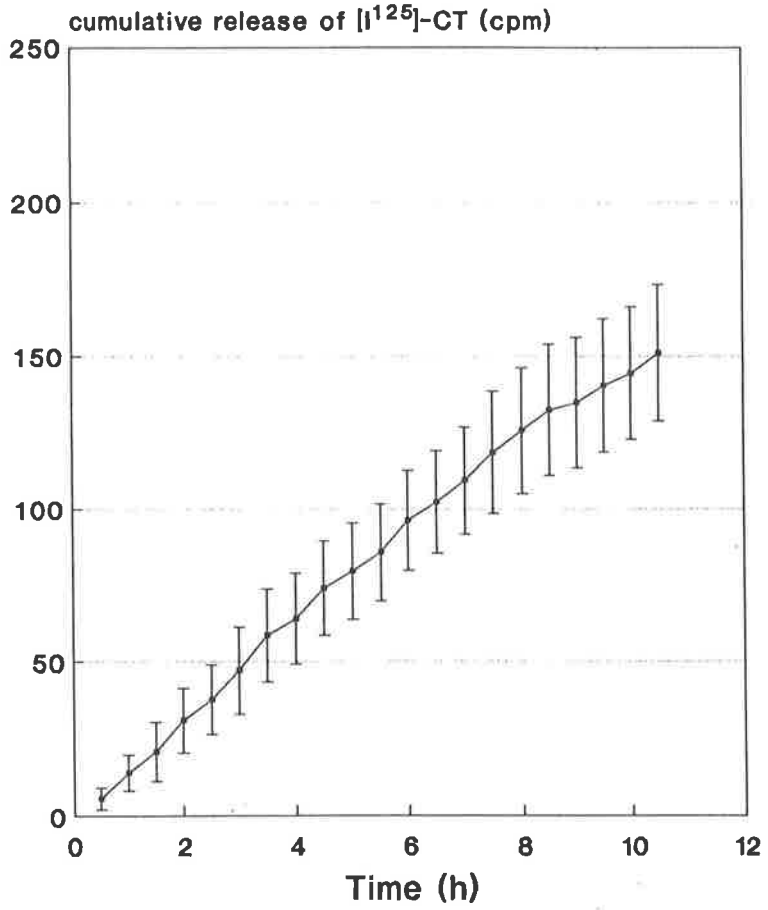

Figure 12. Mean cumulative effluxion (± one SD) of [I^{125}]-CT through teeth with dentine windows (first 10.5 h). Plotted as cumulative cpm of [I^{125}]-CT released versus time.

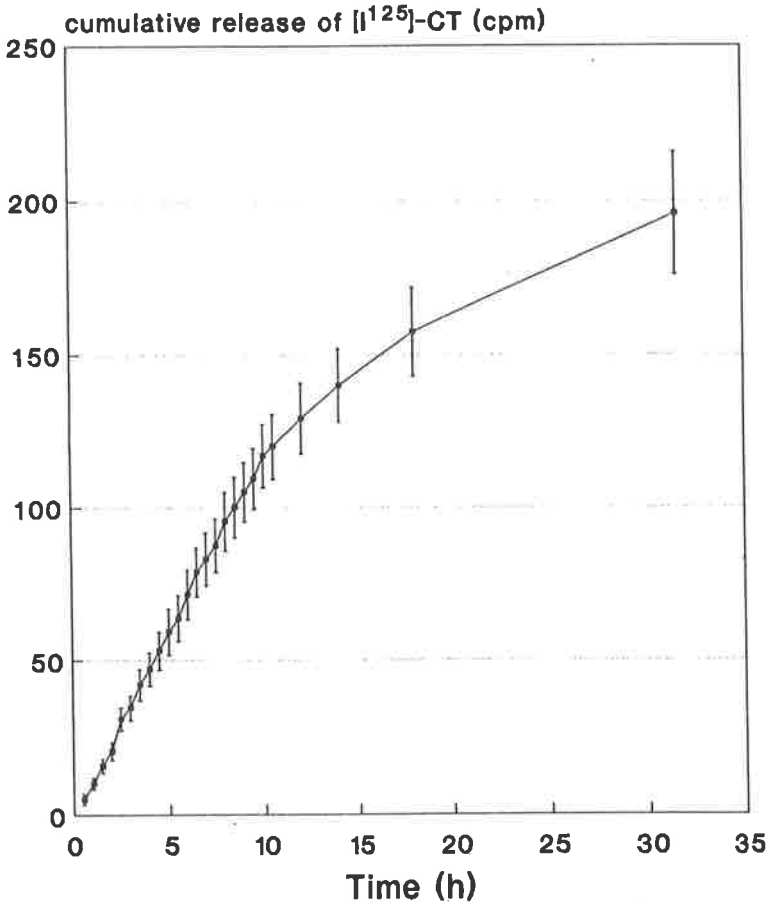

Figure 13. Mean cumulative effluxion (± one SD) of [I^{125}]-CT through teeth with cementum windows (up to 31.5 h). Plotted as cumulative cpm of [I^{125}]-CT released versus time.

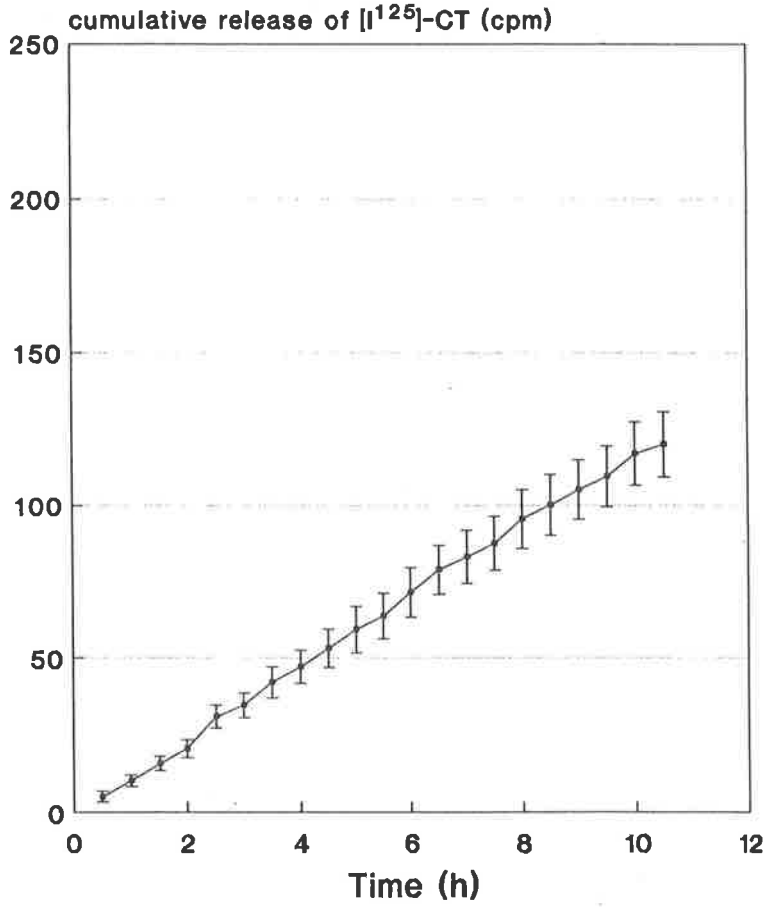

Figure 14. Mean cumulative effluxion of [I^{125}]-CT (± one SD) through teeth with cementum windows (first 10.5 h). Plotted as cumulative cpm of [I^{125}]-CT released versus time.

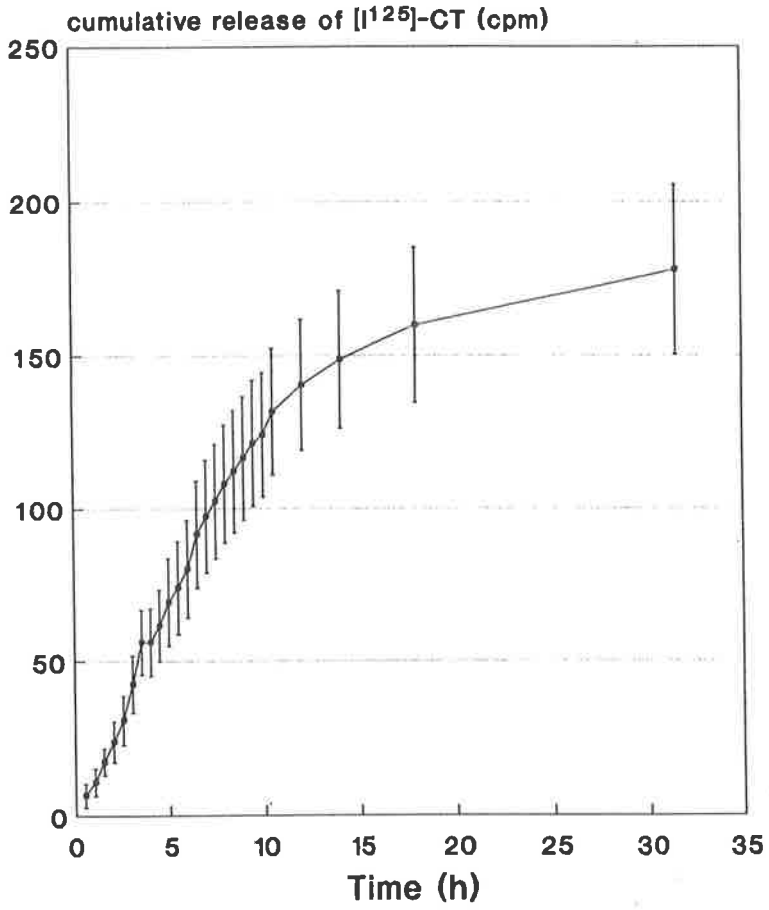

Figure 15. Mean cumulative effluxion of [I^{125}]-CT (± one SD) through control teeth (up to 31.5 h). Plotted as cumulative cpm of [I^{125}]-CT released versus time.

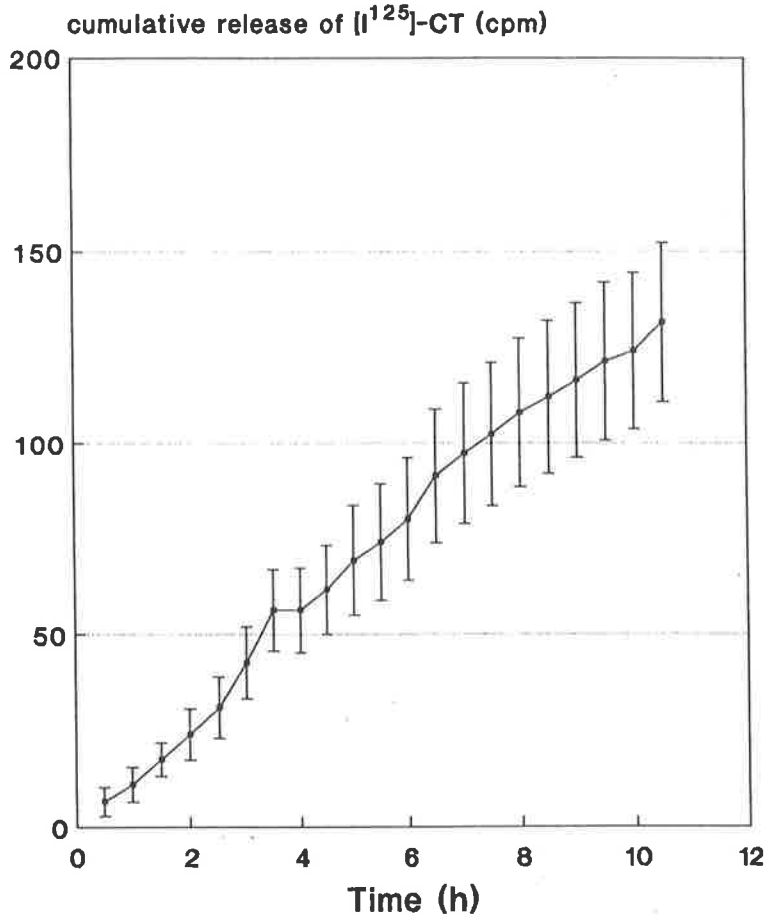

Figure 16. Mean cumulative effluxion of [I^{125}]-CT (± SD) through control teeth (first 10.5 h). Plotted as cumulative cpm of [I^{125}]-CT released versus time.

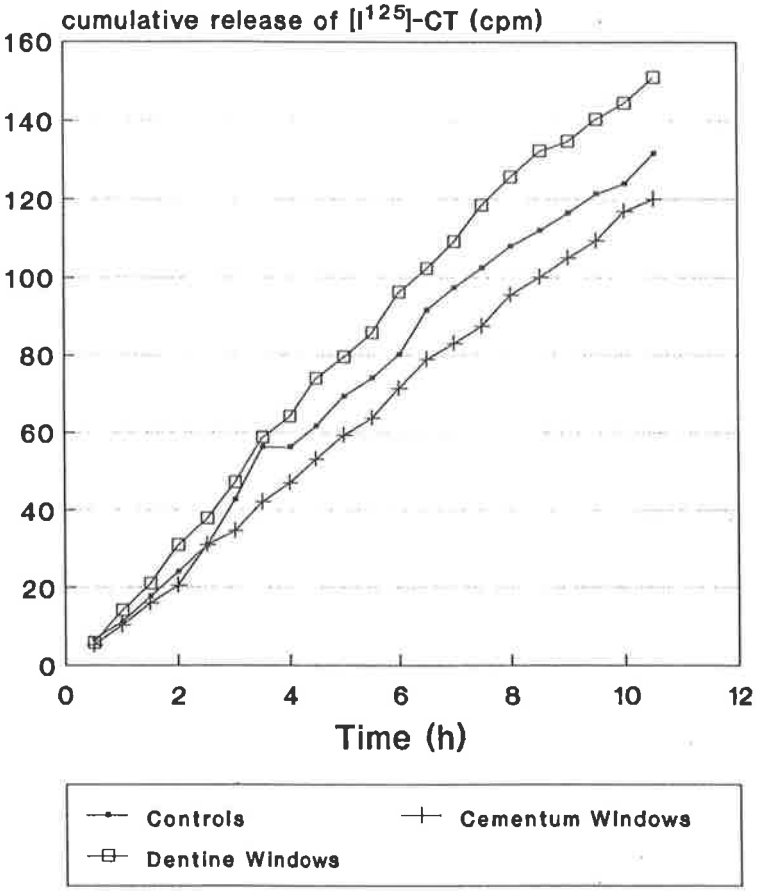

Figure 17. Comparison, between experimental groups, of mean cumulative effluxion of [I^{125}]-CT during the first 10.5 h. No significant differences were detected between the groups ($P < 0.05$). These results compare favourably with those displayed in Figure 9 which show low early cumulative release of [I^{125}]-CT.

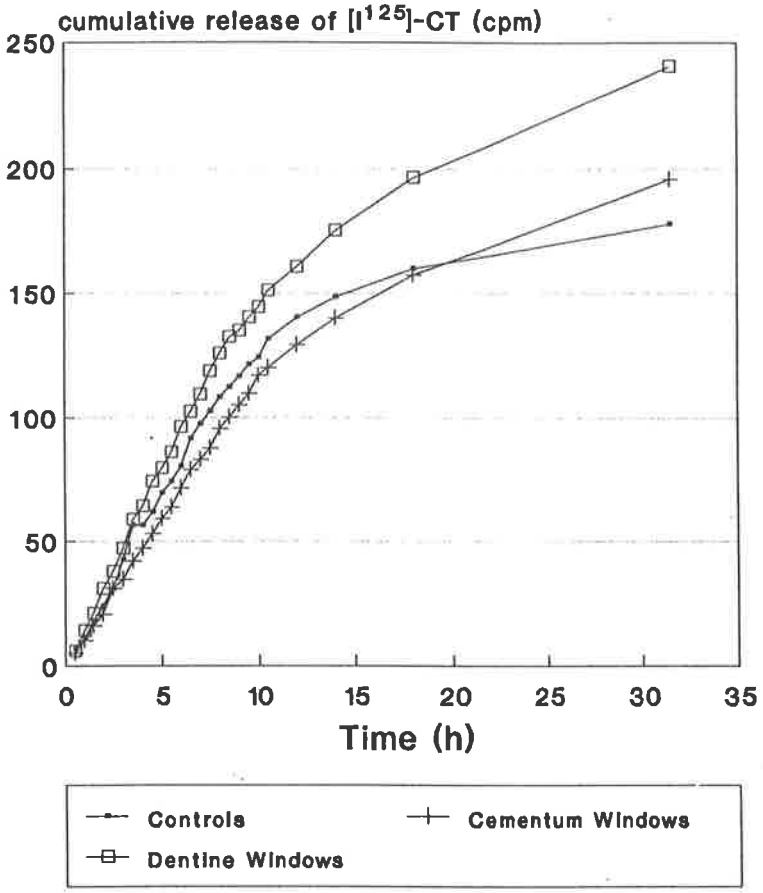

Figure 18. Mean cumulative effluxion of [I^{125}]-CT through dental roots. Comparison between groups during first 31.5 h.

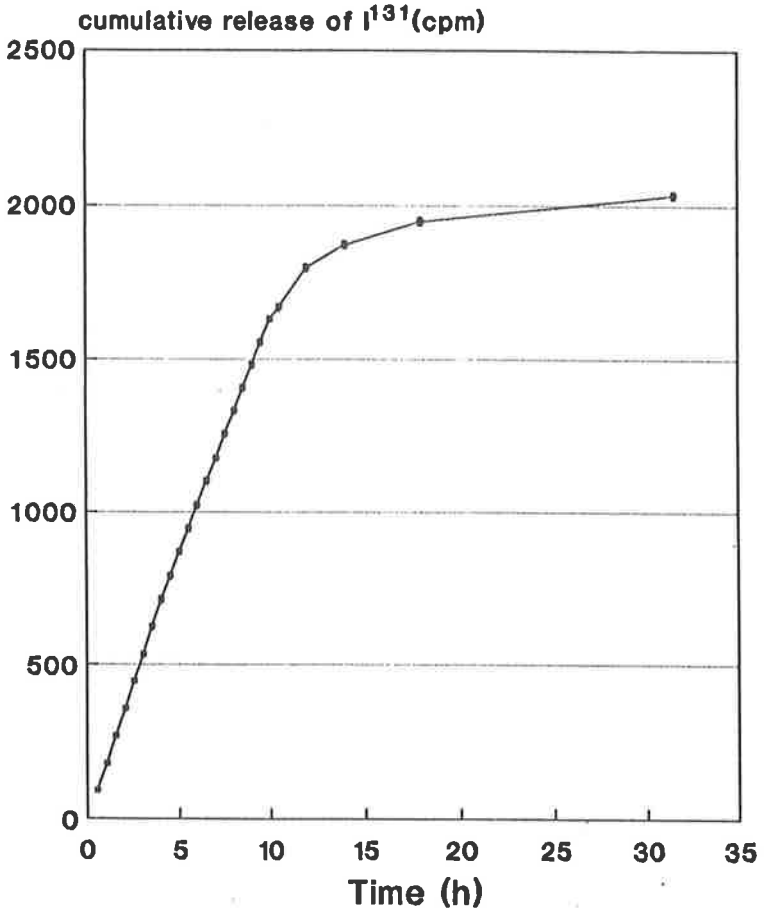

Figure 19. Mean cumulative effluxion of [I^{131}]-iodide (± one SD) through teeth with dentine windows (up to 31.5 h), plotted as cumulative cpm of [I^{131}]-iodide released versus time.

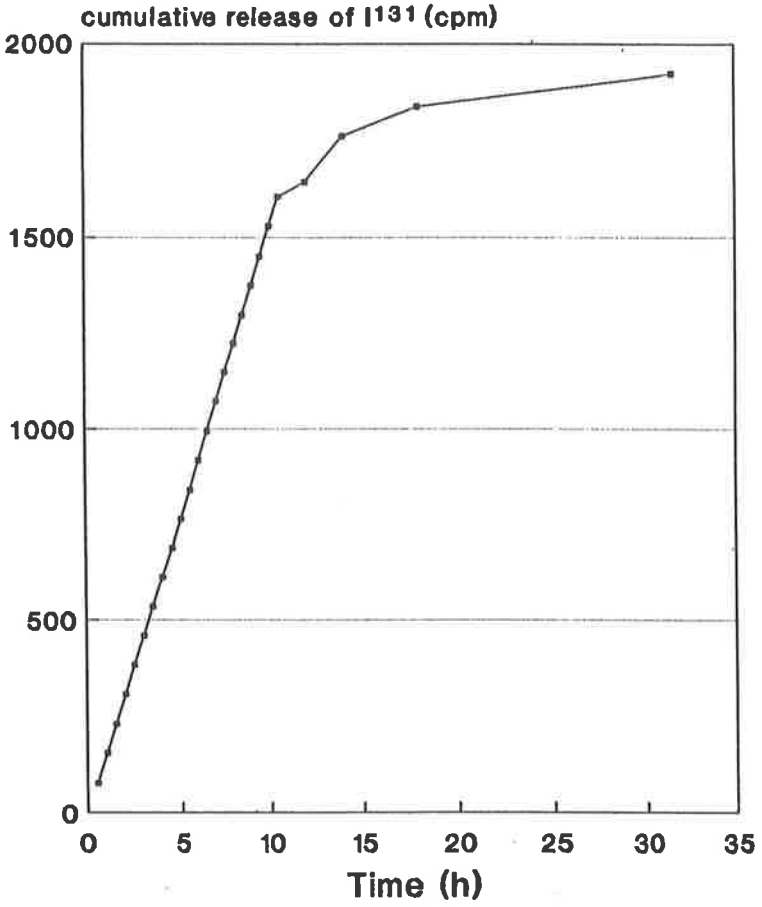

Figure 20. Mean cumulative effluxion of [I^{131}]-iodide (± one SD) through teeth with cementum windows (up to 31.5 h), plotted as cumulative cpm of [I^{131}]-iodide released versus time.

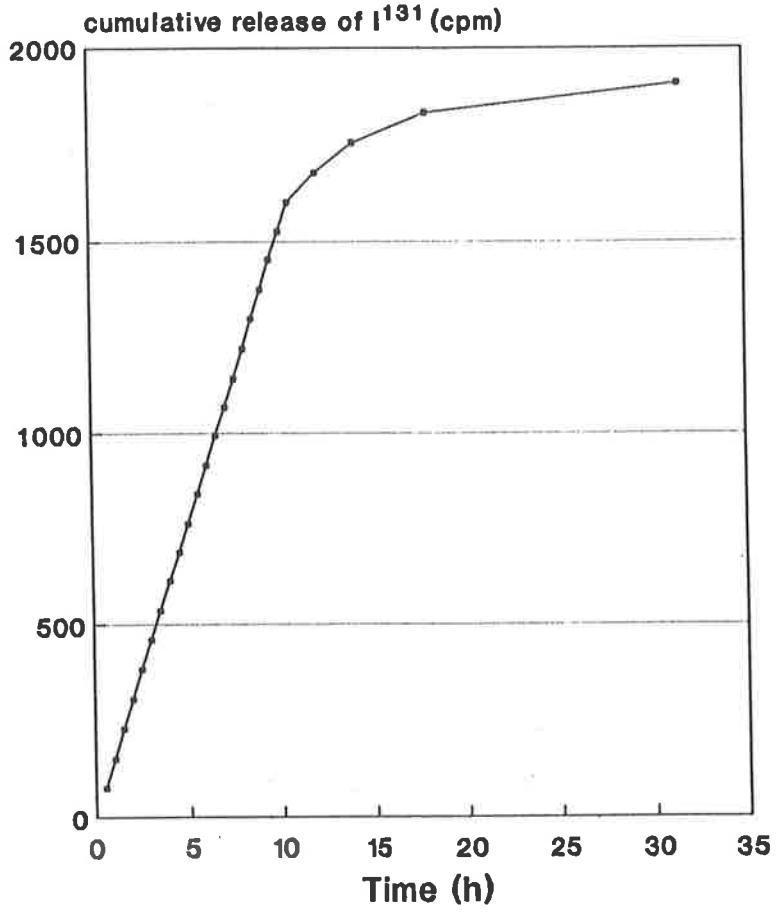

Figure 21. Mean cumulative effluxion of [I^{131}]-iodide (± one SD) through control teeth (up to 31.5 h), plotted as cumulative cpm of [I^{131}]-iodide released versus time.

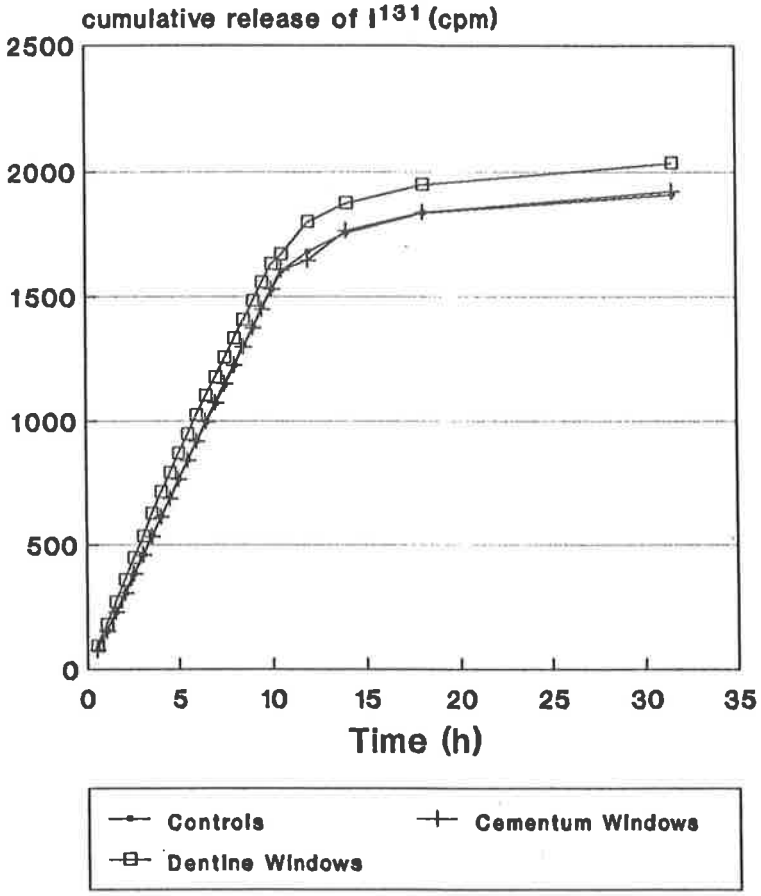

Figure 22. Mean cumulative effluxion of [I^{131}]-iodide through teeth. Comparison between experimental groups.

3. DISCUSSION

The notion that intracanal placement of CT into endodontically-prepared root canals to treat external-inflammatory root resorption has been described (Pierce et al 1988a). For this mode of therapeutic delivery to be successful, CT must: (i) be capable of diffusing through the dental root in order to be available to the tooth-resorbing cells on the external dental-root surface, and (ii) be biologically active at the resorption site. In this study, only the first parameter was investigated. An *in vitro* model which will allow the kinetics of diffusion of CT through endodontically-prepared dental roots to be examined was chosen. By using macerated teeth, the parameters which will affect diffusion were restricted to: (i) that of the physical structure of the dental-root dentine (e.g. cross-sectional area), and (ii) the effect of cementum on CT diffusion. Therefore, the issues examined in this section were:

(i) Is CT is capable of diffusing through dental roots?

(ii) Does diffusion occur over a prolonged period? And,

(iii) Does the presence of cementum delay or inhibit diffusion?

In the experiments, standardized windows were cut in presealed dental-root surfaces. Diffusion of CT through these dental roots, was measured both as a rate of effluxion and cumulative release.

Our investigations showed that CT, when placed topically into root canals as a single "reservoir", can diffuse through the dental root. This diffusion continued to occur for in excess of nine days, albeit with reducing rates of effluxion. This long-term effluxion of CT may prove therapeutically useful for a sustained CT-mediated

suppression of osteoclast activity on external-resorption sites. The maintenance of any biological activity of the CT has not been considered in this study.

Using a radiolabelled CT, cumulative release of [I^{125}]-CT from dental roots was low during the first 10.5 h followed by a steady, prolonged effluxion for up to nine days. This biphasic mode suggested that separate mechanisms may be involved for the diffusion of [I^{125}]-CT through dental roots before and after 10.5 h. Therefore, studies of short (0 - 10.5 h) and long term (10.5 h to nine days) diffusion were chosen.

3.1 Diffusion of [I^{125}]-CT through the dental root: short term.

3.1.1 Initial delay period and rates of effluxion

Following an initial delay of 2 h in the detection of [I^{125}]-CT on the external dental-root surfaces, subsequent rates of effluxion during the first 10.5 h was higher than over the later 9 day period. These higher rates of effluxion were not sustained over these nine days (Figure 10b).

Initially, a 2 h delay prior to the detection of pronounced cumulative [I^{125}]-CT on the external dental-root surface was evident from teeth with exposed dentine windows (Figure 9). The presence of cementum delayed the appearance of [I^{125}]-CT outside the tooth by 2 h (i.e. 4 h total) in each of these experimental groups.

When the data from the subsequent experiments, which focused more specifically on the cumulative effluxion of any [I^{125}]-CT during the first 10.5 h, were expressed as rates of [I^{125}]-CT diffusion and compared with the rates of effluxion of [I^{125}]-CT through the teeth in the first experiment (Figure 10a); at similar time

intervals, the biphasic trend was also observed (Figure 10b). That is, peak rates of effluxion of [I^{125}]-CT from the subsequent experiments occurred by 2 h to 3 h in teeth with cementum-free windows, after which the rates steadily declined. As in the first series of experiments, the presence of cementum delayed the appearance of label on the external dental-root surface by about 1 h.

These subsequent experiments also showed that although low levels of label could be detected at the external dental-root surface by 0.5 h in all experimental groups, by 5 h (Figures 11-16) between about 20 and 30 percent less [I^{125}]-CT had accumulated at the root surface of teeth with intact cementum (Figure 17).
By 10.5 h a slight, but sustained difference between cumulative label outside the cementum-free and intact dental roots could be detected (i.e. approximately 15 to 20 percent of intact teeth). Thus, as in the first experiment, this study demonstrates higher short-term rates of effluxion of [I^{125}]-CT from the dental root could not be sustained.

3.1.2 Cumulative release

The cumulative release of [I^{125}]-CT from dental roots in all experimental groups during the first 10.5 h was low compared with the total released over the nine-day experimental period but it did represent 20 (± 5)% of the total hormone released over the longer period (Figure 9). An early exposure of actively-resorbing cells on the dental-root surface to inhibitory medicaments would be of therapeutic advantage.

A subsequent series of experiments, investigating short-term cumulative release of [I^{125}]-CT, also confirmed that small cumulative amounts did appear by 10.5 h under all conditions, that is, with or without cementum and with or without sealant

(Figures 11-16).

Using all the data for cumulative [I^{125}]-CT release for each group cumulative effluxion of this diffusant could be broadly interpreted as constant. This rate was different from that of free diffusion represented by the marker I^{131}]-iodide. Such analysis denies the biphasic evidence previously discussed but it does emphasize that cementum (and even sealant) will permit the permeation of [I^{125}]-CT to the root surface of a resorbing tooth.

As far as the effect of dental-root sealant was concerned, the results of a pilot study indicated that the acrylic-paint sealant was superior to Visiobond, sticky wax, poly-vinyl acetate glue and nail varnish (see Appendix 1). Any leakage of [I^{125}]-CT from dental-roots completely sealed with acrylic paint represented only 20 (± 5)% when compared with total values of cumulative effluxion of [I^{125}]-CT from experimental teeth with standard windows during the first 10.5 h (Figure 9).

Early (10.5 h), rapid transport of [I^{125}]-CT (one fifth of total nine-day effluxion) was not sustained and it can be construed to be a result of bulk-laminar flow of [I^{125}]-CT, unimpeded by binding, through the lumina of the dentinal tubules.

3.2 Diffusion of [I^{125}]-CT through the dental root: longer term.

3.2.1 Rates of effluxion

Following an initial 5 h to 6 h phase of a relatively rapid rate of effluxion of [I^{125}]-CT from dental roots during the first 10.5 h a slower, prolonged rate of diffusion of label from the dental-root surface (especially through the standard windows) was evident for up to nine days (Figure 10a).

At 12 h to 15 h, discrete plateaus in the diffusion rate of [I^{125}]-CT from the dental root were evident. After this period the rates of diffusion steadily declined (Figure 10a).

The discrete plateaus may represent a phenomenon associated with substantially-sealed teeth. Any bulk flow of [I^{125}]-CT through the sealant may occur once a critical concentration of the hormone beneath the sealant was reached.

The cumulative release of [I^{125}]-CT, by nine days, was retarded (by about 30%) in the experiments where cementum was intact suggesting substantial differences in the rates of diffusion over the longer period (Figure 9).

3.2.2 Cumulative release

More than 80 (± 5)% of [I^{125}]-CT was released from the exposed windows of sealed dental roots between 10.5 h and 9 days. The longer-term data suggest that cementum does contribute to the cumulative release of [I^{125}]-CT release. Furthermore, this retarding effect of cementum becomes more pronounced as time progresses for up to nine days (Figure 9).

Using the linear relationship between the cumulative effluxion of [I^{125}]-CT through the dental roots and time, with the origin at zero, there were no significant differences, over the full nine-day period, in the diffusion of [I^{125}]-CT through the dental roots in all experimental groups ($P < 0.05$). Again, this analysis denies an understanding of the possible mechanisms responsible for the characteristics of [I^{125}]-CT diffusion through dental roots, but it does emphasize the availability of CT following intracanal placement.

3.2.3 Mechanisms of release

Unlike the early (0 to 6 h) rapid, bulk transport through dentinal tubules, the major mechanism affecting diffusion will occur by the following interaction of [I^{125}]-CT molecules with binding sites on the mineralized tubule walls. This binding of [I^{125}]-CT may result in a delayed initial diffusion of the hormone through the dental root. The subsequent chapter focuses on the mechanisms of prolonged appearance of [I^{125}]-CT at the dental-root surface. Thus, the results from this study suggest that a biphasic mechanism exists for [I^{125}]-CT diffusion through the dental root. That is:
(1) an initial, unimpeded (unbound) diffusion of [I^{125}]-CT through the dentinal tubule lumen, followed by;
(2) a delayed transport of [I^{125}]-CT dependent upon binding kinetics.

3.3 Diffusion of I^{131} through dental roots

Iodine^{131}, a tracer molecule known not to bind to dental-root mineral (Pashley et al 1977) was added to the solution containing [I^{125}]-CT. This solution in turn was placed into dental roots in the diffusion experiment focusing on short-term diffusion. This was done to monitor bulk flow of PBS solvent through the dental root. Cumulative effluxion of [I^{131}]-iodide from dental roots was similar for both experimental groups and displayed a linear relationship with time during the first 10.5 h (Figures 19-22). After 10.5 h, the cumulative effluxion of label decreased (Figures 19-22). The reduced rate of effluxion of [I^{131}]-iodide, after 10.5 h, will be as a result depletion of the reservoir of solute in the dental roots.

PART 3: CALCITONIN BINDING TO DENTAL-ROOT MINERAL

A series of studies investigating binding characteristics of CT to ground dental-root powder.

1. MATERIALS AND METHODS

1.1 Binding of [I^{125}]-CT to dental-root mineral

To provide a standard tooth mineral which has been "loaded" with [I^{125}]-CT, macerated, ground dental-root powder was exhaustively exposed to [I^{125}]-CT in PBS.

Intact premolar teeth that were extracted for orthodontic reasons from teenagers and then stored in PBS for up to 3 wks, were used. The crowns of the teeth were removed using a high- speed bur and discarded. The dental roots were macerated; allowed to dry; and then ground into a fine powder using a mortar and pestle.

Five quantities (4.45, 14.49, 21.26, 29.80 and 40.12 mg) of this macerated, ground dental-root powder were each placed in plastic centrifuge tubes (Adelab Scientific, Adelaide, Aust.). An aliquot of 1μCi/ml [I^{125}]-CT (Amersham Int., England) (0.04 ml) was added to each tube and stirred vigorously for 5 to 10 sec on a test-tube shaker. The tubes were stored at 4°C for 24 h and then centrifuged at 13,000 rpm for 5 min. Some supernatant (0.01 ml) was removed from each tube and the radioactivity recorded. The remaining supernatant was discarded. PBS (1 ml) was added to each tube, stirred on a test tube shaker for 5 to 10 sec and the tooth

mineral pelleted by centrifugation. Again, 0.01 ml from each tube was removed and the radioactivity in these 0.01 ml aliqots was measured. Two further PBS washings were performed. The radioactivity in the residual pellets of powder in each tube was then recorded. Such pellets represent the [I^{125}]-CT "loaded" dental-root mineral used in the subsequent study.

1.2 Tightness of binding of [I^{125}]-CT to dental-root mineral

To determine the tightness of binding of [I^{125}]-CT to dental-root mineral, non-radiolabelled CT was allowed to compete for dental-root binding sites already occupied by [I^{125}]-CT.

The five centrifuge tubes containing [I^{125}]-CT loaded dental-root powder from the experiment above (i.e. "[I^{125}]-CT Binding to dental-root mineral") were used. An aliquot (1 ml) of one mg/ml of non-radiolabelled porcine CT (Calcitaire, USV Pharmaceuticals, USA) in PBS buffer was added to each tube and the tubes were then vigorously stirred for 5 to 10 sec on a test-tube shaker. The total radioactivity in each tube was recorded and the the tubes were then stored for 24 h at 4°C. After storage, the tubes were centrifuged at 13,000 rpm for 5 min. The experimental recordings (i.e. time zero) commenced immediately after this centrifugation. Aliquots of supernatant (0.05 ml) were removed from each tube at subsequent 1, 3, 6, 10, 15 and 23 h (i.e. time intervals of +1, +2, +3, +4, +5 and +8 h). The radioactivity in each aliquot was recorded. The residual supernatants in the tubes were then discarded. One ml of PBS was added to the vials after the final 23 h elution; the tubes were then stirred on a test-tube shaker for 5 to 10 sec and centrifuged for 5 min at 13,000 rpm. The supernatants were discarded and the PBS washing repeated. The radioactivity of [I^{125}]-CT in the residual pellets of powder were then recorded

to provide a residual 23 h-bound CT value. The values for displaced [I^{125}]-CT in the supernatants over 23 h were plotted against time interval.

1.3 Competition of bovine serum albumin for calcitonin binding sites in dental-root mineral

To determine whether large proteins can compete with [I^{125}]-CT for binding sites on dental-root mineral, bovine serum albumin (BSA) was allowed to bind to dental-root mineral prior to the introduction of radiolabelled hormone.

Samples of macerated, ground dental-root powder (6.41, 11.87, 33.21, 46.82 and 57.69 mg) were placed in centrifuge tubes. One ml of 1mg/ml BSA in PBS buffer was added to each tube, stirred for 5 sec and incubated at 4°C for 24 h. The tubes were centrifuged at 13,000 rpm for 5 min and the supernatant discarded. An aliquot of 1μCi/ml of [I^{125}]-CT (0.04 ml) was added to each tube, stirred for 5 to 10 sec in a test-tube shaker and incubated at 4°C for 24 h. The tubes were centrifuged (13,000 rpm for 5 min) then 0.01 ml of supernatant was removed and its radioactivity counted. The remaining supernatant was discarded. One ml of PBS was then added to the tubes and stirred. The tubes were again centrifuged at 13,000 rpm for 5 min. Aliquots of supernatant (0.01 ml) were removed from each tube and their radioactivity recorded. Two further PBS washings were performed. The radioactivity of residual pellets of powder in the tubes were recorded. On the basis of dental-root mineral weight, modified Scatchard plots were prepared.

1.4 Exchange of calcitonin molecules on binding sites

In order to further confirm any tightness of binding of CT to dental-root mineral, the displacement or exchange of non-radiolabelled CT by radioactive CT was determined.

Ground, dental-root powder samples (6.30, 12.30, 25.64, 42.75 and 60.60 mg) were pretreated with 1mg/ml non-radiolabelled porcine CT in PBS buffer at 4°C for 24 h. The rest of the experiment proceeded as for the preceding experiment (i.e."Competition of Bovine Serum Albumin for CT binding sites in dental-root Mineral"). Thus, displaced CT was represented by the binding of [I^{125}]-CT to various amounts of tooth mineral. The varying weights of tooth mineral corresponded to varying numbers of binding sites since, based on the initial "loading" experiment, the tooth mineral was exhaustively exposed to excess CT.

2. RESULTS

2.1 Binding of [I^{125}]-CT to dental-root mineral

Using 0.04 ml of 1μCi/ml [I^{125}]-CT, increased weights of powder bound increasing amounts of I^{125}-CT. This held true for up to approximately 40 mg of powder. In spite of exhaustive washing in PBS, the unbound ligand was substantially removed with the first wash leaving less than 10% in the two subsequent washes. The curvilinear nature of the CT to tooth weight plot (Figure 23) indicates that saturation of binding sites on dental-root mineral is exhausted and if reversible is likely to be a time-limiting event. However, the data provided a straight line on a modified Scatchard plot with the X intercept at -230 and Y intercept at -5 (Figure 24) which indicates a simple binding. The k value for the data was calculated to be -2.17 x 10^{-2}. The data from this experiment are displayed in Table IV.

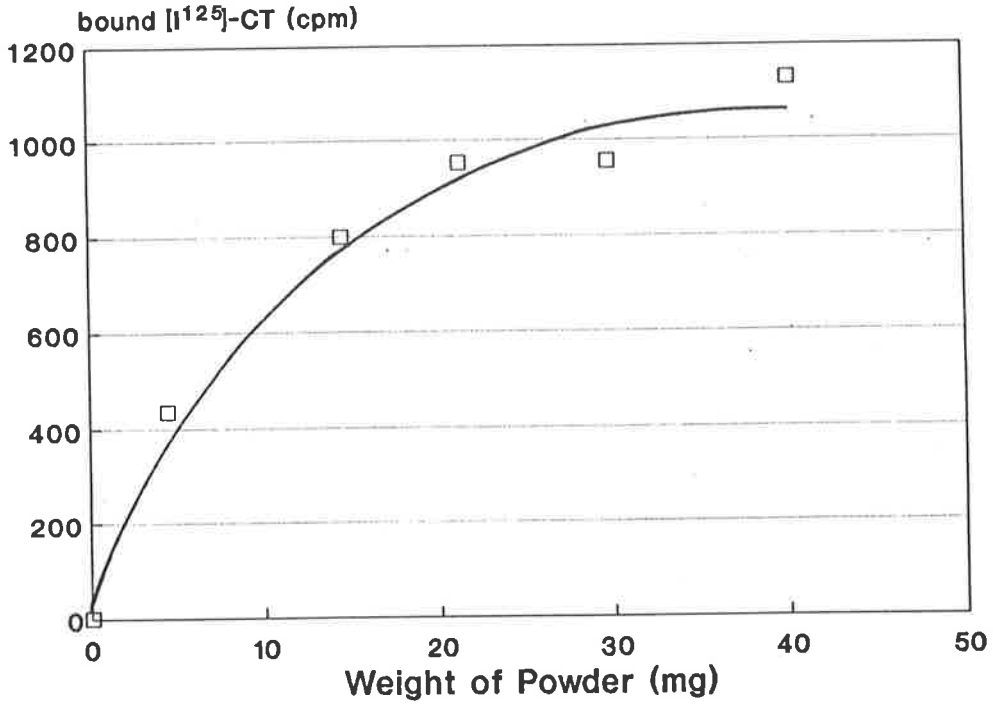

Figure 23. Binding of [I^{125}]-CT to various weights of dental-root powder.

TABLE IV. Binding of [I^{125}]-CT to Dental-Root Mineral

TEST-TUBE	1	2	3	4	5
Weight of Powder (mg)	4.45	14.49	21.26	29.80	40.12
cpm of I125CPM-CT in initial 0.01ml	2542	2488	1974	1916	1558
WASH 1	198	247	332	277	457
WASH 2	6	20	21	31	55
WASH 3	9	16	8	7	13
Residual cpm in powder	437	799	952	955	1128
cpm I125powder/ I125 in solution (x0.01)	4.30	8.02	12.06	12.46	18.11

Note: 1) cpm of I^{125}-CT in initial 0.01ml - is the cpm of [I^{125}]-CT in the 0.01 ml of supernatant removed from the centrifuge tubes after 24 h. **2) Wash 1-3** - is the cpm of unbound [I^{125}]-CT in each of the three PBS washings. **3) cpm I^{125} powder/I^{125} in solution (x0.01)** - is the ratio of bound with unbound [I^{125}]-CT expressed as 10^{-2}. This calculation is used to prepare Scatchard plots.

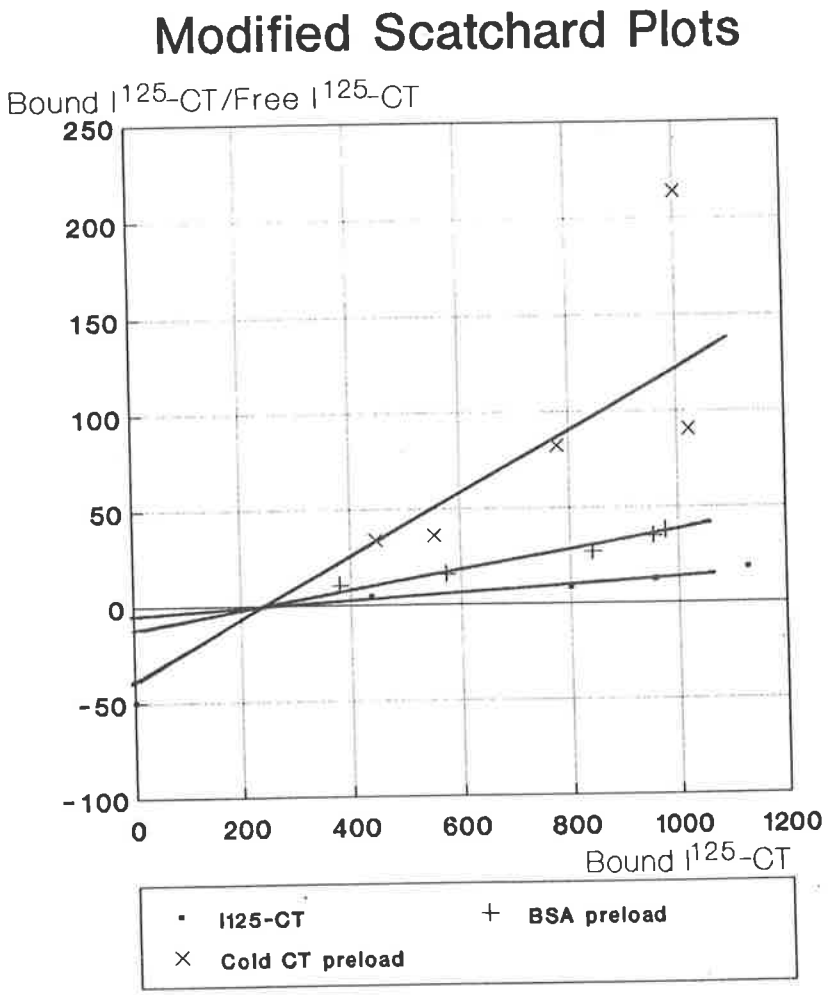

Figure 24. Modified Scatchard plots for dental-root powders: (1) exposed to [I^{125}]-CT ([I^{125}]-CT); (2) preloaded with Bovine Serum Albumin for 24 h prior to introducing [I^{125}]-CT (BSA preload); and, (3) preloaded with non-radiolabelled CT for 24 h prior to introducing [I^{125}]-CT ("Cold" CT Preload).

2.2 Tightness of binding of [I^{125}]-CT to dental-root mineral

Dental-root powders preloaded with [I^{125}]-CT for 24 h continued to release CT into CT-free media over a 23 h period as the time interval increased from 1 h to 8 h (Figure 25). However, the proportion of radiolabel thus released over this period was only a small fraction (< 10%) of the total [I^{125}]-CT originally bound. A constant value of less than 10% of the total [I^{125}]-CT originally bound was released for each of the time intervals in all but the 40.12 mg sample. The amounts released from this largest sample showed greatest decline between 15 and 23 h. This represented an unsaturated dental-root powder (as determined in the previous "Binding of [I^{125}]-CT to dental-root mineral" experiment).

The rate of [I^{125}]-CT released from the dental-root powders into media decreased over 23 h (Figure 26). At the conclusion of the experiment, approximately 45 to 50% of the total originally-bound [I^{125}]-CT remained with the dental-root powder.

The data from this experiment are displayed in Table V.

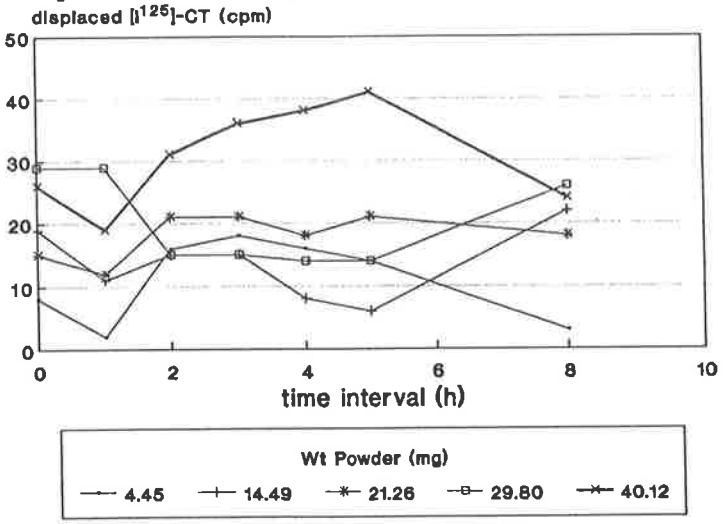

Figure 25. Total amounts of [I^{125}]-CT displaced from different weights of dental-root powder by non-radiolabelled CT over different time intervals.

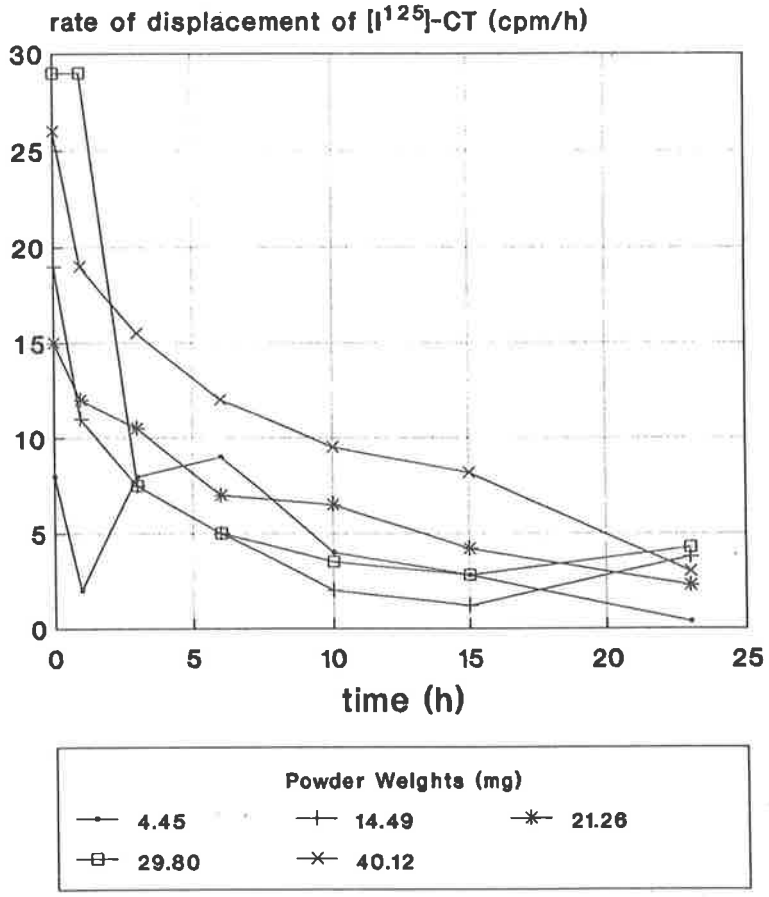

Figure 26. Rates of [I^{125}]-CT displaced from different weights of dental-root powder by non-radiolabelled CT over 23 h.

TABLE V. Tightness of Binding of [I^{125}]-CT to Dental-Root Mineral

Powder Weight (mg)	4.45	14.49	21.26	29.80	40.12
Total cpm of System	396	762	857	863	1058
Time interval (h)					
0	8	19	15	29	26
+1	2	11	12	29	19
+2	16	15	21	15	31
+3	18	15	21	15	36
+4	16	8	18	14	38
+5	14	6	21	14	41
+8	3	22	18	26	24
Residual cpm in Powder	210	377	412	387	506
Residual cpm in solution	115	226	302	292	307
% I125-CT bound	53.00	49.47	48.07	44.84	47.82

Note: 1) Total cpm of system - is the total cpm of [I^{125}]-CT in each centrifuge vial at the beginning of the experiment. 2) The cpm of [I^{125}]-CT in the supernatants are displayed at different time intervals. The time between each subsequent time window is expressed as a time interval (i.e. +1 is 1 hr, +2 is 3 h, +3 is 6 h, +4 is 10 h, +5 is 15 h, and +8 is 23 h). 3) **Residual cpm in powder** - is the cpm of bound [I^{125}]-CT remaining after 24 h exposure to unlabelled CT. 4) **Residual cpm in solution** - is the remaining cpm of [I^{125}]-CT in the residual supernatant at the end of the experimental period. 5) **%I^{125}-CT bound** - is the percentage of bound [I^{125}]-CT remaining at the end of the experimental period as compared to the initial amount of bound radiolabelled hormone.

2.3 Competition for calcitonin binding sites by BSA and unlabelled CT

In contrast to the previously described experiments, various weights of dental-root mineral were "preloaded" with BSA and the subsequent competition for exchange with [I^{125}]-CT was measured. Using this principle of competition, another similar experiment focused on the exchange of bound, radiolabelled CT with non-radiolabelled CT. Both experiments demonstrated a reduced uptake of [I^{125}]-CT per weight of powder, into the preloaded powder systems (Figure 27). BSA reduced uptake by 78% to 87% for weights of dental-root powder between 6.41 mg and 57.69 mg (Figure 28). Similarly, over a saturation range of 6.3 mg to 60.64 mg, non-radiolabelled CT reduced uptake by 75% to 91% (Figure 29).

The use of a more sensitive analysis, the modified Scatchard plot, resulted in straight lines with the X intercept at 230 and Y intercept at -10 for BSA preloading. The corresponding values for the powder system preloaded with unlabelled CT were 230 and -35 respectively (Figure 24). The k values for these two experiments were -4.34 x 10^{-2} for BSA preloading and -15.21 x 10^{-2} for unlabelled CT preloading. The data from the experiments demonstating competition for CT binding sites by BSA and unlabelled CT are displayed in Tables VI and VII respectively.

TABLE VI. Competition for CT Binding Sites by BSA

TEST-TUBE	1	2	3	4	5
Weight of Powder (mg)	6.41	11.87	33.21	46.82	57.69
cpm I125-CT (/0.01 ml)	907	907	778	683	646
WASH 1	252	207	330	306	365
WASH 2	17	5	31	34	31
WASH 3	0	12	14	4	13
Residual cpm in powder	379	573	840	953	975
I125 powder/ I125 solution (x0.01)	10.45	15.79	26.99	34.79	37.73

Note: 1) cpm I^{125}-CT (/0.01 ml) - is the cpm of unbound [I^{125}]-CT in the initial 0.01 ml of supernatant removed after 24 h. **2) WASH 1-3** - the cpm of unbound [I^{125}]-CT in each of the three PBS washings. **3) I^{125} powder/I^{125} solution (x0.01)** -is the ratio of the cpm of bound [I^{125}]-CT to unbound hormone multiplied by 10^{-2}. This calculation is used to prepare modified Scatchard plots.

TABLE VII. Competition for CT Binding Sites by Unlabelled CT

TEST-TUBE	1	2	3	4	5
Weight of Powder (mg)	6.30	12.30	25.64	42.75	60.64
cpm I125-CT (/0.01 ml)	347	385	237	117	282
WASH 1	111	115	163	185	202
WASH 2	11	15	11	27	36
WASH 3	0	4	10	0	6
Residual cpm of Powder	446	552	778	1000	1021
I125 powder/ I125 solution (x0.01)	33.57	35.84	82.07	213.67	90.51

Note: 1) cpm I^{125}-CT (/0.01 ml) - is the cpm of unbound [I^{125}]-CT in the initial 0.01 ml of supernatant removed after 24 h. **2) WASH 1-3** - the cpm of unbound [I^{125}]-CT in each of the three PBS washings. **3) I^{125} powder/I^{125} solution (x0.01)** -is the ratio of the cpm of bound [I^{125}]-CT to unbound hormone multiplied by 10^{-2}. This calculation is used to prepare modified Scatchard plots.

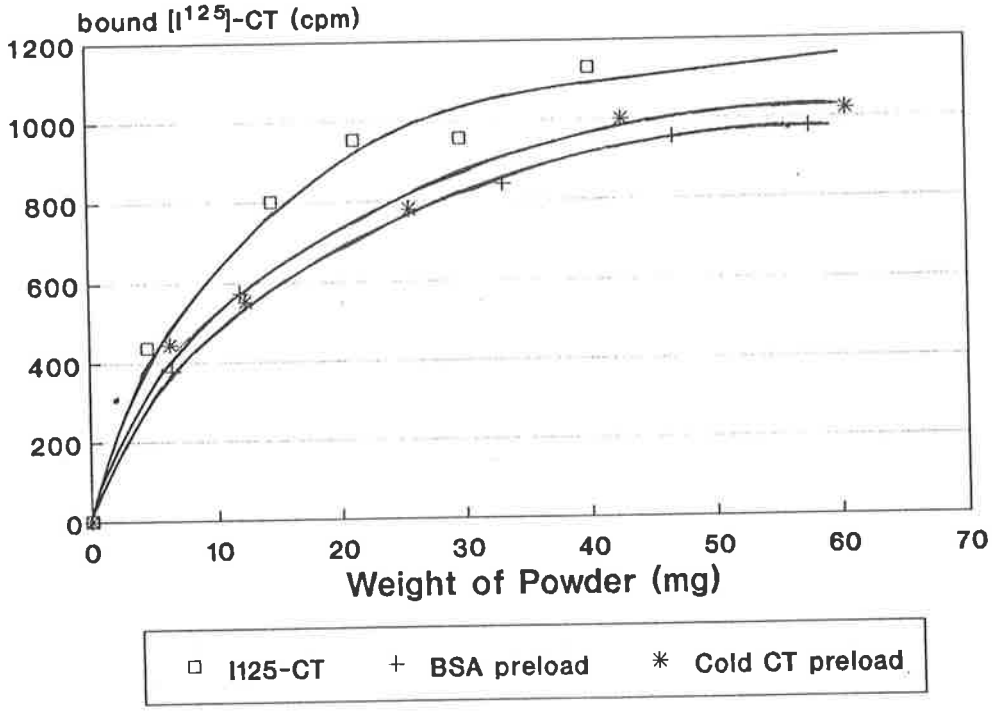

Figure 27. Amount of [I^{125}]-CT bound to different weights of dental-root powder after: (1) 24 h exposure to the hormone ([I^{125}]-CT); (2) 24 h exposure of the radiolabelled hormone to dental-root powders preloaded with BSA for 24 h (BSA preload); and (3) 24 h exposure of the radiolabelled hormone to dental-root powders preloaded with non-radiolabelled CT for 24 h ("Cold" CT preload).

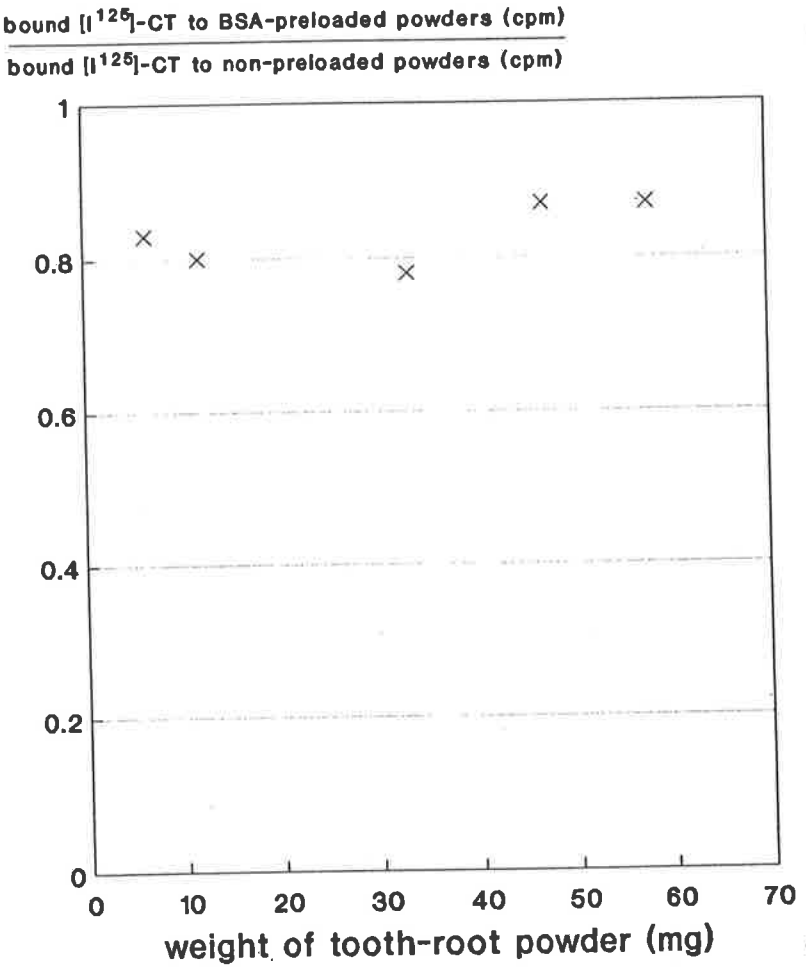

Figure 28. Ratio of the amount of [I^{125}]-CT bound to different weights of BSA preloaded dental-root powder after 24 h, to the amount of radiolabelled hormone bound to similar weights of non-preloaded powders.

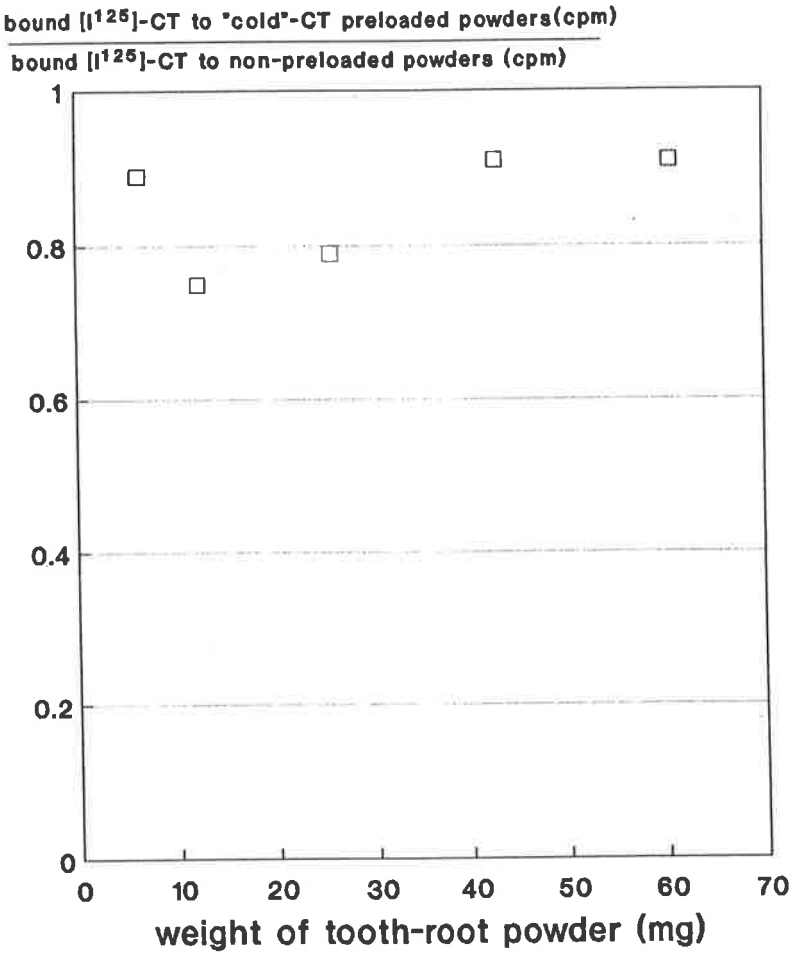

Figure 29. Ratio of the amount of [I^{125}]-CT bound to different weights of non-radiolabelled CT preloaded dental-root powder after 24 h, to the amount of radiolabelled hormone bound to similar weights of non-preloaded powders.

3. DISCUSSION

Part 2 demonstrated that CT diffusion through the dental root is retarded when compared with the diffusion of I^{131}. One of the mechanisms responsible for these diffusion kinetics is likely to be the interaction, or binding, of CT diffusant with the exposed mineral in the dental root. To quantify and control for the many variables in tubule structure (see Part 1 on SEM) powdered, macerated dental roots were chosen. Residual collagen in the dental-root matrix which has been subjected to sodium hypochlorite, will be further exposed during mechanical powdering of dental roots. The exposure of this denatured collagen may be a complicating factor in identifying specific binding sites for CT. Nevertheless, it does have parallels in the traumatized teeth which have been treated by endodontic chemo-mechanical debridement and intracanal CT to arrest root resorption.

The use of a standard pool of dental-root powder averted discrepancies in surface area of powder between each experiment. That is, the number of available binding sites per weight of powder was kept constant.

3.1 Binding of [I^{125}]-CT to dental-root mineral

Binding of CT to dental-root powder was proportional to the weight of powder (Figure 23). A likely explanation for the observed curvilinear relationship is that, at high weights of powder (increased binding sites), availability of the finite amount of CT for location on binding sites is a limiting factor. In addition, the binding of CT to dental-root mineral is likely to be a time-dependent event. That is, as CT binds to dental-root mineral, more time is required for the residual ligand to interact with the diminishing number of free CT binding sites.

In spite of the earlier allusions towards a multiple binding relationship between CT and dental-root mineral, the modified Scatchard plot (see section 3.3 later) gives a straight-line relationship and therefore indicates simple, or non-cooperative, binding overall.

3.2 Tightness of binding of [I^{125}]-CT to dental-root mineral

For CT, or any other therapeutic substance, to be valuable therapeutically in treating external-inflammatory root resorption when placed into root canals, there needs to be a replenishable source of active hormone to resorption sites for a prolonged period. A tight, but reversible, binding of the diffusant to the dental-root mineral would provide a valuable slow-release mechanism for the sustained suppression of dentinoclasts at the resorption sites. If a diffusant that can reversibly bind to the dental root is placed topically into a root canal, then this diffusant may be exchanged or "shuffled along" the course of a dentinal tubule to the external-root surface by similar molecules from the inner side of the dentinal tubule.

The tightness of binding of [I^{125}]-CT binding to dental-root mineral was evaluated in the present study by allowing non-radiolabelled CT to compete for the dental-root binding sites "preloaded" with [I^{125}]-CT. The rate of exchange as measured by the appearance of [I^{125}]-CT into media was used as an indicator of tightness of binding. The amount of [I^{125}]-CT release from saturated dental-root mineral will initially be dependent upon the tightness of binding. For example, low [I^{125}]-CT release implies tight binding. As time progresses, an equilibrium is reached between bound and unbound CT. That is, in the present studies, non-radiolabelled CT and displaced [I^{125}]-CT will compete for the same binding sites, now occupied by either [I^{125}]-CT or non-radiolabelled CT. In the current study, this equilibrium was

expected to be reached by 24 h exposure of radiolabelled powders to non-radiolabelled CT. The subsequent removal of aliquots of media would not affect this equilibrium as the ratio of [I^{125}]-CT to non-radiolabelled CT in the media would not be altered. However, the total radioactivity, per unit time, detected in each subsequent time interval decreased (Figure 26).

After introduction of non-radiolabelled CT to unsaturated powders, no displacement of [I^{125}]-CT would be expected initially. This is due to binding of non-radiolabelled CT to unoccupied binding sites. When the binding sites become saturated, [I^{125}]-CT will be displaced by non-radiolabelled CT. After 24 h exposure of unsaturated, [I^{125}]-CT preloaded powders to non-radiolabelled CT, an equilibrium is also reached in the centrifuge vials. That is, free exchange between radioactive and non-radioactive CT on the binding sites with like molecules in the media. The equilibria in saturated and unsaturated powders, however, are different owing to the different ratios of [I^{125}]-CT to non-radiolabelled CT in the vials. The data collected in this experiment indicated that only a low amount of [I^{125}]-CT was displaced from dental-root mineral during each time interval. Indeed, after the 23 h experimental period there was still approximately 50% of [I^{125}]-CT still bound to the dental-root powders. This indicated that CT will bind tightly to macerated dental-root mineral but with a capacity for slow exchange. In the dentinal tubule, where a reservoir of CT in the canal, and a consumption or dispersion at the exterior root surface drives the gradients, the "shuffle effect" can thus be achieved.

3.3 Competition for CT binding sites by BSA and unlabelled CT

The tightness of binding can not only be expressed by competition of like molecules, but also as a generalized competition of unrelated molecules, such as BSA, for binding sites.

Radiolabelled CT on preloaded dental-root mineral will be displaced by non-radiolabelled CT within the weight ranges of 4.45 mg to 40.12 mg. It has already determined that dental-root powder will bind [I^{125}]-CT by 24 h at levels which are proportional to weight. In this competition experiment where similar levels of non-radiolabelled CT were bound, complete replacement with [I^{125}]-CT, by simple exchange, was not achieved after 23 h. Only between 75% and 91% replacement occurred over a saturation range of 6.3 mg to 60.63 mg (Figure 29). This indicates that replacement, or exchange, of bound CT in a dentinal tubule would permit a journey from one receptor to another, with final effluxion from the outer ends of the tubules occurring by consumption or dispersion. This journey was referred to earlier as a "shuffling along" effect.

Since hydroxyapatite is a standard support material for protein purification, the role of such mineralized phosphate in dentine may be reasonably considered. Generalized protein binding and its consequences for competition with CT was therefore investigated.

Competition for [I^{125}]-CT binding sites by BSA resulted in similar displacement of [I^{125}]-CT as did non-radiolabelled CT (78% to 87%) after 23 h (Figure 28). However, modified Scatchard plots for both non-radiolabelled CT and BSA was a more sensitive indicator of competition.

The affinity of binding sites for ligand can be discussed in terms of the Scatchard plot. This plot is used to examine the interaction between enzymes and ligands. The Scatchard equation can be described as:

$$C_{BL}/ C_L = K.(C^\circ_B - C_{BL})$$

C_L = the molar concentration of free ligand

C_{BL} = the molar concentration of ligand-occupied binding sites

C°_B = the total site concentration

K = the binding constant for the ligand

On plotting C_{BL}/C_L against C_{BL}, and if a straight line is obtained, the implication is that K is constant and the type of binding is of the simple non-reactive type with identical binding sites. The intercept on the C_{BL}/C_L axis gives KC°_B, the product of the binding constant and the number of sites. The intercept at the C_{BL} axis gives C°_B, the number of binding sites.

The experimental models of protein binding to dental-root mineral, do exhibit a number of similarities to enzyme-ligand interactions. These include, a ligand or substrate ([I^{125}]-CT), a number of binding sites (dental-root mineral), and other molecules which can compete for the same binding sites (non-radiolabelled CT and BSA). Therefore, the use the Scatchard plot to examine the binding characteristics of [I^{125}]-CT, and competition from unlabelled CT and BSA was deemed to be relevant for interpretation of our data. Because this equation has been used to describe an as yet unidentified receptor-mediated binding of CT to dental-root

powder, this formula has been referred to as a "modified " Scatchard plot.

Modified Scatchard plots for non-radiolabelled CT and BSA shared a common X intercept with the [I^{125}]-CT plot (Figure 24). This indicated that the binding sites for radiolabelled CT, non-radiolabelled CT and BSA are common.

The K values for the non-radiolabelled CT and BSA powders were -15.21 x 10^{-2} and -4.34 x 10^{-2} respectively. This indicated that binding of [I^{125}]-CT to dental-root mineral was reduced 600% by non-radiolabelled CT and 100% by BSA.

The data from these final two experiments indicate that: (i) the CT-binding sites in the dental root are not specific. That is, other molecules are able to compete with CT for the binding sites, and (ii) there is a low rate of exchange of CT on binding sites. This further strengthens the argument that there is a tight, but reversible, binding of CT to the dental root.

GENERAL DISCUSSION

CT, an experimental therapeutic agent to treat external inflammatory root resorption, may be delivered to resorption sites via diffusion through dentinal tubules, in a similar manner as that shown by Abbott (1985) for the active components of Ledermix paste.

The mechanisms for diffusion of polypeptides such as CT are unknown.

Amongst the features responsible for controlling the mechanisms of diffusion are: (i) the physical constraints of the tooth, (ii) the dentinal tubule contents, (iii) the diffusion characteristics of CT in dentine, and (iv) the interaction of CT with tooth-root mineral, that is, binding.

(i) The physical constraints of the tooth

In order to investigate the first two parameters, a SEM study was performed. Observations of tubule dimensions in the current study included a larger number of tubules adjacent to the pulpal surfaces compared with smaller, less abundant tubules adjacent to the external root surface. In the pulpal third of the dental roots the range was 1.8μm to 2.5μm. In the middle third the range was 0.8μm to 1.8μm and in the outer third the range was 0.8μm to 1.2μm. In the pulpal third of the dental roots there were 35 000 per mm^2 to 45 000 tubules per mm^2 compared with 23 000 per mm^2 to 27 000 per mm^2 in the middle one third and 19 000 per mm^2 to 22 000 per mm^2 in the outer one third. These observations are similar to those reported by Garberoglio and Brannström (1976).

The removal of cementum to expose windows of dentinal tubules, by a dental high-speed bur, produced a smear layer (see Appendix 2). This amorphous layer of crystalline

and organic debris can restrict diffusion (Pashley et al 1978b). This debris was removed by a topical 10 minute application of 15 percent EDTAC (Figure 30). After removal of cementum and smear layer the contribution of dentinal tubule dimensions and mineral surface to the diffusion of CT can be examined. Parallels may be drawn between teeth with cementum-free windows in the current experiments and the resorbing dental root *in vivo* as active resorption sites are usually devoid of cementum. In addition to restricting molecular diffusion, cementum can also impede the passage of bacteria from the dentinal tubules to the PDM (Andreasen 1980a) thus preventing external inflammatory root resorption (Lindskog et al 1985). This protective mechanism is lost when cementum is removed either by trauma or resorption.

(ii) Dentinal tubule contents

Prior to the placement of intracanal anti-resorption agents, the root canals must be chemo-mechanically debrided. Mechanical filing with an irrigation regime of a combination of 1 percent sodium hypochlorite and 15 percent EDTAC has been shown to be effective in removing loose organic contents and smear layer from root canal walls (Goldman et al 1981). However, studies on the efficacy of such a debridement regime throughout the length of dentinal tubules are lacking.

The current SEM study demonstrated the presence of loose organic contents in the dentinal tubules after endodontic preparation of teeth stored in either PBS or DMEM. However, the aim of the present study was to determine the contribution of mineral alone. Therefore the contribution of dentinal tubule contents to the diffusion of CT through dental roots requires future investigation. The current study, in part, has determined the method of *in vitro* storage of teeth for such an investigation. Maceration of endodontically-prepared teeth provided a tooth structure in which tubule dimensions,

exposed mineral and cementum were the only factors affecting diffusion.

(iii) The diffuson characteristics of calcitonin in dentine

The diffusion of molecules through dentinal tubules is dependent on the diameter of the tubules, length of tubules, the surface area of the tooth root and the thickness of cementum. Further factors that influenced the diffusion characteristics of CT through the dental root have been described. They include: the surface area available for diffusion; the time available for diffusion; the permeability constant, concentration, molecular weight and ionic charge of CT.

Although investigators have reported that components of Ledermix paste (Abbott 1985) and tetracycline (Ciarlone et al 1989) show a diffusion gradient as they diffuse through the dental root, it must be remembered that these gradients rely on the concentration at the source and consumption at the other end. The current study did not address these intratubular issues. Moreover, gross movement of solute and solvent through these tubules will be affected by their cross-sectional diameter and contents.

Figure 8 is representative of the tooth diffusion model used. This model is suitable to investigate the contribution of tubule dimensions, exposed mineral and cementum on the passage of therapeutic agents, such as CT, through the dental root.

The diffusion characteristics of CT through macerated, endodontically-prepared dental roots include: (i) an initial period of delay prior to detection of the hormone on the external surfaces followed by an early rapid rate of CT release during the first 10.5 hs, and (ii) diffusion of the hormone for in excess of nine days. The diffusion of CT once it had reached the external dental-root surface was retarded by the presence of cementum and sealant over the nine day experimental period. This observation concurred with the report of Abbott (1985) that described cementum as a significant, but not impermeable,

barrier to molecular diffusion. External inflammatory resorption sites are usually devoid of cementum (Andreasen & Hjörting-Hansen 1966b) thus promoting the early availability of the hormone to resorption sites following placement into root canals. The initial delay period is probably a result of the intratubular passage of CT through the dentinal tubules and, in some cases, through cementum also. Following this initial delay, the early rapid rate of effluxion of CT from dental roots may be attributed to the unimpeded passage of CT through dentinal-tubule lumina. During this first 10.5 h period, approximately 20 percent of the total CT reservoir has reached the external dental-root surface. The following period of slow, sustained release (10.5 h to 9 days) may be a result of molecular binding of CT to the dental-root surfaces. Therefore, a biphasic mechanism of release may be present. The prolonged delivery of CT to resorption sites on the external dental-root surface may be therapeutically useful for the treatment of external inflammatory root resorption.

The diffusion of demeclocycline from Ledermix paste placed in root canals to external dental-root surfaces occurs at steadily declining rates over a period of 14 wks (Abbott 1985). During the first nine days of Abbott's investigation, a biphasic diffusion response was not evident. This could be due to a more rapid binding of these molecules with dental-root surfaces enhanced by the paste delivery system of Ledermix paste.

The local concentration required for inhibition of osteoclast activity is not known and thus CT was used in this series of experiments to examine the kinetics of diffusion of such a polypeptide hormone through dental roots. In addition, the biological activity of the radiolabelled CT was not investigated. Such parameters require future investigation.

The ability of CT to traverse the cementum layer suggests the hormone will be available to osteoclasts at sites of surface resorption not yet totally denuded of cementum.

Thus the early placement of CT into root canals may play a preventive role in sites of early external inflammatory root resorption.

The rate of release of CT from dental roots may be modified by incorporating the hormone into some form of slow release delivery vehicle such as that described by Tronstad et al (1985) or by incorporating the hormone into some form of paste system. These delivery systems have not been considered in the current study.

(iv) The interaction of calcitonin with dental-root mineral

The interaction of CT with dental-root material (i.e. binding) will influence diffusion of the polypeptide through the dental root. Given the heterogenous nature of the macerated dentinal tubule surface, there are likely to be different levels of binding of the polypeptide to the individual components of dental-root material. Bernardi and Kawasaki (1968) suggested that the binding of CT and other polypeptides to hydroxyapatite may take place due to the interaction of the polypeptide carboxyl groups and the calcium ions of the hydroxyapatite crystals. The present study considered the overall binding of CT to dental-root material in general rather than specific binding levels.

Binding of CT to dental-root mineral will depend on its molecular weight, ionic charge, and avidity and affinity for binding sites. The binding of CT to tooth-root material is analogous to enzyme-ligand interactions. Therefore enzyme-ligand kinetic formulae such as the Scatchard plot can be used to describe the binding of CT to tooth-binding sites. The current experiments investigated the interaction of CT to macerated and powdered dental roots. These experiments indicated that CT did bind to dental roots and that the binding is saturable, reversible, and non-specific. The diffusion of CT through the dental root could therefore be modified by the ability of this polypeptide to

bind to the dentinal-tubule surface. In addition, the presence of other polypeptides that are able to compete for binding sites may further modify diffusion.

The concept of binding of medicaments to tooth-root mineral acting as a slow release mechanism of delivery has been considered by Abbott (1985) and Ciarlone et al (1988). Abbott (1985) investigated the diffusion of demeclocycline, a tetracycline drug, through endodontically prepared dental roots, with or without cementum and with or without an apical seal over a 14 wk period. The author attributed the slow release partly to reversible binding of demeclocycline to dental-root mineral. The author suggested that the placement of demeclocycline into endodontically-prepared root canals could provide a sustained therapeutic action to treat external inflammatory root resorption.

Ciarlone et al (1988) suggested that binding of tetracycline to tooth-root surfaces could be used to treat periodontal disease. The authors found that tetracycline binding to dental-root mineral was reversible, saturable and concentration dependent. They also stated that agents that bind to dental-root surfaces too tightly may not be therapeutically valuable as they will not be available to the sites of pathology. The current study demonstrated that CT does bind to dental-root mineral but can be displaced by like molecules and dissimilar proteins such as bovine serum albumin. Therefore a shuffling effect of CT from the root canal to the external dental-root surface along binding sites is a possible explanation for the slow release of CT from dental roots.

When total consumption of CT at the external root surfaces has occurred, the residual CT bound within dentinal tubules may serve as a protective mechanism against resorption by clastic cells. However, it was not the intention of this study to investigate this intratubular parameter.

In summary, the binding of CT to dental-root mineral is of a simple non-interactive nature despite the heterogeneous nature of the dentinal tubule surfaces. The binding is

also saturable, reversible, and non-specific. The diffusion of CT through the dental root is modified by the ability of this polypeptide to bind to the dentinal tubule surface. In addition, the presence of other polypeptides that are able to compete for binding sites may further modify diffusion.

Directions for further research

In the current experiments, the effects of dentinal-tubule contents on CT diffusion through dental roots was not considered. Therefore, the diffusion of CT through dental roots containing such contents requires investigation.

The concentration of CT required for an inhibitory effect on osteoclasts is not known. Further investigations are therefore required to (i) determine the biological activity of CT once it has diffused through dental roots, and (ii) establish therapeutic guidelines for the correct dose of CT required, to be placed into root canals, to treat external-inflammatory root resorption.

CONCLUSIONS

Calcitonin, a polypeptide hormone, has been shown to exhibit delayed diffusion through endodontically-prepared dental roots. Since calcitonin is a potent inhibitor of external-inflammatory root resorption, this route of delivery to external resorption sites may prove therapeutically valuable in treating this condition. Furthermore, protein binding to tooth-root mineral is a characteristic which may be exploited to enhance the slow-release therapeutic delivery of such agents to resorption sites.

APPENDIX 1. Microleakage evaluation of sealing materials

AIM:

The aim of this pilot study was to find a material which, when applied to both dry and wet dental-root surfaces, with or without the presence of free organic material, would remain impervious to the penetration of small to medium sized polypeptides even after immersion in PBS for at least 14 days.

METHOD:

The study was divided into two parts to investigate both wet and dry surface conditions.

(1) Macerated teeth (dry dental-root surface devoid of loose organic material)

7 single-rooted teeth were endodontically prepared, macerated, apically sealed and coated from cervical enamel to apex with test sealant materials. The method is described below.

a) endodontic preparation: A regular endodontic access cavity was prepared using a high speed handpiece and a tapered fissure bur with water spray. The pulp was removed with a barbed broach (Nervadelin CC-cord, Vereinigte Dentalwerke, KG) and the canal filed to a size 25 file (Kerr standard, Kerr manufacturing Co., Romulus, Mich., USA). A 1% sodium hypochlorite solution (Milton Pharmaceutical Co., Villawood, NSW) was used for irrigation during instrumentation, and a class I cavity was cut at the apical ends of all teeth using the same bur.

b) maceration: The teeth were boiled in water for 1 h in a microwave oven after which they were placed in an ultrasonic bath for 30 min in a 4% sodium hypochlorite solution (White King Antiseptic Bleach Cleaner, Kiwi Australia, Chadstone, Vic.). The teeth were then allowed to dry naturally overnight.

c) apical seal: The apical class I cavities were filled with a glass ionomer/composite resin sandwich technique according to the following method (McLean et al, 1985): The surface was cleaned with a 20 sec application of a 25% polyacrylic acid conditioner (Ketac Conditioner, ESPE, Fabrik Pharmazeutischer Praparate GMBH & Co.KG, Seefeld/Oberbay, W. Germany), followed by a 60 sec wash with water. Cavities were air dried with compressed air, and Ketac Fil (ESPE, Fabrik Pharmazeutischer Praparate GMBH & Co.KG, Seefeld/Oberbay, W. Germany) was placed within the cavities and allowed to dry for at least 30 min. The surface was recleansed with Ketac Conditioner for 10 sec, washed for 60 sec and air dried. Visiobond (ESPE, Fabrik Pharmazeutischer Praparate GMBH & Co.KG, Seefeld/Oberbay, W. Germany) was applied and light cured for 10 sec, this was followed by the placement of Visiodispers (ESPE, Fabrik Pharmazeutischer Praparate GMBH & Co.KG, Seefeld/Oberbay, W. Germany) over the glass ionomer cement and light cured for 60 sec.

d) coating with test materials: The teeth were divided into three groups each of two teeth and a fourth group of one tooth (that is, a total of 7 teeth).

Group 1 Sticky wax: The entire root surface was covered with two layers of yellow sticky wax (ASH Model Cement, England).

Group 2 Visiobond: The root surfaces were preconditioned with Ketac Conditoner for 10 sec, washed for 60 sec, air dried, and coated with 3

layers of Visiobond. Each layer was light cured for at least 20 sec on each surface of the root.

Group 3 Acrylic Paint: Dental floss was ligated above the neck of the teeth to serve as a mechanism for immersing them in a white water-based acrylic paint (Dulux Weathershield exterior gloss acrylic [white], Dulux Australia, Clayton, Vic.). The first layer was allowed to dry for at least 2 h according to manufacturer's instuctions, and the final coat was left to dry overnight.

Group 4 Poly-vinyl acetate glue ("Solver" Aquatect, W.P. Crowhurst PTY.LTD., Adelaide, Aust.): Two coats were applied to the root surfaces, one 10 min after the other.

{Note: The use of nail varnish as a dental-root sealant in a pilot study proved totally inadequate in preventing the permeation of [I^{125}]-CT and therefore was not included in this experiment.}

(2) Teeth stored in DMEM (wet dental-root surface with organic material present)

Six freshly extracted teeth were stored in Dulbecco's modification of Eagle's medium (DMEM), with antibiotics, for 24 h at 4°C. The teeth were endodontically prepared and apically sealed in a similar manner to the macerated teeth. During this process, the teeth were kept moist by holding them with gauze soaked in the storage medium. The roots of the teeth were carefully blotted dry with tissue paper to remove excess moisture so as to still maintain a wet surface. Each of four teeth was coated with two coats of acrylic paint. The second coat was placed after 2 h for two of the teeth and 24 h for the second. The fifth tooth was coated with yellow

sticky wax and the sixth with 3 layers of Visiobond. A summary of the sealants used is presented in Table VIII.

Table VIII. SUMMARY OF SEALANTS TESTED

Tooth	Macerated	DMEM Storage	Sealant
1	yes	no	Visiobond
2	yes	no	Visiobond
3	yes	no	Sticky wax
4	yes	no	Sticky wax
5	yes	no	Crowhurst
6	yes	no	Dulux paint
7	yes	no	Dulux paint
8	no	yes	Visiobond
9	no	yes	Sticky wax
10	no	yes	paint (2 h)
11	no	yes	paint (2 h)
12	no	yes	paint (24 h)
13	no	yes	paint (24 h)

All 13 teeth (both macerated and non-macerated) were supported by a layer of red wax around their crowns and suspended in 2.5 ml phoshate buffered saline (PBS) in plastic vials). The wax also prevented any possible evaporation of media. The root canals of the teeth were thoroughly washed with PBS from a syringe. Toluidine Blue dye (1%), prefiltered of any solid impurities was syringed into the root canals. The dye was replenished every second day. After 14 days, the PBS in each vial was analysed using a spectrophotometer at 600 nm for the presence of any dye.

RESULTS:

The spectrophotometric results are presented in Table IX.

Table IX. SUMMARY OF LEAKAGE OF SEALANTS

SPECIMEN	TREATMENT	SPECTROPHOTOMETRIC READING (600nm)
1	macerated/Visiobond	0.054
2	macerated/Visiobond	0.018
3	macerated/wax	0.015
4	macerated/wax	0.026
5	macerated/PVA	0.100
6	macerated/paint	0.000
7	macerated/paint	0.000
8	DMEM/Visiobond	0.154
9	DMEM/wax	0.060
10	DMEM/paint (2 h)	0.540
11	DMEM/paint (2 h)	0.927
12	DMEM/paint (24 h)	0.034
13	DMEM/paint (24 h)	0.043

The results revealed Toluidene Blue microleakage in all specimens except the macerated/acrylic paint groups. The DMEM/acrylic paint (2 h drying) groups provided high spectrophotometric recordings which was attributed to the turbidity of the solution caused by unset or decomposing paint. A further DMEM/Acrylic paint group was then given an extra 22 h (i.e. 24 h total) to set. This averted the problem of a murky PBS solution after 14 days but nevertheless, leakage was still evident.

Poly-vinyl acetate was difficult to use and therefore unsuitable for our purpose. Sticky wax sealed satisfactorily but proved awkward to handle and therefore was deemed unsuitable as a dental-root sealant.

CONCLUSION:

Acrylic paint, applied to the surfaces of macerated teeth, provides an effective seal against Toludine Blue diffusion through the dental root.

APPENDIX 2. Effectiveness of EDTAC in removing smear layer

AIM:

To determine whether application of EDTAC, into standardized windows cut on dental-root surfaces, is effective for removal of smear layer.

METHOD:

Six endodontically-prepared, macerated teeth were divided into three groups each of two teeth.

Group 1. 5 min EDTAC: Five separate windows were made on the proximal surfaces of the teeth in the mid root region with a No.1 inverted cone bur to the depth of the actual bur head (1mm approx.). The windows were treated with EDTAC (see Appendix 4.) soaked in a cotton pellet for 5 min. The teeth were washed for 5 min under running water.

Group 2. 10 min EDTAC: As for group 1 only the EDTAC is applied for 10 min.

Group 3. Controls: Five separate windows were made on the proximal surfaces of the teeth in the mid root region with a No.1 inverted cone to the depth of the actual bur head. The windows were then washed for 5 min with running water.

All six specimens were dehydrated and prepared for SEM investigation (see Part 1, Materials & Methods). The windows on the specimens were observed using SEM to investigate whether the depth of cut was sufficient to expose the dentinal tubules

(i.e. cementum was completely removed) and the patency of the tubules themselves.

RESULTS:

Group 1 - EDTAC 5 min: The cementum was completely removed from all of the windows. Most of the dentinal tubules were visible, however, there was still a significant amount of gross debris, smear layer, and partially (and some totally) occluded dentinal tubules.

Group 2 - EDTAC 10 min: The cementum was completely removed from all of the windows. All the tubules were clearly visible without the presence of any superficial debris or smear layer (Figure 30).

Group 3 - Control: It was impossible to determine whether the cementum layer was completely removed due to the presence of gross debris and smear layer. Few tubules were patent (Figure 31).

CONCLUSION:

1. Use of a number 1 inverted-cone bur to a depth of 1 mm on the mid-root surface of single-rooted premolar teeth was effective in penetrating to dentine and created a surface smear layer.

2. This smear layer could be removed by a 10 min application of EDTAC.

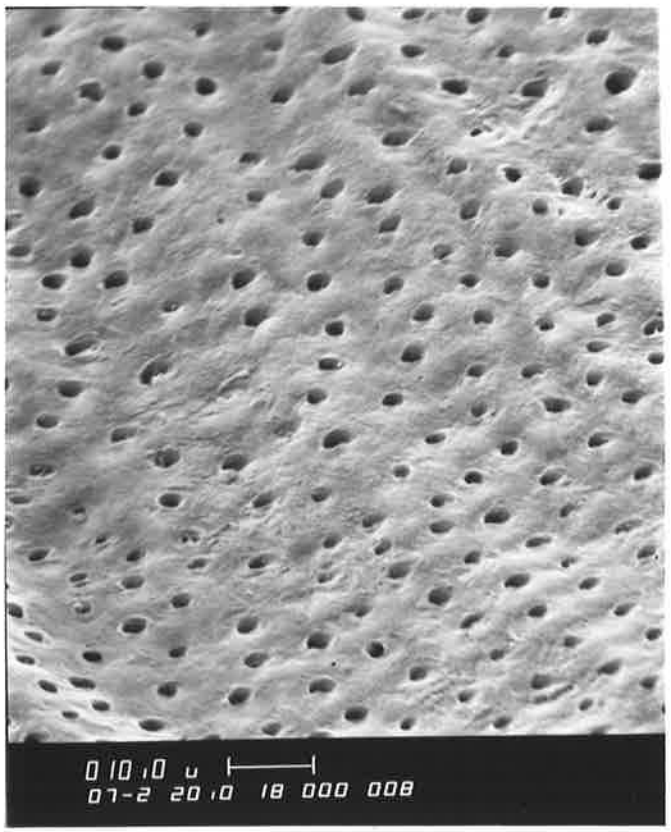

Figure 30. Floor of a standardized slot cut to a depth of 1 mm on external dental-root surface after 10 min exposure to EDTAC. Note the presence of patent dentinal tubules and absence of smear layer. (Bar = 10μm)

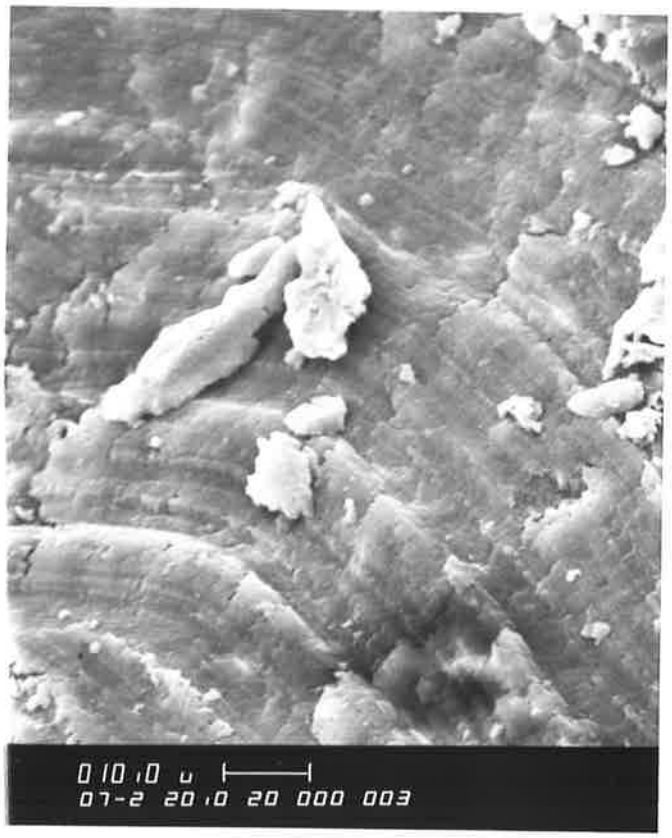

Figure 31. Floor of a standardized slot cut to a depth of 1 mm on external dental-root surface. Note that the surface is covered by gross debris and smear layer. (Bar = 10μm)

APPENDIX 3. Effect of cutting cementum surface with a scalpel blade

During preparation of standardized windows of exposed dental-root surface in painted roots, a scalpel blade was used to cut and remove the dental-root coating of acrylic paint. Although minimal forces were used, we needed to ensure that dentinal tubules were not exposed by this procedure. If dentinal tubules were exposed by scalpel scratches during the preparation of exposed cementum windows then leakage of [I^{125}]-CT may occur via the scalpel scratches at the periphery of the window rather than through cementum.

AIM:

To determine whether cutting into the cementum using a scalpel blade is sufficient to expose dentinal tubules.

METHOD:

Three endodontically prepared and macerated single-rooted premolar teeth were used in this study. A scalpel was used to place five scratches on the root surfaces of each tooth at the mid-root level. The forces applied were much greater than that used to expose a cementum window in the diffusion study. The teeth were then mounted with a silver adhesive to mounting stubs, vacuum coated with gold-palladium, and examined using SEM.

RESULTS:

None of the scratches penetrated the cementum surface. Figure 32 shows a typical scalpel scratch on the dental-root surface

Figure 32. External dental-root surface (top right) subjected to heavy scalpel blade scratch (centre). Note absence of dentinal tubules. (x 700, 1 cm = 10 μm)

CONCLUSION:

Removal of a window of acrylic paint sealant, with a scalpel blade, from the dental-roots of teeth in Part 2 is unlikely to expose dentinal tubules.

This simple experiment showed that removal of a window of paint with a scalpel blade did not expose dentinal tubules. This allowed us to assume that leakage through the teeth with exposed cementum windows used in the diffusion study was in fact through the cementum.

APPENDIX 4. Experimental materials and sources

SOLUTIONS

Dulbecco's Modification of Eagle's Medium (DMEM)

(Flow Laboratories, Australia)

Ingredients followed by concentration (in mg/litre) in parentheses:

L-Arginine HCL (84.00); L-Cystine disodium salt (56.78); L-Glutamine (584.0); Glycine (30.00); L-Histidine HCL H_20 (42.00); L-Isoleucine (104.8); L-Leucine (104.8); L-Lysine HCl (146.2); L-Methionine (30.00); L-Phenylalanine (66.00); L-Serine (42.00); L-Threonine (95.20); L-Tryptophan (16.00); L-Tyrosine disodium salt (89.50); L-Valine (93.60); D-Ca pantothenate (4.00); Choline chloride (4.00); Folic acid (4.00); i-Insitol (7.00); Nicotinamide (4.00); Pyridoxal HCl (4.00); Riboflavin (0.40); Thiamin HCl (4.00); $CaCl_2.2H_20$ (246.9); $Fe(NO_3)_3.9H_20$ (0.10); KCl (400.0); $MgSO_4.7H_20$ (200.0); NaCl (6400); $NaHCO_3$ (3700); $NaH_2PO_4.2H_20$ (141.3); D-glucose (4500); Phenol red sodium salt (15.00); Sodium pyruvate (110.0).

{**Note:** Penicillin [50IU/ml] and streptomycin [50μg/ml] were added to the DMEM in all experiments}.

Phoshate-buffered saline (PBS) (ANAX Australia)

0.2M Na_2HPO_4, 0.2M NaH_2PO_4, 0.15M NaCL, (pH 7.4)

EDTAC solution (Nygaard-Ostby 1957)

143g Ethylenediamine tetra-acetic acid (di-sodium salt)

0.84g Cetyltrimethylammoninum bromide

1000cc Distilled water

NaOH - added until pH reaches 7.4

Milton's Solution (Milton Pharmaceutical Co., Villawood, NSW)

Available chlorine 1% as sodium hypochlorite (NaOCl), and sodium chloride 16.5%.

MATERIALS

Barbed Broach - Nervnadeln CC-cord, Vereinigte Dentalwerke, KG, Munchen, Germany.

Bovine, Albumin - Sigma Chemical Co., St Louis, Mo. USA>

Burs - Tungsten carbide Jet 330, Tapered fissure 169L. Komet, Germany.

Sodium hypochlorite (4% solution) -

(White King Antiseptic Bleach Cleaner) - Kiwi Australia, Chadstone, Vic.

Polyacrylic acid (25%) conditioner (Ketac Conditioner) -

ESPE, Fabrik Pharmazeutischer Praparate GMBH & Co.KG, Seefeld/Oberbay, W. Germany.

Ketac Fil - ESPE, Fabrik Pharmazeutischer Praparate GMBH & Co.KG, Seefeld/Oberbay, W. Germany.

Visiobond - ESPE, Fabrik Pharmazeutischer Praparate GMBH & Co.KG, Seefeld/Oberbay, W. Germany.

Visiodispers - ESPE, Fabrik Pharmazeutischer Praparate GMBH & Co.KG, Seefeld/Oberbay, W. Germany.

Dulux Weathershield exterior gloss acrylic [white] -

Dulux Australia, Clayton, Vic.

Poly-vinyl acetate glue ("Solver" Aquatect) -

W.P. Crowhurst Pty.Ltd., Adelaide, Aust.

Filter paper (Whatman No.1) -

Whatman International Ltd., Maidstone, England.

Ledermix Paste - Lederle Pharmaceuticals, Wolfrathausen, West Germany.

Waxes - Sticky wax - ASH, Amalgamated Dental Trade Distributors, London, England,

Red base plate wax - Investo Manufacturing Co., Camellia, NSW.

Blue periphery wax - Surgident Periphery Wax, Columbus Dental, St. Louis, Mo., USA.

EQUIPMENT

Micrometer - Micro 2000, Moore & Wright, UK.

Gamma counter - LKB-Wallac Compugamma 1282, Wallac OY, Turku, Finland

Scanning Electron Microscope -

Siemens ETEC Autoscan, ETEC Corp., USA.

Centrifuge - Microcentaur, Clements medical & scientific equipment, Adelaide, Australia.

Weighing scales - Mettler Balance, Model H54AR, Watson Victor Ltd, Australia.

CALCITONIN

I^{125}-CT - (3-[I^{125}]iodotyrosyl^{12}) human CT (Amersham International, Buckinghamshire, England)

code: IM.175

specific activity: 2000Ci/mmol, 74TBqmmol

batch: B58

molecular weight: 3542

radiochemical purity: >90% with <3% free [I^{125}]iodide and <5% CT containing a methionine sulphoxide residue (determined by reverse phase HPLC).

position of labelling: [I^{125}]-CT is labelled with [I^{125}] at tyrosine residue 12.

storage and stability: stored at 2-4°C. expect <5% decomposition in 4 weeks from initial analysis.

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