INVESTIGATIONS OF A MICROBIAL MODEL FOR THE DETERMINATION OF LEAKAGE OF ROOT CANAL FILLINGS

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CHAPTER 3 THE EFFECT OF SMEAR LAYER ON MICROBIAL
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DECLARATION

This thesis is the original work of the author

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SUMMARY

This study was a laboratory based investigation of a microbial model for the determination of leakage of root canal fillings using facultative anaerobes, obligate anaerobes, mixed micro-organisms and lipopolysaccharide as markers. Five hundred extracted human teeth with straight, single root canals were prepared chemo-mechanically and obturated with gutta-percha using different techniques and root canal sealers. These teeth were divided randomly into three groups for three major studies; the effect of smear layer on leakage, leakage immediately after obturation and also after long-term storage. A microbial model was developed. The cut end of a tube was sealed around the coronal part of each root canal so that the markers placed therein could move only through the obturated canal space. Each root was placed in a glass bottle containing sterile media and aliquots of the markers were placed in each coronal chamber. The model system was stored in the incubator at 37°C and any change of the medium in the apical chamber checked for 30 days (lipopolysaccharide) or 90 days (micro-organisms). There were no statistically significant differences in leakage between teeth with smear layer intact or smear layer removed and among different root canal sealers. However, teeth in the long-term storage study showed significantly less leakage than those tested immediately after obturation. Lateral condensation of cold gutta-percha with various sealers could not prevent leakage of butyric acid. Moreover, lipopolysaccharide penetrated obturated root canals faster than the micro-organisms tested.

INTRODUCTION AND AIMS

Endodontology is the branch of dentistry concerned with the morphology, physiology and pathology of the human dental pulp and periradicular tissues. Its study and practice encompasses the basic clinical sciences including the biology of the normal pulp, the aetiology, diagnosis, prevention and treatment of diseases and injuries of the pulp and associated periradicular conditions (Walton & Torabinejad 1989).

The objective of endodontic therapy is restoration of the treated tooth to its proper form and function in the masticatory apparatus, in a healthy state (Weine 1989). The quality of nonsurgical root canal treatment depends upon a triad of basic phases, including diagnosis, cleaning and shaping of the canal and the obturation of the root canal system. Although each phase is a separate portion, in the overall situation it must be meticulously carried out to obtain success. If any phase is faulty, the entire system may fail.

Recently, it has been suggested that leakage of the root filling from the coronal direction is an important factor (Saunders & Saunders 1994a) and also the quality of the coronal restoration itself (Ray & Trope 1995). Nowadays, the sealing ability of a new dental material or filling technique is routinely investigated. Large numbers of *in vitro* leakage determination have been used for the research in endodontics but there is considerable variation in the methodology employed (Wu & Wesselink 1993). These tests include dye tracers such as methylene blue (Dummer *et al.* 1993) and India ink

(Saunders & Saunders 1994b), radioisotopes (Matloff *et al.* 1982), electrochemical circuits (Jacobson & von Fraunhofer 1976), saliva (Magura *et al.* 1991), bacteria (Torabinejad *et al.* 1990) and endotoxin (Trope *et al.* 1995).

Micro-organisms and their by-products are considered to be the major causes of pulpal and periradicular pathosis (Nair *et al.* 1990). When strict anaerobic techniques were applied to endodontic samples, many infections that were previously considered to be caused by aerobic or facultative bacteria are now known to be polymicrobial infections dominated by anaerobic bacteria (Haapasalo 1989, Sundqvist *et al.* 1989, Wayman *et al.* 1992, Haapasalo 1993, Tani-Ishii *et al.* 1994). Since inadequacies with dye studies have been highlighted (Mortensen *et al.* 1965, Krakow *et al.* 1977, Goldman *et al.* 1980) the use of a microbial leakage marker may be more relevant clinically.

In root canal treatment, root canals are usually filled by gutta-percha in combination with a sealer. Review of the large number of leakage studies published points to general agreement that leakage occurs between the root filling and the root canal wall (Hovland & Dumsha 1985, Wu & Wesselink 1993). Therefore anything that might influence the adaptation of the root filling to the canal wall is of great significance in determining the degree and extent of leakage and ultimately the prognosis of the root canal therapy. One important factor is the presence or absence of the smear layer which might influence the adaptation of the root filling to the canal wall (Saunders & Saunders 1992b, Gutmann 1993). In contrast, some authors have questioned the advisability of removing the smear layer because it may block dentinal tubules from bacterial penetration (Pashley *et al.* 1981, Safavi *et al.* 1989). The clinical significance

of the smear layer in root canal therapy remains controversial (Czonstkowsky *et al.* 1990), and the relevance of its removal has been the subject of several investigations.

The sealing ability of a root canal sealer may be related to some of its physical properties. These include dimensional stability under changing temperatures which may be important when a warm gutta-percha technique is used and its solubility in oral and tissue fluid. This is especially relevant in the coronal part of the root canal system which may be easily contaminated by fluids and may contribute to failure of root canal treatment.

The aims of this *in vitro* investigation, were to:

- develop a reproducible model for the study of coronal leakage of root canal fillings in natural teeth using facultative anaerobes, obligate anaerobes, mixed micro-organisms and lipopolysaccharide as markers

- investigate the effect of smear layer on coronal leakage in relation to different root filling techniques

- assess the coronal leakage of root canals obturated with different types of root canal sealers

- determine the effect of storage time on microbial coronal leakage of root canal fillings with different types of root canal sealers.

CHAPTER 1 REVIEW OF THE LITERATURE

1.1 PATHOLOGICAL CHANGES IN THE DENTAL PULP

According to Ingle *et al.* (1994), the causes of pulp inflammation, necrosis and dystrophy are:

- micro-organisms and their end-products

- trauma

- iatrogenic factors

- chemical materials

- idiopathic factors.

Recent studies have indicated that the important cause of periradicular inflammation is most likely due to the persistence of bacterial infection and their end-products in the root canal system (Nair *et al.* 1990, Sjögren *et al.* 1990, Lin *et al.* 1991).

Factors that make the pulp unique and alter its ability to respond to irritation are:

- it is almost totally surrounded by dentine, which limits the area for expansion and restricts its ability to tolerate oedema

- it has an almost total lack of collateral circulation, which severely limits its ability to cope with bacteria, necrotic tissue and inflammation

- the pulp possesses a unique cell, the odontoblast, as well as cells that can differentiate into hard tissue-secreting cells which form more dentine and /

or irritation dentine in an attempt to protect itself from injury (Simon *et al.* 1994).

1.1.1 Reversible Pulpitis

Mild-to-moderate injuries of short duration cause reversible pulpitis. These include mechanical removal of caries or an unlined restoration. If the cause can be removed, the pulp should recover. In contrast, if the cause remains, the symptoms may persist or the inflammation may become more widespread, eventually leading to an irreversible pulpitis and pulpal necrosis (Cohen 1994).

1.1.2 Irreversible Pulpitis

An irreversible pulpitis can be subdivided into *symptomatic* and *asymptomatic irreversible pulpitis.* If the exudate that is being formed from pulp inflammation can be vented, the tooth may remain quiescent. Conversely, if the exudate remains within the hard confines of the root canal, pain will probably occur (Cohen 1994).

1.1.3 Pulp Necrosis

Pulp necrosis may result from an untreated irreversible pulpitis and can be partial or total. Untreated necrosis may spread beyond the apical foramen causing periradicular disease. Yamasaki *et al.* (1994) investigated changes in pulpal and periradicular tissues histologically and histometrically after pulpal exposure in rats. The results showed that pulpal necrosis extended gradually from the coronal part of the pulpal tissue to the apex, with inflammation starting in the periradicular

tissue at an early stage. As the periradicular lesion developed, resorption of alveolar bone and cementum was also found.

1.1.4 Periradicular Lesions

1.1.4.1 *Acute inflammation*

In acute inflammation, the polymorphonuclear (PMN) leukocytes are the first cells to emigrate to the site of infection, drawn there by chemotactic agents that are expressed either by the bacteria itself or by other mediators of inflammation. The monocytes are then attracted to the site (Simon 1994).

1.1.4.2 *Chronic inflammation*

Granuloma or *cyst* are the chronic responses of the periradicular tissues to irritants. Microscopically, the apical periodontal cyst consists of a pathological cavity usually lined by a relatively thick, stratified squamous epithelium. Depending on the stage of the development, the lesions consist of varying amounts of macrophages, lymphocytes, plasma cells, polymorphonuclear (PMN) leukocytes, mast cells and fibroblasts (Langeland *et al.* 1977, Weiner *et al.* 1982). Piattelli *et al.* (1991) examined 12 samples of periapical granuloma using light and electron microscopes and monoclonal antibodies. The results showed that monocytes / macrophages, lymphocytes, and plasma cells were nearly always the most abundant cell populations.

Types and pathogenicity of the bacteria in the root canal and the status of the host's defence may also determine the development of the abscess and the granuloma. If pyogenic (pus forming) organisms such as *Prevotella intermedia* and *Porphyromonas endodontalis* predominate, suppuration and abscess formation are likely to develop. If, on the other hand, less virulent organisms are the source of inflammatory stimuli, diffusion of bacterial antigens may result in an infiltration of the periradicular tissues by macrophages, T cells and other inflammatory elements, thus causing a granuloma to develop (Trowbridge & Stevens 1992).

The tissue response to irritants can be a non-specific and / or a specific immunological inflammatory reaction (Torabinejad *et al.* 1985, Stashenko 1990). Macrophages and lymphocytes play essential roles in cell-mediated mechanisms involved in chronic inflammation. This inflammation is often associated with irreversible destruction of parenchymal tissue, and fibrous connective tissue fills the resultant defect. Proliferation of fibroblasts and collagen production are enhanced by the secretion of cytokines by T cells and macrophages (Trowbridge 1990).

If tissue necrosis occurs in inflammatory periradicular lesions, it is usually localised near the apical foramen, or centrally in the body of the lesion, where the tissue encounters the highest level of bacterial toxins or harmful metabolic end-products diffusing through the root canal. Necrosis may also occur after direct contact with cytotoxic filling materials at the apical foramen or in the periradicular area (Nair 1987, Lin *et al.* 1991).

Periradicular inflammation is able to cause resorption of apical alveolar bone of the endodontically involved tooth, and occasionally the root cementum and dentine of this tooth and the adjacent one by means of locally produced leukotrienes (LTs), prostaglandins (PGs) particularly PGE_2 and cytokines such as interleukin-1 and tumour necrosis factor (TNF) from stimulated inflammatory and immunologically responsive cells (Stashenko 1990). A study by McNicholas *et al.* (1991) showed that acute periradicular lesions have higher concentrations of PGE_2 than chronic periradicular lesions.

1.1.5 Odontogenic Pain

The sensitivity of the dental pulp is controlled by myelinated (A-delta) and unmyelinated (C) afferent nerve fibres. A-delta fibres are large, located in the region of the pulp-dentine junction and affect the relatively low stimulation threshold. C fibres are small, high threshold fibres and run subjacent to the A-delta fibres. C fibre pain is associated with tissue injury and is modulated by inflammatory mediators, vascular changes in blood volume and blood flow, and an increase in tissue pressure. When C fibre pain dominates over A-delta fibre pain, pain is more diffuse and that signifies that irreversible local tissue damage has occurred (Cohen 1994).

1.1.6 Treatment

Irreversible pulpitis, pulp necrosis and periradicular lesions do not normally heal without treatment. As a result, conventional root canal treatment is directed towards removal or extirpation of the necrotic pulpal tissue, chemomechanical

preparation of the root canal system to eliminate the bacterial contamination and obturation with an inert material to allow healing of the periradicular tissue.

1.2 THE HISTORY AND DEVELOPMENT OF ROOT CANAL TREATMENT

There is an abundance of fascinating information on the early development of endodontics. Like the rest of dentistry and medicine, endodontics began as an offshoot of magic and superstition.

In 3700 BC, during the first Egyptian dynasty, part of the Ebers papyrus prescription was a remedy for "bennut blisters" probably gingival abscesses or sinuses arising from dead teeth. Another part was a cure for "Uxedu" which was probably the swelling associated with an alveolar abscess (Curson 1965).

The Chinese believed that these alveolar abscesses were caused by a white worm with a black spot on its head which could always be seen when the tooth was extracted. The remedy was to destroy the worms and the prescription for this contained arsenic. The use of this drug was taught in most dental schools as recently as the 1950s. The "worm theory" was current until the middle of the eighteenth century (Harty 1990).

Pulpal treatment during Greek and Roman times was aimed at destroying the pulp by cauterisation, either with a hot needle, with boiling oil or with a fomentation of opium and hyoscyamus. Archigenes of Syria, who lived in Rome towards the end of the first

century AD, found that pain could be relieved by opening directly into the pulp chamber with a trephine of his own design in order to obtain drainage and then cauterising with a red hot iron.

Galén (131-201), a great physician and anatomist practising in Rome, was the first to refer to the nerves of the teeth and had some understanding of the underlying pathology of toothache. He believed that dental caries was produced by an abnormal condition of the blood. He also recognised the need to gain access to the pulp chamber with a small drill. To prevent a carious tooth from producing pain or becoming suppurated, he advised the carious hollow to be filled with black veratrum mixed to a paste with honey (Guerini 1909).

Hippocrates considered "affections" of the teeth to depend in part on natural dispositions, that was, on congenital weakness of the dental system. In one of his treatises dealing with dental decay he wrote "If the tooth is not loose but painful, it is necessary to desiccate it by cauterising" (Guerini 1909). Serapion, who lived in the tenth century, described the number of dental roots and expressed an opinion that the upper molars required their three roots in order to keep them firm, while two roots were sufficient to keep the lower molars in place. In case of persistent odontalgia because of caries, he advised the application of opium into the carious cavity.

Avicenna, one of the greatest luminaries of medicine amongst the Arabs, considered that dental pains were due to an excessive accumulation of "humours" in the roots. He advised therefore that the tooth be drilled and afterwards filled with drugs based on

arsenic. Abulcasis (1050-1122), however, still used cautery for persistent dental pain because of a worm in a tooth. Mesue Vulgar, a disciple of Avicenna, cauterised a dental fistula to its base with cautery in the form of a probe, or extracted the tooth, which by reason of its diseased root he considered to be the cause of the fistula.

Guy de Chauliac (1300-1368), the greatest surgeon of the Middle Ages, wrote at length on a whole range of medical topics. In most respects he followed the teaching of Galén and the Arabian writers. He believed in the presence of dental worms, but it was clear from his writing that anaesthetic inhalations were used. His treatment for a painful tooth was still cauterisation into the pulp chamber, this procedure being done through a cannula to protect the surrounding tissues (Curson 1965). Bartolomeo Montagnana, who taught surgery in the University of Padua, considered the extraction of a tooth as the best means of curing odontalgia. In his day, there was a tendency to advocate the extraction of a tooth, a philosophy to which the ancients had held.

By the sixteenth century, quite a number of significant ideas were being formed concerning the cause and treatment of toothache, but the tooth worm theory was still considered as the principal cause of dental pain. The macroscopic anatomy of the teeth was brought to a high degree of perfection by Bartholomeus Eustachius (1520-1574). He wrote the first monograph devoted to the anatomy of the teeth, and dealt accurately with the macroscopic and developmental anatomy and the physiology of teeth. He was the first to describe the periodontal membrane, by which the roots were "tightly connected" with the alveolar bone (Guerini 1909).

Ambroise Paré (1517-1592) considered the cause of toothache to arise from an internal or external source. He treated proximal caries by filing between the teeth, removing sufficient substance and filling with a cork or a piece of well-fitted lead. Jacques Guillemeau (1550-1613), a pupil of Paré, was the first author to refer to the use of inorganic materials for the restoration of natural teeth.

In the seventeenth century, the development of the science of optics, which revealed the true nature of the development and structure of the teeth, was to a large extent, responsible for a better understanding of dental disease. The first notable improvements in optical lenses were made by Anthony van Leeuwenhoek (1632-1723), the father of microscopy. Using single biconvex lenses, he discovered the dentinal tubule, which he described in a communication to the Royal Society of London in 1678. He examined scrapings from teeth and observed micro-organisms. He also observed, in the enamel of a well-formed human tooth, lines running parallel to each other, following the contour of the tooth. Friederich Ruysch (1638-1731), Professor at Amsterdam, traced the vascular ramifications within teeth and demonstrated the existence of capillary vessels using a method of intra-vessel injection which he had devised.

Pierre Fauchard (1690-1761), the founder of modern scientific dentistry, wrote *The Surgeon Dentist* which provided accurate descriptions of the pulp cavities and root canals of different teeth. He did not believe in the existence of dental worms, but considered that they might be present accidentally. He used the term dental caries, which he believed was caused by a "humour which insinuates itself into the teeth". His treatment for dental pain was opening the tooth using a trepan, leaving it to drain for

two or three months, putting cotton-wool soaked in oil of cinnamon or of cloves into it, and later filling the pulp chamber with lead foil, tin or gold. Although he did not mention root canal therapy, he described a pulp extirpation procedure using a small pin (Cruse & Bellizzi 1980a).

In 1756, Philip Pfaff was the first to carry out capping of an exposed dental pulp without previous cautery, prior to insertion of a filling. Later, in 1757, Bourdet treated the dental pulp by cauterisation. In the case of partial displacement of the tooth which severed the nerve, he removed the pulp to the root-end with a three-sided, pointed instrument. He also described an "intentional endodontic treatment" in which he extracted a painful tooth, thoroughly removed the decay and, after completely filling the root canals and cavity, he replanted it (Cruse & Bellizzi 1980a).

By the end of the eighteenth century, Friedrich Hirsch wrote of diagnosing dental infections by tapping suspected teeth, the one in which the percussion provoked pain, was the diseased tooth. The treatment he recommended was perforation of the tooth at its neck followed by repeated insertion of a red hot probe. The cavity was then filled with lead. In 1809, Edward Hudson, an Irishman practising in Philadelphia, was the first dentist to place fillings in root canals. He packed the canals with gold foil, using instruments of his own design. But the first American reference to gold as a filling material was in the *American Journal of Dental Science*, in 1839, written by John Baker, one of George Washington's dentists (Cruse & Bellizzi 1980a).

At the beginning of the nineteenth century, the "vitalistic theory" of teeth was proposed by Charles Bew. He believed that there was blood flow into the pulp through the apical foramen, and out of the root canal through the dentinal wall and periodontal membrane. Since the mid-nineteenth century the phrase "root canal therapy" has been used to describe pulpal treatment.

The first root canal broach was developed from a watch spring by Edwin Maynard, in 1838. This enabled dentists to treat teeth with small canals, such as premolars and molars. In addition, he developed hoe-like instruments that could be used for enlarging and for shaping root canals. The first root canal sealer was used in 1856. It was oxychloride of zinc which was a composition of zinc oxide and zinc chloride (Grossman 1976).

In 1864, in America, Barnum devised a thin sheet of rubber to isolate a tooth during gold foil operations. This became the indispensable rubber dam we know and use today. Bowman of St. Louis has been given credit by many authors as the first to have used gutta-percha as the sole material for filling a root canal. He was also the co-inventor of the rubber dam clamp forceps in 1873. In 1867, the year that Joseph Lister used antiseptic during surgical procedures, Leber and Rottenstein proved the existence of bacteria. They found these organisms on tooth surfaces, in carious lesions, and in the dentinal tubules. Their findings led to the conclusion that tooth decay could cause gangrene of the pulp (Cruse & Bellizzi 1980a).

In 1874, Adolf Witzel of Germany described a treatment for embalming the pulp which he called pulp mummification. Rogers suggested in an article published in *Dental Cosmos* in 1878 that pathogenic organisms might be the most common cause of diseases of the pulp. He concluded that successful treatment required the total destruction of these organisms. By that time, arsenic was known to seep beyond the pulp chamber and to damage the periodontium. Consequently, in 1883, Mills described a procedure for pulpal extirpation without arsenic. This consisted of gaining access to the pulp chamber, applying a pointed hickory peg against the pulp, and giving it a sharp tap with a mallet (Grossman 1976).

In 1884, Richmond taught a method of filling root canals by sterilising the root canals with phenol and iodine, and sealing the foramen with a sterile solid material to prevent extrusion of fillings and then obturating with an antiseptic cement. In the same year the value of cocaine as a topical anaesthetic became known. It was first used to obtain pulpal anaesthesia in 1892 by Briggs of Boston. In 1888, Miller, the father of oral microbiology, formulated the bacteriological basis for endodontic treatment. He described the formation of dental alveolar abscesses as a continuation of pulpal infection (Cruse & Bellizzi 1980b).

The discovery of X-rays by Roentgen in 1895 and the first radiograph of the teeth by Koenig in 1896, further popularised root canal therapy. Previous unknown pathological conditions were now revealed and the inadequacy of procedures employed to obturate the root canal became obvious (Harty 1990).

In the early twentieth century, Johnston was the first to use the term endodontics, which was the combination of the Greek words "endon" (within) and "odontas" (a tooth). In 1908, Rhein devised a technique to determine canal length and degree of obturation. Black (1836-1915) also suggested a measurement control to determine the length of the canal and the size of the apical foramen so that over filling procedures, commonly encountered by many at that time, could be prevented.

By 1910 "root therapy" had reached its zenith and no tooth was extracted if its preservation was at all possible. As a result, crown and bridgework were placed on badly broken down teeth, non-vital or inadequately root-filled teeth. A lecture, *The Role of Sepsis and Antisepsis in Medicine*, given by William Hunter in 1910 condemned the concept of fixed crown and bridgework with periradicular and pulpal diseases. The principle of the focal infection theory was that organisms from diseased teeth would spread through the blood stream or lymphatics to other organs or tissues and caused serious systemic disease. As a consequence of this theory, teeth, both pulpless and vital, were removed in wholesale numbers. This was especially the case if the disease was of unknown origin or the illness was chronic. Root canal therapy fell into disrepute and this situation prevailed in America and Britain until the 1940s (Grossman 1976).

In the area of pulp conservation, Hermann began using a calcium hydroxide mixture, called Calxyl, for filling root canals in 1920. Later, in 1930, he also advocated using Calxyl for pulp capping, pulpotomy, pulpectomy and for treatment of infected canals. He showed that the amputated, viable pulp, when covered with Calxyl, could form a

bridge of reparative dentine over the severed pulp. Rickert, in 1925, proposed the use of sealer in conjunction with the gutta-percha cone for treatment of the non-vital tooth canal (Bellizzi & Cruse 1980).

The hollow tube concept of Rickert and Dixon (1931) led to the belief that if a prepared root canal was left unfilled or incompletely filled, tissue fluid from the periradicular tissues would percolate into this space where it would stagnate. The breakdown products would diffuse back into the periradicular region and cause chronic irritation. This theory was questioned by Torneck (1966, 1967). He showed that the inflammatory reaction around the openings of implanted tubes was associated with the contents of the tube and not the tube *per se*. If the contents were irritant or contaminated with micro-organisms then the potential for repair was less favourable than if the lumen of the tube was clean and sterile. This realisation that the "apical seal" was important led to the search for a filling material that was stable, non-irritant and provided a perfect seal at the apical foramen.

Fish and MacLean (1936) had maintained that the presence of tissue fluid within the root canal could enable any residual organisms within the canal or the dentinal tubules to remain viable and perpetuate periradicular irritation. Gradually the concept that a tooth without a pulp was not necessarily infected began to be accepted. Furthermore, it was realised that the function and usefulness of the tooth depended on the integrity of the periodontal tissues and not on the vitality of the pulp (Marshall 1928).

By 1953, many antibiotics and combinations of various antibiotics had been used in endodontic procedures. In the same year, Auerbach re-emphasised the importance of thorough cleaning of the pulp chamber rather than dependence on drug therapy only. This led to the acceptance of the principle of combining instrumentation with disinfection to render a canal clean and ready to be filled (Bellizzi & Cruse 1980).

As we look back on the progress of endodontics various sciences have contributed to a much better understanding of the physiology and pathology of the pulp, enabling us to examine and compare it in health and disease. A knowledge of pulpal anatomy and the use of a sterile technique, has enabled us to achieve thorough cleaning of the root canal and three-dimensional obturation of the whole pulp canal system. The discovery of x-rays has made the diagnosis of pulpal and periradicular disease more accurate, the discovery of local anaesthetics has conquered the pain associated with endodontic treatment and the biological approach to treatment has made endodontic practice a more exact science and art.

1.3 PREPARATION OF THE ROOT CANAL SYSTEM

Schilder introduced the concept of "cleaning and shaping" of root canal system in 1974. This concept is now considered the most important step in root canal treatment. The objective of cleaning is to eliminate all contents of the root canal system before and during shaping. These include organic substrates, microflora, bacterial end-products, food, caries, pulp stones and previous root canal filling materials from the canal space.

Shaping refers to the enlargement and tapering of the canal to:

- permit vertical pluggers to fit freely within the root canal wall

- generate the hydraulics required to transform and capture a maximum cushion of gutta-percha and a microfilm of sealer into all foramina (West *et al.* 1994).

According to Schilder (1974), five mechanical objectives for successful cleaning and shaping were:

- develop a continuously tapering conical form in the root canal preparation

- make the canal narrower apically, with the narrowest cross-sectional diameter at its terminus

- make the preparation in multiple planes

- never transport the foramen

- keep the apical foramen as small as is practical.

1.3.1 Canal Irrigation

Root canal cleansing is supported by copious irrigation. The requirements of an ideal irrigant (Walton & Rivera 1996) are that it should:

- dissolve organic debris

- have low surface tension

- lubricate endodontic instruments

- eliminate micro-organisms

- remove the smear layer

- be relatively non-toxic

- other factors that should be considered include: moderate cost, adequate shelf life, and ease of storage.

1.3.1.1 *Types of irrigation solution*

Sodium hypochlorite

Sodium hypochlorite (NaOCl) is the most popular and the most widely advocated irrigant. It can fulfill the first four actions of the ideal irrigant. Proprietary products such as Chlorox and Purex bleach are common sources of concentrated sodium hypochlorite (5.25%). The concentration of sodium hypochlorite is considered to be at its most effective between 1% and 5%. A 2.5% solution is commonly recommended (West *et al.* 1994). An advantage over full strength (5.25%) sodium hypochlorite is that the lower concentration is more pleasant to use, without a strong odour and with much less toxicity. Its action is enhanced significantly by copious flushing and by warming the solution to 37°C (Moorer & Wesslink 1982).

Chelating agents

Ethylene diamine tetra-acetic acid (EDTA) was introduced into endodontic practice by Östby in 1957. It functions by forming a calcium-chelate solution with the calcium ion of dentine, the dentine thereby becomes more friable and easier to instrument (Grossman *et al.* 1988). It also removes the smear layer produced on the canal wall during instrumentation. This solution should not be used exclusively but must be accompanied by irrigation with sodium hypochlorite (Saunders & Saunders 1990a).

Lubricants

Lubricants are an aid in passing instruments to working length when exploring and negotiating small, constricted canals. RC Prep (Premier Dental Products, Norristown, PA, USA) is a gel-like preparation. It combines EDTA with urea peroxide in a base of carbowax, therefore, it provides excellent lubricant, chelating and disinfectant properties (Miserendino 1994). It is, however, difficult to remove from the wall of the root canal during irrigation with NaOCl. Recently, a new formulation of RC Prep has been marketed which does not include carbowax.

Sterile water, local anaesthetic solution or isotonic saline

The beneficial bactericidal and tissue-dissolving requirements are not present in these irrigants. These bland solutions can be employed, however, as the final irrigant after using sodium hypochlorite (Saunders & Saunders 1990a).

Chlorhexidine gluconate

Chlorhexidine gluconate is a broad-spectrum antibacterial agent. It acts by adsorbing onto the cell wall of the micro-organisms and causing leakage of intracellular components (Davies 1973). A study by Vahdaty *et al.* (1993) indicated that chlorhexidine gluconate and sodium hypochlorite were equally effective antibacterial agents at similar concentrations against *Enterococcus faecalis* in bovine dentinal tubules.

1.3.2 Canal Preparation Technique

1.3.2.1 *Stepback or Telescopic technique*

Mullaney (1979) defined the outline of the principles of this technique into 3 phases, which are:

- establishing the apical stop and preparing the apical section of the root canal system

This phase consists of apical enlargement at the working length to a master apical file (MAF, the last size file used to length). One of the most important parts of this phase is the reuse of files one size smaller than the last one used, to prevent dentine shavings from building up and causing blockage of the canal. This repetition of instrumentation is referred to as "recapitulation".

- stepping back

This phase is aimed at modifying the canal space to increase accessibility to the apical preparation (Tidmarsh 1982). It is achieved by shortening one larger size files by 1 mm sequentially to produce a coronal taper. The patency of the apical segment must be ensured by continued use of MAF to full working length after each step back.

- completion of preparation

The middle and coronal thirds of the canal can be prepared either by filing or by using Gates-Glidden burs. The ledges created along the length of the canal by this technique must be removed by circumferential filing with the MAF at the full

working length. The completed preparation should be conical in shape with its narrowest portion apically and its widest diameter at the access cavity.

1.3.2.2 *Anticurvature filing technique*

Abou-Rass *et al.* (1980) described a "danger zone" where perforation is most likely and which lies on the inner or concave aspect of a curved root. They advocated a technique termed anticurvature filing for instrumentation of fine curved canals to avoid lateral perforation instead of the standard circumferential filing method. They emphasised that during shaping procedures, files should be pulled from canals as pressure is applied to the outside canal wall. Lim and Stock (1987) recommended a circumferential filing technique which involves preparation of the bulkier parts of the canal wall, that is, the buccal, lingual and mesial aspects, while less frequent filing of the furcal wall, in the ratio of 3:1.

1.3.2.3 *Balanced force technique*

The use of the "balanced force" technique with a modified tipped file was originally recommended by Roane *et al.* in 1985. This technique involves placement of the instrument into a canal space using a clockwise rotation with light apical pressure, followed by counterclockwise rotation and apical pressure that achieves cutting of the dentine of the canal wall.

The clockwise rotation of each instrument must be limited to no more than 180 degrees in order to prevent overinsertion of the apical portion of the instrument into dentine. The counterclockwise rotation should be 120 degrees or greater to completely

enlarge the canal to the file diameter, free the instrument and prepare it for placement to a deeper depth when the next clockwise rotation is supplied. This technique has been shown to work effectively without precurving instruments (Southard *et al.* 1987).

Powell *et al.* (1986) showed that hand preparation of simulated root canals was significantly better with modified tipped instruments than that with non-modified. In addition, a significant difference in the preparation was evident with these modified instruments. Less transportation was observed and the original canal curvature was maintained (Sabala *et al.* 1988).

Preparation of the canal from the crown to the apical constriction

Recently, considerable support has been given to techniques which prepare the coronal two-thirds of the root canal prior to the apical section. The reasons are:

- the greatest number of micro-organisms in the root canal lie in the coronal portion (Shovelton 1964). Therefore initial preparation of this section of the root canal system helps to reduce the number of micro-organisms that may be forced into the periradicular tissues.

- early flaring of the coronal part of the preparation eliminates cervical dentine structures and reduces canal curvatures. Consequently, the files are easier to control and the working length is less likely to change during preparation of the apical third of the canal (Goerig *et al.* 1982).

- disinfecting irrigating solution can penetrate deeper into the inner recesses of the canal, thereby effectively cleaning the coronal part of the canal before the apical third is approached (West *et al.* 1994).

1.3.2.4 *Crown-down pressureless technique*

This technique was first described by Morgan and Montgomery in 1984. The preparation method involves early radicular canal flaring with Gates-Glidden burs. This is followed by incremental removal of canal contents and dentine shavings from the access cavity to the working length, with a straight file used in larger to smaller sequence. Fava (1983) recommended a combination of the crown-down pressureless and the stepback technique, which is referred to as the *double-flare technique,* but could only be prepared in straight root canals.

1.3.2.5 *Stepdown technique*

Goerig *et al.* (1982) advocated a stepdown technique, as a modification of stepback technique. The coronal two-thirds of the canal is enlarged with hedstroem files and Gates-Glidden burs with light apical pressure and lateral pressure directed away from furcation. This results in straight-line access to the apical third of the root canal. The apical preparation is then completed with a stepback preparation. This part of the canal system is approached only after the coronal two-thirds has been prepared.

1.3.2.6 *Modified double-flare technique*

Saunders and Saunders (1992a) recommended a modified double-flared technique for curved canals which consisted of a combination of the double-flare technique with

balanced force instrumentation. The preparation was commenced in the coronal part of the root canal. A size 40 non-cutting tipped file was instrumented in the straight part of the canal, using the balanced force method. This part of the canal was then enlarged sequentially, up to a Gates-Glidden size 090 drill.

Using the balanced force technique, a size 20 file was taken to the working length. The apical section was prepared sequentially to a MAF varying between size 40 and size 45 file. A stepback technique using balanced forces was then used to prepare the remaining curved portion of the canal. All instrumentation was accompanied by lubrication with Hibiscrub (ICI Ltd., Macclesfield, UK) and copious irrigation with sodium hypochlorite. They found that this technique produced a better quality of overall preparation than when unmodified or modified tipped files were used with stepback technique.

1.3.2.7 Ultrasonic and Sonic technique

In 1976 Martin developed a system using ultrasonic energy for cleaning and preparing the root canal. Both ultrasonic (20-40 KHz) and sonic (1.5-3 KHz) have a component which allows a continuous flow of irrigating solution to pass along the file and over their oscillating tips. The tip of the file is unconstrained and shows the largest oscillation. A transverse oscillation of the ultrasonic file consists of nodes where the oscillation is minimal, and antinodes where the oscillation is greatest (Stock 1991). In contrast, the sonically powered file has one node nearest to the driver and one large antinode at the tip (Walmsley et al. 1989).

With endosonics, activation of NaOCl has been shown to be improved compared to the other intracanal irrigants (Cheung & Stock 1993). The efficacy of the system may be attributed to acoustic microstreaming within the irrigant (Ahmad *et al.* 1987). In the ultrasonic and sonic systems, acoustic microstreaming occurs along the length of the file. The amount and force of streaming, however, is dependent upon two factors:

- amount and freedom of file movement

- the length of time the file is used in the canal (Krell *et al.* 1988).

The recommended technique for the use of vibratory instruments is circumferential anticurvature filing (Saunders & Saunders 1990a). The effect of instrumentation procedures on the shape of the root canal preparation has been reported with various results. Manual preparation was found to be more effective than ultrasonic preparation (Reynolds *et al.* 1987). In other studies, ultrasonic preparation resulted in cleaner canals (Martin & Cunningham 1985).

1.3.2.8 *Noninstrumented technique*

Lussi *et al.* (1993) developed a device which was able to build up controlled cavitation in the root canal. Under reduced pressure, alternating pressure fields generated microscopic and macroscopic cavitation bubbles. Subsequently, these vapour-filled cavitation bubbles collapsed, creating hydrodynamic turbulence. These two phenomena allowed the irrigant (NaOCl) to penetrate the canal system. They found that in an *in vitro* study, the treatment resulted in similar or better cleanliness when compared with machine and hand instrumentation. It has not been demonstrated, however, whether this ultimately results is better clinical success and more research is required.

A variety of techniques and instruments have been introduced for preparing root canal systems. Regardless of the method employed to prepare the canals, the intensive efforts made towards obtaining total debridement and complete patency of the complex root canal system is essential for success in endodontic therapy.

1.4 OBTURATION OF THE ROOT CANAL SYSTEM

1.4.1 Root Canal Filling Materials

Total obliteration of the canal space and perfect sealing of the apical foramen at the dentine-cementum junction and accessory canals with an inert, dimensionally stable, and biologically compatible material are the goals for consistently successful endodontic treatment (Nguyen 1994).

According to Grossman *et al.* (1988), an ideal root canal filling material should:

- be easily introduced into the root canal

- seal the canal laterally as well as apically

- be dimensionally stable after insertion

- be impervious to moisture

- be bactericidal, or at least, should discourage bacterial growth

- be radiopaque

- not stain tooth structure

- not irritate periradicular tissue or affect tooth structure

- be sterile, or easily and quickly sterilised before insertion

- be easily removed from the root canal if necessary.

Root canal filling materials currently in use or under clinical investigation may be grouped into:

1.4.1.1 *Solid materials*

Silver and other metal cones

Silver obturating cones were introduced in 1933 by Jasper and have been used with popularity to fill root canals for over 50 years. They were easily inserted into narrow or tortuous canals to the exact length and they were clearly visible on the radiograph (Wesselink 1990).

Silver cones have now fallen into disrepute. The incompressibility of a silver cone means that there must inevitably be a lack of conformity to the shape of canal walls. Nicholls (1984) pointed out that a false sense of security was easily created by the apparently dense radiographic appearance of a silver cone root filling. He felt this had served to encourage inadequate preparation of the root canal and abuse of the material. Should the apical seal so achieved be less than ideal, resorption of the sealer would occur with subsequent prolonged exposure of the silver point to tissue fluid. This hazard would also exist if the defect was in the coronal restoration. Seltzer *et al.* (1972) and Brady and del Rio (1975) found that this situation led to corrosion of the silver cone with the formation of highly cytotoxic silver compounds. An additional disadvantage is that, in some instances, removal of silver cones may be extremely

difficult or even impossible if a canal has to be retreated or restored with a post and core.

There has been some effort to investigate new and efficacious solid core filling materials that do not have corrosion potential. Palmer *et al.* (1979) and Messing (1980) introduced titanium cones. Gutta-percha-coated and Teflon-coated silver cones have also been tested by West *et al.* (1979) and Negm *et al.* (1980). However, except for corrosion toxicity, these metal cones have the same disadvantages as silver cones.

1.4.1.2 *Semisolid materials*

Gutta-percha is a naturally occurring substance, being the coagulated exudate from the mazer wood tree. It is a polymer of isoprene which was introduced to dentistry by Bowman in 1867. The characteristic feature of the organic polymer molecule is a chain of covalently bonded atoms, built by the repetitive chemical linking of small, simple units. Their large size and chainlike structure result in fields of attraction between individual molecules (Van der Waals bonding).

Natural rubber and gutta-percha are both high molecular weight polymers and both are structured from the same basic building unit. The natural rubber has "cis" polyisoprene, with its CH_2 groups on the same side of the double bond (Fig. 1.1), whilst gutta-percha has "trans" polyisoprene, with its chain forming CH_2 groups on the opposite side of the double bond (Fig. 1.2).

50
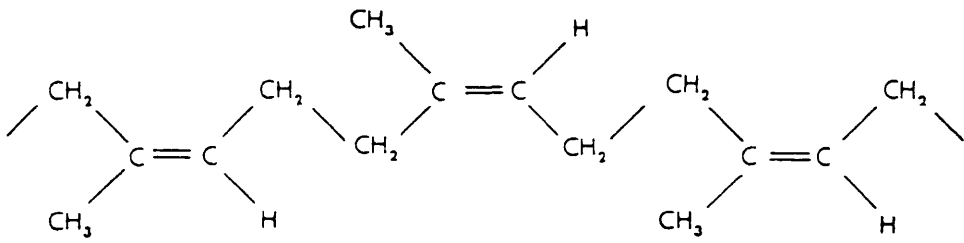
Fig. 1.1 NATURAL RUBBER ("Cis" polyisoprene).

Alpha gutta-percha　　　　*trans*-Polyisoprene

Beta gutta-percha　　　　*trans*-Polyisoprene
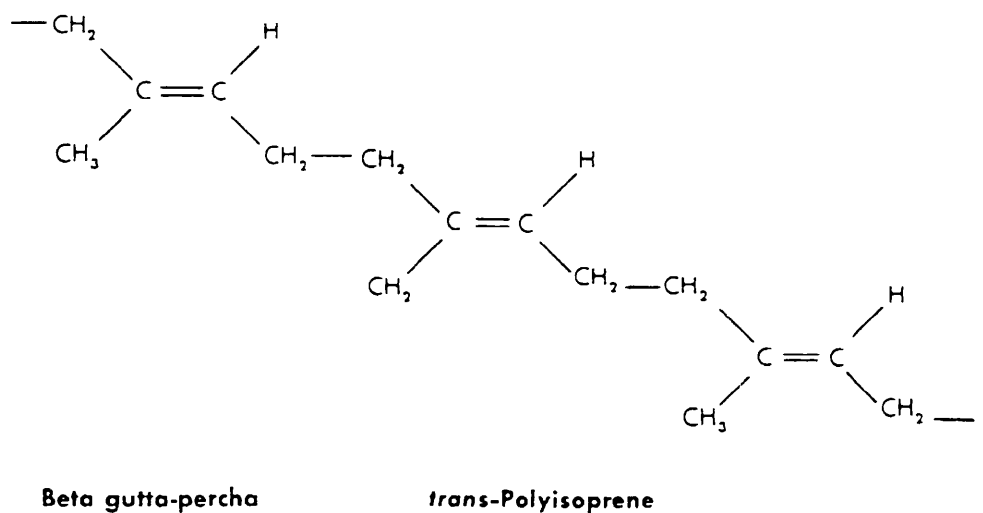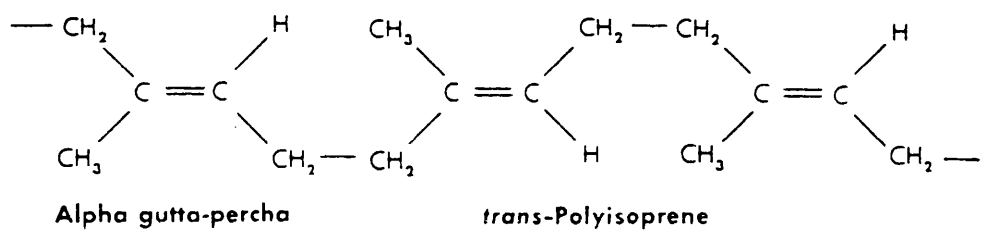
Fig. 1.2 GUTTA-PERCHA ("Trans"polyisoprene).

The "cis" form is more kinked which allows mobility of one chain against another. This gives natural rubber its elastic character. The "trans" form is more linear and crystallises more readily. Consequently gutta-percha is harder, more brittle and less elastic than natural rubber. At sufficiently high temperatures the linear polymer melts to form an amorphous rubbery substance, whereas at sufficiently low temperatures the same polymer is a rigid solid with its chain locked in position.

Gutta-percha can exist in two different crystalline forms, "alpha" and "beta" modifications. Most commercial gutta-percha exists as the "beta" crystalline structure which has a melting point of 64°C. There is apparently no difference in the mechanical properties of "alpha" and "beta" gutta-percha, but there are differences in thermal properties, because the two forms can be converted into each other at specific transition points during a heating cycle. In addition, the coefficient of thermal expansion of gutta-percha may influence the quality of obturation in root canal treatment. These phase transformation temperatures are affected by molecular weight, purity, addition of other materials, the degree of crystallinity, thermal and mechanical properties (Goodman *et al.* 1974).

Schilder *et al.* (1974) found that dental gutta-percha exhibited two crystalline transformations upon heating from room temperature to 100°C: 42°C-49°C for the "beta" to "alpha" transition and 53°C-59°C for the "alpha" to amorphous transition. Their results depended on the specific brand of gutta-percha used. Rootare *et al.* (1976) examined eleven different brands and deduced that different brands not only had different ingredients but also different thermal manipulation during manufacture.

Friedman *et al.* (1977) described the approximate composition of gutta-percha cones as:

66%   zinc oxide (filler)

20%   gutta-percha (matrix)

11%   heavy metal sulphate (radiopacifier)

3%   waxes or resins (plasticizer)

Spångberg (1969) studied the reaction of guinea pig bony tissue to dental gutta-percha and other obturating materials. Gutta-percha was well tolerated in both short and long-term studies. In an *in vitro* tissue culture investigation by Spångberg and Langeland in 1973, into the toxicity of root canal sealers, gutta-percha points were used as controls. In contrast to the sealers the gutta-percha produced little cell lysis.

Advantages of gutta-percha as a filling material are:

- compatibility, gutta-percha adapts well to the irregularities and contour of the canals using lateral and vertical pressure especially when heated

- ability to become plastic when warmed or in contact with organic solvents

- inertness

- dimensional stability

- tissue tolerant

- non-discolouration of tooth structure

- radiopacity

- it can be easily removed from the canal when necessary.

Disadvantages of gutta-percha as a filling material are:

- lack of rigidity; gutta-percha will bend easily when subjected to lateral pressure which make it more difficult to use in smaller sizes

- lack of adhesive quality; gutta-percha does not adhere to the canal walls consequently sealer is required

- lack of length control; gutta-percha permits vertical distortion by stretching which may tend to induce overextension during the condensing process.

1.4.1.3 *Pastes*

Pastes have been used as the sole root canal filling material because of the simplicity and speed of technique. The paste is either pumped (using a gutta-percha point or a plugger), injected by pressure syringe or spun into the canal using a Lentulo spiral. A disadvantage of this technique is the lack of control during insertion, often resulting in overextention and the difficulty of eliminating entrapped air within the filling. Paste-type filling materials will be described with root canal sealers.

1.4.2 Root Canal Sealers and Pastes

Nguyen (1994) described the roles of root canal sealer as:

- a binding agent to cement the well-fitted primary cone into a canal

- a filler for the discrepancies between the cone and the canal walls

- a lubricant to facilitate the sealing of the primary cone into the canal.

A root canal sealer is used in combination with a (semi)solid root canal material or cone, root canal pastes are meant to be used without a (semi)solid material. Laboratory research projects have reported that comparison made between different root filling techniques without the use of sealer showed only minimal differences in their sealing ability, whereas almost every study has revealed that, with the addition of a root canal sealer, the results with all techniques were remarkably enhanced (Marshall & Massler 1961). Investigations by Kapsimalis and Evans in 1966 studied the comparative sealing properties of several commonly used endodontic filling materials using radioactive isotopes. Gross leakage was observed in those specimens where sealer cement was omitted. Limkangwalmongkol *et al.* (1991) demonstrated that laterally condensed gutta-percha alone showed considerably more apical leakage than when used in conjunction with a root canal sealer.

The requirements and characteristics of a good root canal sealer (Grossman *et al.* 1988) are:

- provides an excellent seal when set
- produces adequate adhesion between it, the canal walls and the filling material
- radiopacity
- nonstaining
- dimensionally stable
- easily mixed and introduced into the canals
- easily removed if necessary
- insoluble in tissue fluids

- bactericidal or discourage bacterial growth

- nonirritating to periradicular tissue

- slow setting, to ensure sufficient working time.

Inevitably no root canal sealer and paste satisfy all these requirements. *In vitro* studies by Spångberg and Langeland (1973) using tissue culture techniques showed that all root canal sealer cements tested were highly toxic during the setting phase. A study by Langeland (1974) showed that all root canal sealers were resorbable by vital tissue to a greater or lesser extent. In a review article in 1982, on the physical properties and sealing action of root canal sealers, Branstetter and von Fraunhofer stated however that no standard specifications for sealers had been agreed regarding optimum property characteristics, despite the large range of materials available and their very widespread use.

Root canal sealers and pastes may be divided into:

1.4.2.1 *Zinc oxide-eugenol based*

Most of the recommended zinc oxide-eugenol cements are based on the Grossman formula (1958) who modified the original formula given by Rickert and Dixon (1931). It is used widely and presents a minimal degree of irritation and a high level of antimicrobial activity. The formula for Grossman's nonstaining cement is as follows:

powder		liquid
42%	zinc oxide reagent	eugenol
27%	staybelite resin	
15%	bismuth subcarbonate	
15%	barium sulphate	
1%	sodium borate, anhydrous	

The main virtue of Grossman's sealer is its plasticity and slow setting time in the absence of moisture, together with good sealing potential because of the small volumetric change on setting. However, zinc eugenolate is soluble in tissue fluids, making zinc oxide-eugenol an unstable material (Nguyen 1994). On the other hand, this ability is an advantage in cases of apical extrusion of the sealer during canal obturation.

Several other types of zinc oxide-eugenol cements are commercially available such as Tubliseal, Kerr pulp canal sealer (both Kerr, Romulus, MI, USA) and Roth sealer (Roth, Chicago, IL, USA). Tubliseal mixes well, has excellent lubricating properties and does not stain the tooth structure. However, it sets rather rapidly, particularly in moist canals. Recently, the company has reformulated the sealer to extend working time (Tubliseal EWT).

Nogenol (COE Mfg. Co., USA) has been advocated to overcome the irritating quality of eugenol. The base is zinc oxide with barium sulfate as the radiopacifier along with a vegetable oil. Set is accelerated by hydrogenated rosin, methyl abietate, lauric acid,

chlorothymol and salicylic acid (Ingle & West 1994). Crane *et al.* (1980) tested

Nogenol subcutaneously against two eugenol-containing sealers. They found that after

24 hours, all sealers caused considerable inflammation but at 96 hours Nogenol was

less irritating than the other two sealers.

1.4.2.2 *Resin-based*

AH26 (De Trey, Zurich, Switzerland) is an epoxy resin base which sets upon mixing

with an activator. It is composed of:

	powder		liquid
60%	bismuth trioxide	100%	bisphenol diglycidyl ether
25%	hexamethylene tetramine		
10%	silver powder		
5%	titanium dioxide		

Because of its silver content, AH26 sealer may cause discolouration of tooth structure.

Therefore, all traces of this sealer must be removed below the free gingival level to

prevent tooth discolouration. Ørstavik and Mjör (1988) showed that AH26 elicited an

initial severe inflammatory reaction which subsided after some weeks.

Diaket (ESPE, Seefeld, Germany) is a resin-reinforced chelate formed between zinc

oxide and diketone. It consists of a fine, pure white powder and a viscous, honey-

coloured liquid. A study by Yates and Hembree in 1980 showed that Diaket was less

effective as a sealer than Tubliseal, but both were found to be more effective than N2.

Hydron (NPD Dental Systems, Melville, NY, USA) is a poly-2-hydroxyethyl-methacrylate. It is a hydrophilic gel which absorbs water and swells when in contact with moisture. As a result, it was developed as an injectable root canal filling material with the addition of a catalyst, benzoyl peroxide, for polymerisation (Benkel *et al.* 1976).

1.4.2.3 *Gutta-percha based*

Chloropercha is a paste made by dissolving gutta-percha in chloroform. Eucapercha has replaced chloropercha because chloroform has been designated a potential carcinogen (Morse *et al.* 1983). Some clinicians used these materials as the sole root canal filling. However, they are mainly intended to be used in combination with gutta-percha points because the solvents are volatile and the root filling shrinks as the solvent evaporates (Wong *et al.* 1982).

1.4.2.4 *Dentine-adhesive materials*

Glass ionomer cements have been found to possess desirable characteristics in terms of biocompatibility (Zmener & Dominguez 1983). Although there are problems with moisture sensitivity and handling difficulties, they were found to be more effective than zinc oxide-eugenol cements for the prevention of leakage in an *in vitro* study (Pitt Ford 1979). Ray and Seltzer (1991) concluded that the Ketac-Endo material (ESPE, Seefeld, Germany) is equal to or superior to Grossman's sealer in terms of flow, radiopacity, setting time, and adaptation to the root canal wall, although they did note that no solvent currently exists which can facilitate removal of this material for retreatment. Saunders *et al.* (1992) showed that Vitrebond (3M Dental Products

Division, St. Paul, MN, USA) bonded as well to gutta-percha as the zinc oxide-eugenol sealer, Tubliseal.

1.4.2.5 *Materials to which medicaments have been added*

(Para)formaldehyde is used as a disinfectant and a corticosteroid is supposed to reduce post-operative pain (Grossman *et al.* 1988). Several cements are commercially available such as N2 (Indrag, Agsa, Locarno, Switzerland) and Endomethasone (Septodont, St. Maur des Fosses, France). If deposited in the periradicular tissue, these materials may give rise to severe inflammatory reactions and they seem not to be biocompatible (Pitt Ford 1985). Lewis and Chestner in 1981 suggested that formaldehyde has both a mutagenic and carcinogenic potential, which raises the question of its use against vital tissue. Brewer in 1975 has indicated the potential danger of the N2 formula to patients. Cases of paraesthesia or severe pain produced as a result of extrusion of formaldehyde or paraformaldehyde sealers from the root canal are well documented (Ørstavik *et al.* 1983). Recently, this material has been banned in some countries.

Calcium hydroxide has been added to root canal sealer on the assumption that the formation of hard structures would be stimulated in a similar manner to the formation of dentine bridges over pulp exposures, after capping with calcium hydroxide (Wesselink 1990). A wide variety of calcium hydroxide-based sealers are available commercially such as Sealapex (Kerr, Romulus, MI, USA), Calcibiotic root canal sealer (CRCS) (Hygenic Corp., Akron, OH, USA) and a recently introduced root canal

sealer, Apexit (Vivadent, Schaan, Liechtenstein) whose manufacturer claims that it has good sealing ability and is biocompatible with the surrounding tissues.

Limkangwalmongkol *et al.* (1991) showed that there were no statistically significant differences in leakage among Tubliseal, Sealapex and Apexit. A comparative study of the apical seal produced by two calcium hydroxide sealers and a Grossman-type sealer by Alexander and Gordon (1985) showed that Sealapex produced an apical seal equal to that produced by the Grossman-type sealer and both were better than CRCS.

1.4.3 Obturation Technique

1.4.3.1 *Single-cone technique*

Nguyen (1994) stated that the single-cone technique may be used when:

- the canal wall is reasonably parallel and the master cone fits snugly in the apical third of the canal

- the canal is too wide and commercially available gutta-percha cones will not fit the canal adequately.

A customised cone is fabricated by rolling warmed gutta-percha cones together into a bundle. The apical third of the cone is softened by dipping into an organic solvent to allow replication of the internal shape of the root canal. Root canal sealer is lightly coated on to the cone and canal wall. The cone should be inserted very slowly to allow the cement to flow back coronally, otherwise it will act as a plunger to force the cement beyond the apical foramen. This technique may well produce an acceptable

apical seal provided it is not disturbed. If a post retained restoration is required later, there is a possibility that the seal may be disrupted because the single gutta-percha cone could entwine itself round the hand or engine-driven instrument used to ream an adequate length of channel for the post (Jeffrey & Saunders 1987).

1.4.3.2 *Solvent technique of canal obturation*

Chloroform dip technique, a cone-fitting method, is used in large canals or to seat a cone 2-3 mm short of the radiographic apex. The apical third of the selected master cone is dipped into the solvent which is chloroform, xylol, eucalyptol or oil of turpentine in order to soften the surface of gutta-percha. The softened cone is inserted into the moist canal until the working length is reached. The master cone is gently positioned into the prepared canal, the wall of which has been lightly coated with the root canal sealer. The standard lateral condensation of cold gutta-percha technique is then performed. This technique is not recommended because studies on apical sealability generally show extensive leakage (Zakariasen & Stadem 1982) and poorer long-term prognosis (Ørstavik *et al.* 1987). In addition, chloroform is a listed group II substance which implies probable carcinogenicity in humans. As a result, it should be avoided whenever possible.

1.4.3.3 *Lateral condensation of cold gutta-percha technique*

Lateral condensation of cold gutta-percha technique is preferred to the single-cone method because most teeth present wide canals or flares that cannot be densely filled with a single gutta-percha cone. The use of the term "lateral" condensation refers to the successive placement of accessory gutta-percha points adjacent to a well-fitted

master gutta-percha cone (master cone should exhibit some degree of apical fit or "tugback" within 1 mm of working length). Space for the accessory points is created with an endodontic spreader, which may be either a finger or a hand instrument. The spreader is inserted next to the gutta-percha cone and "condenses" the master cone and any previously placed accessory points onto the wall of the prepared canal. Lateral and apical pressure is applied by revolving the spreader through 90 degrees. Upon removal of the spreader, a space is present in which an additional accessory point of corresponding size is placed. This process is repeated until the spreader cannot penetrate more than 1 or 2 mm into the canal orifice. The excess gutta-percha is removed from the pulp chamber by searing with a hot metal instrument, and the slightly softened mass further plugged into the coronal end of the canal.

The success achieved with this technique depends mainly on canal cleaning and, in particular, canal shaping. The shape of the prepared canal must be a continuously tapering funnel. The spreader should be placed without binding on the canal wall and to within 1 mm of the working length. Failure to do so will prevent the placement of the master cone, spreading instrument and accessory points to their proper distance leading to inadequate condensation. Allison *et al.* (1979) observed *in vitro* that "the group in which the spreader tip could be inserted to within 1 mm of the prepared length had considerably less microleakage than did the group in which the distance between the spreader tip and prepared length was greater".

The lateral condensation technique does not create the merging of the gutta-percha cones into a homogeneous mass. The added auxiliary cones are compressed laterally

against each other and the canal walls. It is rather time-consuming but if properly executed gives a dimensionally stable root filling with a dense radiographic appearance. It is the most widely practised and accepted obturation technique (Allison *et al.* 1979). Virtually every investigation has included this method as the control group against which other techniques are assessed.

The term "lateral condensation" is not an accurate description of the procedure. Vertical forces are also created during the insertion of the spreader and with the canal being tapered, the vectors of force created are a composite of both lateral and vertical (Glickman & Gutmann 1992). Meister *et al.* (1980) showed that excessive force during lateral condensation of the gutta-percha attributed to 84% of vertical root fractures. Martin and Fischer (1990) indicated the warm lateral condensation technique created less stress during obturation than did cold lateral condensation.

1.4.3.4 *Lateral condensation of warm gutta-percha technique*

In an attempt to enhance the adaptability of root canal filling materials into canal walls, the Endotec (L.D. Caulk / Dentsply, Milford, DE, USA) and the Thermopact (Degussa, Neuilly sur Seine, France) have been recommended. The Endotec was developed by Martin and Fischer in 1990 as a lateral heat spreader. The Thermopact device, however, has been designed for use with both warm lateral and vertical condensation.

With this technique, the gutta-percha is fused and condensed into a denser, more homogeneous mass, creating a three-dimensional obturation of the root canal space,

whereas in the traditional cold lateral condensation technique, the gutta-percha points are merely laminated together, leaving possible voids for potential leakage. A leakage model *in vitro* study by Kersten (1988), showed that the Endotec warm lateral condensation has the least leakage, compared with Ultrafil and lateral condensation. The forces required to condense the thermo-softened gutta-percha are less stressful with the Endotec condenser than those used in cold lateral condensation, as demonstrated by photoelastic stress studies by Martin and Fischer in 1990. This would be an advantage in the prevention of root fractures.

1.4.3.5 *Vertical condensation of warm gutta-percha technique*

Vertical condensation of warm gutta-percha technique was suggested by Schilder in 1967. This technique involves the placement of the master cone which has good tugback 1-2 mm short of the working length into the canal which is lightly coated with root canal sealer. The fitted master cone is softened with a heat transfer instrument and a suitably-sized cold plugger is then applied to this softened mass which is forced apically. The canal should be a continuously tapering funnel and wider coronally to permit the introduction of a graded series of pluggers with which to condense the increments of gutta-percha in an apical direction.

The process is continued moving the heat transfer instrument in an apical direction and so softening the gutta-percha further into the canal while removing portions at the cervical end. Vertical pressure is used to condense the gutta-percha in the middle portion of the canal which in turn is spread and condensed laterally. When condensation of the apical section is completed, small increments of gutta-percha are

inserted, heated and plugged apically. Under the force of vertical condensation, the softened gutta-percha and entrapped sealer are compressed into the irregular configurations and canal ramifications, creating a good three-dimensional seal.

1.4.3.6 *Thermomechanical compaction technique*

In the thermomechanical compaction technique, introduced by McSpadden (1980), gutta-percha is plasticized by frictional heat and inserted by means of a compactor that forces the material apically. The mechanical compactor is available in colour-coded assorted sizes and resembles an inverted hedstroem or K-type file, with the flutes facing the tip of the instrument. It is operated in a latch-type slow speed contra-angle handpiece at a minimum rotational speed of 8,000-10,000 revolutions per minute.

The principle of this obturation technique is that if the compactor is placed alongside a pre-fitted gutta-percha cone in a prepared root canal, the frictional heat generated between the rotating compactor and the canal walls will be sufficient to plasticize the gutta-percha. The softened gutta-percha will then be moved apically by the flutes on the instrument while simultaneously being spread laterally. Wong *et al.* (1981) in an *in vitro* investigation comparing thermomechanical compaction with warm vertical and cold lateral condensation techniques used without sealer, found that thermomechanical compaction produced a filling which replicated a standard artificial root canal better than that achieved by lateral condensation but poorer than that by vertical condensation.

The major disadvantage of this technique is that the compactor can be used only in widely prepared straight canals. In addition, Kerekes and Rowe (1982) reported that although thermomechanical compaction was superior to lateral condensation in filling irregularly shaped canals, instrument fracture usually occurred. They felt that more attention should be paid to producing compactors of better quality to prevent this instrument breakage. The procedure itself takes less than 10 seconds to complete, but requires a lot of training and experience. It is recommended that the clinician practice this technique on models and on extracted teeth prior to clinical use (Tagger *et al.* 1984).

1.4.3.7 *Hybrid technique*

Tagger (1984) has devised a technique for using thermomechanical compactors as an adjunct to lateral condensation of cold gutta-percha. The prepared canal is filled initially by laterally condensing cold gutta-percha and root canal sealer. The middle and coronal sections of the canal are further modified by the use of thermomechanical compaction. The gutta-percha is plasticized by frictional heat and the irregularities such as fins or intercommunications in the canal are filled without affecting the previously condensed gutta-percha at the apical third of the canal.

1.4.3.8 *Thermoplastic injection-moulding gutta-percha technique*

In 1977 Yee *et al.* introduced obturation of the root canal systems using thermoplasticized gutta-percha in conjunction with a pressure syringe. Subsequent studies by Moreno (1977), Torabinejad *et al.* (1978) and Marlin *et al.* (1981) have supported the use of the thermoplasticized technique and its ability to achieve:

- a detailed replica of the intricacies of the root canal system

- a seal equal to, if not superior to, that produced by other techniques

- a quality root canal fill in a significantly shorter time.

As a result of these studies, two types of delivery systems were developed. The Obtura system, originally developed by the Unitek Corporation and improved and marketed as the Obtura II system (Texceed, Costa Mesa, CA, USA). It is a high-heat (160°C) delivery system in which the molten gutta-percha is injected through disposable, suitably sized, silver needle applicator tips. The thermoplasticized gutta-percha extrudes through the needle tip at a temperature of 55°C-60°C. The system consists of an electric control unit with digital read-out of temperature, a gun-like syringe with silver needle applicator tips and specially designed gutta-percha sticks. The problem of protection, both for the clinician and the patient, from the hot applicator tip is overcome to a certain extent by a polystyrene sleeve, but great care is still required when positioning the heated needle into the prepared canal. The applicator tip can be bent with a special tool, to help gain access to the root canal but these silver needles are fragile and may be broken easily.

A thermoplasticized low-temperature gutta-percha was developed with a slightly different delivery system because of criticism of the high temperature of the gutta-percha in the Obtura system. The Ultrafil System (Hygenic Corp., Akron, OH, USA) is a low-heat (70°C) system in which cannules containing gutta-percha are heated and placed in a syringe prior to the delivery. The temperature of the extruded gutta-percha through the needle tip ranges from 38°C-44°C.

The system contains a sterilisable injection syringe, a portable heating chamber with preset temperature and three different types of disposable gutta-percha cannules with attached needles. The gutta-percha used contains the same ingredients as gutta-percha points, however, there appears to be a greater proportion of paraffin. Clinicians can choose one of three types of gutta-percha, depending on the consistency required. The "Regular Set" requires no condensation because of its low viscosity. For the "Firm Set" gutta-percha, condensation is optional. Both of them are used primarily for simple injection techniques where the gutta-percha will not be compacted manually. To counteract the concern about expansion and shrinkage of the injected gutta-percha, the "Endoset" was introduced, where condensation is required.

The success of these obturation techniques depends on a number of factors.

- The root canal preparation should be a continuously funnel shape with a slight flare at the coronal portion to accommodate the needle and a definite apical constriction to obtain a three-dimensionally well condensed gutta-percha filling with restriction of apical extrusion.

- The applicator tip should be placed as close to the apical portion as possible without binding against the canal walls, ideally the selected needle tip should be able to reach to within 3-5 mm from the apical constriction.

- The gutta-percha must be heated properly prior to injection into the canal with either system, the gutta-percha must flow out of the system in a thin hairlike strand, stretching 6-8 inches from the tip of the needle.

- With either system, injection of the gutta-percha must be passive to prevent excessive internal pressure built up within the cannule. Pressure is placed on

the syringe trigger only and gutta-percha should be allowed to flow out of the needle at its own rate. A tactile sensation of gutta-percha pushing the applicator tip out of the canal should be felt.

- A slow-setting root canal sealer should be used. The sealer will not only enhance the seal but also assist in the flow of the gutta-percha.

- The segmental method of obturation may be used by injecting a small amount of gutta-percha in the apical third and condensing that first with an appropriately sized plugger. Once the apical seal is verified with a radiograph, the remainder of the canal is rapidly back-filled and condensed with larger pluggers.

- To deliver and condense the softened gutta-percha properly, the clinician must be thoroughly familiar with the mechanics of each unit by practising the technique.

Michanowicz and Czonstkowsky (1984) investigated the sealing properties of a low-temperature gutta-percha (70°C) with and without sealer, compared with the lateral condensation with sealer. The apical seal was tested for leakage with 5% methylene blue dye. Results showed that low-temperature gutta-percha created a good seal, especially if used in conjunction with a sealer. A further study was undertaken by Czonstkowsky *et al.* (1985), using the radioactive isotope C-14 starch for quantitative determination of apical leakage. The same obturation techniques were chosen as in the previous study. The degree of leakage exhibited in the three groups was minimal. Low-temperature injection gutta-percha with sealer showed the least amount of leakage, followed by lateral condensation with sealer. Low-temperature injection gutta-percha

without sealer showed a slightly larger isotope leakage than the other groups. This study confirmed that the use of a sealer can improve the quality of the apical seal.

Evans and Simon (1986) reported that both the lateral condensation and the injected thermoplasticized gutta-percha techniques do not provide an apical seal to ink penetration when used without a root canal sealer, even with the smear layer removed. As a result, it is recommended that the use of injected thermoplasticized gutta-percha be accompanied by the use of a sealer whether or not the smear layer has been removed.

1.4.3.9 *Thermafil endodontic obturators*

The placement of gutta-percha onto a stainless steel file was first introduced by Johnson in 1978. This technique has been refined, improved and commercially marketed as the Thermafil endodontic obturators (Tulsa Dental Products, Tulsa, OK, USA). The Thermafil obturator device is a specially designed flexible metal (stainless steel or titanium) or plastic carrier coated with alpha phase gutta-percha. It is adapted with a sliding silicone rubber stop for precise length measurement and control during canal insertion at obturation time. This alpha phase gutta-percha, once heated, exhibits a wetting phenomenon and becomes sticky and more adhesive. This wetting phenomenon allows the gutta-percha to be transported into the canal towards the apex without being stripped from the flutes of the carrier. This carrier is not the primary cone for obturation. It acts as a carrier and condenser for the thermally plasticized gutta-percha and seems to be capable of forcing the softened gutta-percha to the apex and laterally to fill the irregularities of root canal walls and accessory canals.

When the gutta-percha-containing carrier has been heated sufficiently in the heat portable chamber, the device is inserted into the prepared canal that has been lightly coated with slow-setting sealer. The shaft of the carrier protruding above the canal orifice is severed with a bur to a point 1-2 mm above the canal orifice. This will allow easier removal in case retrieval is necessary. A small condenser is then used to condense vertically the gutta-percha around the shaft.

Thermafil obturators are sized from 20 to 140 and colour coded to correspond to endodontic instruments. The canal preparation has less flare in its occlusal half compared with that in the vertical condensation or the thermoplasticized gutta-percha injection methods. A study by Gutmann *et al.* (1993), compared the use of plastic thermafil obturation technique with lateral condensation technique, using radiographic evaluation. This showed that Thermafil provided a better overall canal obturation, whilst, in the apical third, both techniques were not significantly different. When the apical orifice was patent, there was a significant propensity for the extrusion of filling materials beyond the apex with the Thermafil technique.

1.4.3.10 *The new Trifecta technique and Trifecta kit*

Hygenic Corporation, OH, USA, have added a new SuccessFill gutta-percha syringe and a set of SuccessFill cores to the Ultrafil kit. In contrast to Thermafil, SuccessFill uses its own titanium cores that have been sized according to ISO standards. The presumption is that this instrument is more effective in resisting slippage and displacement of the gutta-percha than a precoated carrier such as Thermafil in order to gain complete apical control and exact placement of gutta-percha.

A SuccessFil syringe containing alpha phase gutta-percha is heated to a sufficient temperature using a special heater. A sterile master apical file is inserted 2-3 mm into the SuccessFil syringe to remove some gutta-percha for the formation of an apical plug. The carrier file is inserted into the root canal which has been lightly coated with the slow-setting sealer. The file is then removed, leaving just gutta-percha and sealer in the canal. Ultrafil "Firmset" or "Endoset" gutta-percha is injected and condensed to obtain a three dimensionally dense and well-compacted obturation.

It is important that endodontic procedures are performed in a way that prevents the extrusion of the root filling material into the periradicular tissues. Clinical and radiographic follow-up examinations by Seltzer *et al.* (1963) found a higher percentage of failures with canals which were over-filled than with those that were filled short of the apex. Mann and McWalter (1987) observed over-extension of root fillings in 50% of cases obturated by injection moulded thermoplasticized gutta-percha and only 20% over-extension with lateral condensation. Over-extension of gutta-percha root fillings produced by the thermomechanical compaction method was also noted by Tagger *et al.* (1984).

Schilder *et al.* (1985) stressed that it was important that vertical pressure be applied in all obturation techniques involving warm gutta-percha in order to compensate for the volume changes which occurred as cooling to 37°C took place. They also found that the lower the temperature to which gutta-percha was subjected the less was the volume change on cooling.

Although dentine is a good insulator, concern exists as to whether the high temperatures from the warm gutta-percha techniques are transferred to the outer surface of the root, which may cause damage to the periodontal ligament. An *in vitro* study by Hardie (1986) showed that temperature rises up to 27°C on the central region of the root surface could occur when using the thermomechanical compaction technique. A further study by the same author in 1987 showed that temperatures at a point in the apical third of the root surface may rise with the various obturation techniques; Engine Plugger (16,000 rev/min): 6°C-19°C, Obtura system: 3.5°C-11°C and hybrid technique (16,000 rev/min): 0°C-10°C. *In vivo*, no apparent tissue destruction to the periodontium was evident in short-term animal experiments (Gutmann *et al.* 1987). However in a ferret study 20-40 days after thermomechanical compaction, some root resorption and ankylosis was observed (Saunders 1988).

The aim of canal obturation is to fill the entire volume of the root canal space completely and densely with biologically inert and compatible filling materials. Regardless of the technique used, all obturation techniques have disadvantages as well as advantages. It will be seen that no single technique is applicable to all teeth. From leakage studies it appears obvious that none of the available root canal fillings prevent leakage and it may be that the importance of leakage testing has been over-emphasised, as a method of determining clinical success of root canal treatment.

1.5 MICROBIOLOGICAL ASPECTS OF PERIRADICULAR DISEASE

Since Miller (1890) demonstrated the presence of bacteria in necrotic pulp tissue, the role of the oral microflora in the pathogenesis of pulpal and periradicular pathosis has become increasingly evident. Under normal circumstances, pulp tissue and its surrounding dentine are protected by the enamel and cementum. The major pathways of pulp contamination are:

1.5.1 Exposed Dentinal Tubules

Bacterial invasion of the pulp is most often the result of dental caries. Therefore the endodontic microflora beneath deep dentinal lesions resembles that found in caries. Researchers (Olgart *et al.* 1974, Nagaoka *et al.* 1995) have shown that the dentinal tubules of viable dentine are not easily invaded by oral micro-organisms. Slow invasion of viable dentine by bacteria might be because of the presence of natural resistance factors in the dentine and the pulp tissue.

Hoshino *et al.* (1992) used anaerobic procedures to demonstrate the early bacterial invasion of unexposed dental pulps, which were covered by clinically sound dentine beneath carious lesions. Six of nine teeth had bacterial invasion, the predominant organisms being obligate anaerobes. They concluded that the organisms isolated in this study had passed through some individual dentinal tubules to invade the dental pulp.

1.5.2 Pulp Exposure

As a consequence of pulpal exposure to oral flora, the pulp and its surrounding dentine can harbour bacteria and their end-products. A study by Baumgartner (1991) concluded that periradicular lesions are maintained by micro-organisms from the root canal advancing and / or their products emanating into the periradicular area and interacting with the apical host tissues. The route of infection appears to be selective for only a few bacterial strains, mostly facultative anaerobic bacteria found in the oral flora. The pathogenic process is related to:

- the virulence of the bacteria

- host resistance

- amount of circulation

- degree of drainage.

As bacteria reach the pulp, polymorphonuclear neutrophils (PMNs) are the first cells to emigrate to the site of infection. They respond chemotactically to the bacteria itself or by other mediators of inflammation. The monocytes are then attracted to the site. When these cells enter the tissues they are called tissue macrophages or histiocytes. In the inflammatory process both PMNs and macrophages function as phagocytes (Simon 1994). Pulpal tissue may stay inflamed for long periods and may undergo necrosis eventually or become necrotic quickly. Yamasaki *et al.* (1994) investigated pulpal and periradicular changes following pulp exposure in rats. The results showed that necrosis extended gradually from the upper portion of pulp to the apex.

Korzen *et al.* (1974) studied the effects of normal oral flora and monoinfection (*Streptococcus mutans*) on the pulp and periradicular tissues of conventional and gnotobiotic rats. The results showed that the severity of pulpal and periradicular inflammation were related directly to the quantity of micro-organisms in the root canals and to how long these tissues were exposed to the micro-organisms. Furthermore, it showed that the degree of inflammation was less severe with monoinfection than with mixed infection.

Möller *et al.* (1981) exposed the pulps of monkey teeth to the oral environment for 7 days and the teeth were then sealed and examined 6 months later. All the exposed teeth became infected with micro-organisms similar to those present in human root canals and 90% of the teeth developed periradicular lesions. Tani-Ishii *et al.* (1994) induced periradicular lesions in rats by pulp exposure and subsequent infection from the oral cavity. The results demonstrated that lesions expanded rapidly between day 7 and day 15 to 20, with slower expansion thereafter.

When strict anaerobic techniques were applied to endodontic samples, many infections that were previously considered to be caused by aerobic or facultative bacteria are now known to be polymicrobial infections dominated by anaerobic bacteria (Haapasalo 1989, Sundqvist *et al.* 1989, Wayman *et al.* 1992, Haapasalo 1993, Tani-Ishii *et al.* 1994). The number of different species in one canal is usually low, ranging from 4-7 species (Haapasalo 1993). The most frequent isolates belong to the genera *Prevotella, Fusobacterium, Peptostreptococcus,* and *Streptococcus* (Haapasalo 1989, Sundqvist *et al.* 1989, Haapasalo 1993, Gomes *et al.* 1994b).

Studies have shown a correlation between the presence of anaerobes and symptoms such as pain, swelling, sinus tract formation and foul odour (Griffee *et al.* 1980, Yoshida *et al.* 1987, Haapasalo 1989). Gomes *et al.* (1994a) determined the particular groups of bacteria which were associated with specific endodontic symptoms and clinical signs in 30 root canals. They concluded that a significant association existed between pain and the presence of *Prevotella* and *Peptostreptococcus* species in symptomatic root canals. Furthermore, Gomes *et al.* (1994b) revealed that positive and significant associations occurred between 31 species pairs from 65 different species isolated. The highly significant associations (P<0.01) were between 20 species pairs such as: *Prevotella* and *Peptostreptococcus* species, between *Bacteroides* and *Fusobacterium* species and between *Prevotella* and *Streptococcus* species.

Sundqvist *et al.* (1989) reported that infection of the root canal with a combination of micro-organisms containing *Prevotella intermedia, Porphyromonas endodontalis* and *Porphyromonas gingivalis* induced purulent inflammation in the apical region. Fabricius *et al.* (1982a) inoculated 75 root canals of monkeys with 11 bacterial species separately, or in combinations, and sealed the access cavities for 6 months. Their bacteriological and histologic examinations showed that mixed infections have a greater capacity to cause apical lesions than monoinfections. Furthermore, they reported that the *Bacteroides* strain did not survive in the root canals when inoculated as pure cultures. *Enterococci* survived as pure cultures, and facultative *Streptococci* induced small periradicular lesions.

Baumgartner and Falkler (1991) used strict aerobic and anaerobic culturing techniques to identify micro-organisms infecting the apical 5 mm of root canals of teeth with carious exposures and periradicular lesions. They were able to isolate and identify 50 strains of bacteria, of which 68% were strict anaerobes. The results suggested that only a limited number of species of bacteria from the oral flora were capable of growth in an infected root canal. Tronstad *et al.* (1987) investigated the presence of periradicular microbial flora of 8 cases which had not healed with nonsurgical root canal treatment. In all 8 cases, bacterial growth was evident on culture. Five of the eight cases contained mixed flora that were dominated by anaerobes while two cases were found to contain anaerobes exclusively.

Iwu *et al.* (1990) studied the bacterial content of 16 periapical granulomas obtained under as aseptic a technique as possible. Fourteen of 16 cultures (88%) yielded positive growth. There were 47 isolates of which 26 (55%) were facultative anaerobes and 21 (45%) were strictly anaerobes. Brook *et al.* (1991) aspirated pus from periradicular abscesses in 39 patients. Bacterial growth was present in 32 specimens of which anaerobic bacteria only were present in 16 (50%) cultures and mixed aerobic and anaerobic flora in 14 (44%). The predominant isolates were *Bacteroides* species (23 isolates).

After pulpal necrosis, the ratio of anaerobes to facultative bacteria increases with time because of the lack of blood flow in the necrotic tissue, the lower oxidation-reduction potential and the establishment of synergistic relationship with other bacteria (Trowbridge & Stevens 1992). Fabricius *et al.* (1982b) found that the ratio

of anaerobes to aerobes increased from 3.9:1 at 90 days to 11.3:1 at 1,060 days. The diversity of the microbiota within a given tooth has also been found to be quite limited in all systems studied. Sundqvist (1992) investigated the antagonistic relationships between micro-organisms from 65 root canals with apical periodontitis. The results showed that there were strong positive associations between *Fusobacterium nucleatum* and *Peptostreptococcus micros*, and between *Porphyromonas endodontalis*, *Selenomonas sputigena* and *Wolinella recta*. There was also a positive association between *Prevotella intermedia* and *Peptostreptococcus micros*, and between *Peptostreptococcus anaerobius* and the eubacteria.

Gram-negative bacteria contain endotoxin, a lipopolysaccharide (LPS), which is a structural component of the outer cell envelope, and can be either secreted in vesicles by growing organisms or released after the death of the organism. Endotoxins play a significant role in the pathogenesis of diseases by activating pathways of inflammation, complement (via the alternative pathway), fever induction, adjuvant activity, cytotoxicity, blood clotting and fibrinolysis (Trowbridge & Stevens 1992). Researchers have shown that there is a significant relationship between the presence of endotoxin in periradicular lesions and the extent of inflammation (Bergenholtz 1977, Dahlén & Bergenholtz 1980, Dwyer & Torabinejad 1981, Schonfeld *et al.* 1982). Warfvinge *et al.* (1985) applied endotoxin to the pulpal floor of Class V cavities in monkeys and observed an accumulation of neutrophils in the pulp beneath the exposed dentinal tubules. The result suggested that endotoxin diffused to the pulp and activate the complement system, thus producing C5a, a chemotactic factor for neutrophils.

Horiba *et al.* (1990) studied the distribution of endotoxin in the dentinal walls of infected root canals. The endotoxin content was significantly higher in samples from the pulpal surface of the root canal to 300 μm in depth than from specimens taken near the cementum. Horiba *et al.* (1991) also found that the endotoxin content from the root canals of 30 teeth with apical periodontitis was higher in symptomatic teeth, teeth with apical rarefactions and teeth with an exudate. Yamasaki *et al.* (1992) measured the amount of endotoxin and identified Gram-negative bacteria in experimental periradicular lesions in rats. The results showed that the amount of endotoxin in the periradicular tissues gradually increased with increasing time and that Gram-negative bacteria were isolated from the same region but did not increase in number concurrently with the increase in the amount of endotoxin. A study by Nissan *et al.* (1995) indicated that endotoxin is capable of diffusing across a smeared free dentine 0.5-mm thick in a relatively short period.

Short-chain fatty acids such as propionic, butyric and isobutyric acid are metabolic end-products of anaerobic bacteria and are released at the infection site. Short-chain fatty acids are virulence factors that effect neutrophil chemotaxis, degranulation, chemiluminescence, phagocytosis and ultrastructural changes (Baumgartner 1991). Eftimiadi *et al.* (1991) showed that short-chain fatty acids could inhibit both T-cell blastogenesis and mixed lymphocyte culture. Butyrate was greater in activity than propionate, which was greater than isobutyrate. They also showed that butyric acid stimulated the monokine interleukin-1, which is a powerful bone-resorbing cytokine.

It has become increasingly apparent that the greatest cause of endodontic and periradicular pathosis is microbial infection of the pulp and root canal. Sophisticated culturing techniques have identified infections of endodontic origin as being polymicrobial and predominantly anaerobic. The number of micro-organisms in the root canal can be much reduced by a thorough chemo-mechanical cleaning followed by a complete filling of the root canal space but remaining bacteria may multiply and cause endodontic failure. Further studies are needed to determine the relationship between leakage of specific micro-organisms or their metabolic end-products from the root canal and periradicular inflammation.

1.6 THE ROLE OF SMEAR LAYER IN ROOT CANAL TREATMENT

Debridement of root canal walls produces a micropellicle of debris called a smear layer that was first described by McComb and Smith (1975). This tenacious layer has been found consistently on canal walls that have been instrumented mechanically. From a chemical viewpoint, the smear layer is composed of two phases:

- organic phase which is mainly composed of dentinal collagen residues, necrotic or viable pulp tissues, odontoblastic processes, blood cells and micro-organisms. This phase serves as a matrix for the inorganic phase

- inorganic phase is made up of tooth structure and some non-specific inorganic contaminants.

Structurally, it is said to be composed of two layers:

- the first layer covering the canal walls is thin, loosely adherent and easy to remove

- the second layer occludes the dentinal tubules and adheres strongly to the canal walls (Mader *et al.* 1984).

The smear layer on the canal wall is typically about 1-5 μm thick. The depth entering the dentinal tubules may vary from a few μm up to 40 μm (Mader *et al.* 1984). According to Gilboe *et al.* (1980), factors which may affect the thickness of the smear layer are:

- dry or wet cutting of the dentine

- the size and shape of the root canal

- the type of instrument used

- the amount and chemical composition of the irrigating solution.

The clinical significance of the smear layer in root canal therapy remains controversial (Czonzstkowsky *et al.* 1990). The presence or absence of the smear layer might influence the adaptation of the root filling to the canal wall. The relevance of its removal has been the subject of several investigations.

Pashley (1984) proposed that smear layer containing bacteria or bacterial products might provide a reservoir of irritants. Thus, complete removal of the smear layer would be consistent with elimination of irritants from the root canal system. In the long-term, the smear layer, a separate structure from the underlying dentine, may crack open and

pull away from the underlying dentinal tubules. Yamada *et al.* (1983) and Baumgartner and Mader (1987) suggested that the smear layer may interfere with the effectiveness of intracanal medicaments. This antimicrobial benefit can be eliminated by the presence of organic material and viable bacteria in the smear layer. However, Ørstavik and Haapasalo (1990) found that the smear layer delayed, but did not abolish the action of some disinfectants.

Other investigators (White *et al.* 1984, Saunders & Saunders 1992b, Gencoglu *et al.* 1993, Gutmann 1993) observed tubular penetration of root canal sealers in the absence of smear layer and indicated that such tubular penetration increased the interface between the filling material and the dentinal wall and thus may decrease subsequent leakage. Saunders *et al.* (1992) noted the presence of a resin reinforced glass ionomer cement, used as a root canal sealer in the dentinal tubules after the removal of the smear layer.

Recently, Holland *et al.* (1995) have shown that the use of EDTA prior to intracanal dressings (calcium hydroxide or camphorated paramonochlorophenol) significantly reduced the leakage observed with a zinc oxide-eugenol sealer and a glass ionomer Ketac-Endo. Gettleman *et al.* (1991) found that AH26 had a stronger bond when the smear layer was removed and Oksan *et al.* (1993) showed that smear layer obstructed the penetration of root canal sealers. Whilst no tubular penetration of the sealers was observed in the control groups, the penetration in the smear-free groups ranged from 40-60 μm. However, Vassiliadis *et al.* (1994) showed that smear layer did not prevent Grossman's sealer penetrating into the dentinal tubules in an *in vivo* study.

A study by Kennedy *et al.* (1986) has shown that with the smear layer intact, apical leakage will be increased significantly. Without the smear layer, the leakage will still occur but at a decreased rate. Cergneux *et al.* (1987) found that there was a decrease in apical leakage of obturated root canals with a zinc oxide-eugenol sealer cement after the smear layer had been removed. These authors considered that the smear layer may have an unstable volume because of its high water content. Mader *et al.* (1984) considered the smear layer to be susceptible to leakage because of its non-homogeneous nature and low density. Karagöz-Kücükay and Bayirli (1994) using SEM studies, showed that teeth obturated with Ultrafil with or without root canal sealer showed no significant differences in leakage. There was a highly significant decrease in leakage in the absence of the smear layer.

Pashley and Depew (1986) found that microleakage decreased after the removal of smear layer, but dentine permeability increased. Meryon *et al.* (1986) have shown that micro-organisms have the ability to break down the smear layer *in vitro*, and the possibility of degradation of the smear layer has been implicated in the failure of retrograde fillings following apical surgery (Pitt Ford & Roberts 1990). On the other hand, other investigators (Michelich *et al.* 1980, Pashley *et al.* 1981, Safavi *et al.* 1989) considered that the smear layer should be left intact, as the smear plugs in the apertures of the dentinal tubules may prevent bacterial penetration and / or fluids by altering dentine permeability.

Michelich *et al.* (1980) have shown that the smear layer will prevent bacterial penetration (*Streptococcus mutans*), but will permit fluid filtration. Williams and

Goldman (1985) showed that smear layer delayed the penetration of *Proteus vulgaris*, but was not a complete barrier to this bacteria. Meryon and Brook (1990) observed that *Actinomyces viscosus, Corynebacterium* species and *Streptococcus sanguis* digested the smear layer and facilitated their penetration. Drake *et al.* (1994) suggested that smear layer produced during root canal therapy may inhibit *Streptococcus anginosus* colonising the root canal wall. One suggested mechanism is that smear layer may block bacterial entry into dentinal tubules. A study by Safavi *et al.* (1989) concluded that removal of the smear layer facilitated passive penetration of *Streptococcus faecium* into the dentinal tubules *in vitro*.

Evans and Simon (1986) reported that the presence or absence of smear layer had no significant effect on the apical seal of injected thermoplasticized gutta-percha or lateral condensation with and without root canal sealer. Love *et al.* (1996) investigated the penetration of smeared and nonsmeared dentine by *Streptococcus gordonii*. The SEM, histological and fluid filtration results indicated that the organism did not cause dissolution of the smear layer and that the smear layer was an effective barrier to tubule invasion. Furthermore, Love (1996) has shown that dentinal smear layers did not enhance or impede bacterial adherence to the dentine matrix. The presence of patent dentinal tubules significantly increased the number of bacteria retained on the surface of dentine.

1.6.1 Removal of the Smear Layer

1.6.1.1 *Chelating agents*

The most commonly used chelating solutions are based on ethylene diamine tetra-acetic acid (EDTA) which reacts with calcium ions in dentine and forms soluble calcium chelates (Grossman *et al.* 1988). Goldman *et al.* (1982) recommended alternate use of sodium hypochlorite (NaOCl) and ethylene diamine tetra-acetic acid (EDTA) to remove smeared dentine. The NaOCl removes organic material, including the collageneous matrix of dentine and EDTA removes the mineralised dentine, thereby exposing more collagen. The combined effect of 17% EDTA 10 mL followed by 10 mL of 5.25% NaOCl final irrigation after root canal preparation has been shown to effectively remove superficial debris and the smear layer (Yamada *et al.* 1983). In addition, Wennberg and Ørstavik (1990) found that treatment of the dentine with EDTA improved the bond strength of Tubliseal.

1.6.1.2 *Organic acids*

Citric acid appears to be effective in removing the smear layer in concentrations of 10%, 25% and 50% (Tidmarsh 1978, Wayman *et al.* 1979, Baumgartner *et al.* 1984, White *et al.* 1984). Its use as an irrigant is based on two properties:

- it acts as a chelating agent on dentine because of its low pH

- because it occurs naturally in the body, it is more acceptable biologically than other acids (Wayman *et al.* 1979).

Georgopoulou *et al.* (1994) have proved that 25% citric acid is bactericidal against anaerobic micro-organisms in the root canals especially against cocci, but it is less effective than 2.5% NaOCl. A study by Yamada *et al.* (1983) showed that a final flush with 25% citric acid followed by 5.25% NaOCl produced canal walls free of organic and inorganic debris similar to a final flush with 17% EDTA followed by 5.25% NaOCl. The citric acid group, however, left precipitated crystals in the root canal. Recently, Pileggi *et al.* (1995) evaluated apical leakage of ultrasonically cut retro-preparations using *Escherichia coli*. The results showed that treatment with 50% citric acid in retro-preparations restored with adhesive materials significantly increased bacterial penetration.

Garberoglio and Becce (1994) evaluated the effect of six endodontic irrigants on smear layer *in vitro* of 53 root canals using SEM. The irrigants were: 1% and 5% NaOCl, a combination of 24% phosphoric acid and 10% citric acid, 0.2%, 3% and 17% EDTA. The results revealed that NaOCl did not remove the smear layer, even when 5% NaOCl was scrubbed on the dentinal walls. The solution of 3% EDTA was as effective as phosphoric-citric acid and 17% EDTA, however, EDTA did not show the marked demineralising effect on the dentinal walls and tubules as the acidic solution.

When the smear layer is not removed, the durability of both coronal and apical seal should be evaluated over a long period, since it may disintegrate and dissolve, creating a void between the root canal wall and the sealer. However, it should also be borne in mind that there is a risk of re-infection of dentinal tubules by microleakage if the seal should fail after removal of the smear layer (Brannström 1984). To reduce

the risk of re-infection, but also to avoid the development of secondary caries, in permanent coronal restorations of root-filled teeth, the cavity should be treated in the same way as cavities in vital teeth. Further studies are certainly needed to establish a correlation between endodontic smear layer and clinical performance of root canal fillings.

1.7 FAILURE OF ROOT CANAL TREATMENT

Over the last 60 years many surveys have been carried out to determine the success or failure of conventional root canal treatment. In different studies the success rate ranges from 48% (Bergenholtz *et al.* 1979) to 95% (Ørstavik *et al.* 1987). The reasons for considerable variation are related to the design of the studies, the endodontic techniques employed, the complexity of the included cases, the observation period and the criteria used for evaluating treatment results (Stabholz *et al.* 1994).

1.7.1 Criteria for Definition of Treatment Results

Clinically, failure of root canal treatment is determined on the basis of radiographic findings and clinical signs and / or symptoms of the treated teeth. Bender *et al.* (1966) suggested the following criteria for successful endodontic therapy:

- absence of pain or swelling

- disappearance of fistula

- no loss of function

- no evidence of tissue destruction

- radiographic evidence of an eliminated or arrested area of rarefaction after 6 to 24 months.

The methods for recalling patients vary considerably within the areas of dentistry and among individual practitioners. Generally, with root canal treatment, review appointments are conducted 6 months after completion of treatment and then every 6 months postoperatively until 2 years after treatment (Ingle *et al.* 1976). Ideally, recall examination and evaluation should take place for a minimum of 4 years, especially in questionable cases (Reit 1987).

Since the radiographic evaluation plays a basic role in the assessment of treatment results, any fallibility associated with the interpretation of radiographs directly distorts the reported rates of success and failure. According to Stabholz *et al.* (1994), the factors that influence fallibility are:

- change in angulation
- quality of film
- lack of radiographic changes
- proximity to anatomic landmarks
- radiolucency of periradicular scar tissue
- personal bias and disagreement between different interpreters.

Reit and Gröndahl (1983) elucidated the significance of observer performance (3 endodontists and 3 radiologists) on radiographic evaluation of periradicular conditions after root canal treatment therapy and on evaluation of root filling seal

quality. It was found that in diagnosing the periradicular tissues and the root filling seal, there was complete agreement in only 39% and 32% of 119 evaluated roots respectively. This could be compared with Goldman *et al.* (1972) who reached consensus in only 47% of the cases.

1.7.2 Causes of Root Canal Treatment Failures

1.7.2.1 *Preoperative causes*

Failure of root canal treatment can be the result of misdiagnosis, poor case selection, or a poor prognosis, all of which are preoperative considerations (Stabholz *et al.* 1994).

1.7.2.2 *Operative causes*

Operative errors caused approximately 76% of root canal treatment failures in the Washington study in 1965 (Ingle *et al.* 1976), making them the major cause of endodontic treatment failure. These included:

- improper access cavity preparation which may lead to inadequate cleaning and shaping of the root canal wall or breakage of endodontic instruments

- level and quality of root canal fillings-poorly condensed obturation, either over-or under-fillings

- iatrogenic factors such as: mechanical perforations or ledging

- asepsis of treatment regimen.

1.7.2.3 *Postoperative causes*

Postoperative causes include:

- occlusal trauma and fracture

- improper final restorations

- periodontal failures.

The teeth that fail are predominantly affected by multiple factors (Matsumoto *et al.* 1987, Grossman 1988). Sjögren *et al.* (1990) studied the influence of various factors that may affect the outcome of root canal therapy in 356 patients 8 to 10 years after the treatment. The results of treatment were directly dependent on the preoperative status of the pulp and periradicular tissues. The possibility of instrumenting the root canal to its full length and the level of root filling significantly affected the outcome of treatment. This was also concluded by Petersson *et al.* (1986) who showed that in teeth with completely obturated root canals, only 7% had a periradicular lesion, as compared to 45% of the teeth with inadequately sealed root canals.

Matsumoto *et al.* (1987) investigated the correlation between prognosis and various factors influencing repair by using clinical and radiographic follow-up evaluations of 223 root canal treated teeth. There was no statistically significant difference between success of repair in teeth yielding positive canal cultures. Factors influencing failure included deep periodontal pockets, periradicular rarefactions, occlusal trauma and teeth with only one or no adjacent teeth.

Byström et al. (1987) evaluated healing of periradicular lesions of pulpless teeth after root canal treatment with controlled asepsis. The canal was not root obturated until all detectable bacteria had been eliminated. Healing of the periradicular lesions of the teeth was followed for 2 to 5 years. The majority of the 79 lesions healed completely or decreased in size. Lin et al. (1991) observed the causes of 150 cases of endodontic treatment failure. They found that there was no correlation between the size of periradicular rarefaction and the occurrence or severity of clinical signs and / or symptoms. Stainable bacteria were demonstrated in 69% of the teeth and were present mostly in the canal. Swelling and pain or a draining sinus tract was often associated with stainable bacteria inside the canal.

Furthermore, Lin et al. (1992) studied 236 cases of endodontic treatment failure and demonstrated that there was a correlation between bacterial infections in the canal system and the presence of periradicular rarefaction. The apical extent of the root canal filling e.g. underfilled, flushfilled, or overfilled, seemed to have no correlation to treatment failure. In contrast, Ödesjö et al. (1990) have shown that, from 967 cases, excess root filling material was related to a significantly higher frequency of periradicular lesions.

Swartz et al. (1983) evaluated 1,007 treated teeth and suggested that a lower success rate was associated with overfilled canals, canals with pre-existing rarefaction, and teeth not properly restored following completion of root canal therapy. Vire (1991) evaluated 116 teeth over a 1-year period. Of the teeth, 59.4% were prosthetic failures which were due primarily to crown fracture. Periodontal

failures constituted 32% and only 8.6% of the failures were due to endodontic causes, but these failures became evident more quickly than those in the other categories.

1.7.3 Roles of Leakage

The most common cause of failure of root canal treatment was considered to be apical leakage around inadequate root fillings (Allen 1964). An inflammatory reaction is initiated by the percolation of periradicular tissue fluid, bacteria and their metabolic end-products, which may result in clinical or radiographic signs of failure.

It is also important to seal the coronal part of the root canal, including the pulp chamber, from salivary contamination. This is particularly important in multirooted teeth, where accessory canals may be present (Vertucci & Anthony 1986). The latter can be responsible for inflammatory changes occurring in the periodontal tissues due to direct spread from infected remnants within the pulp chamber (Sinai & Soltanoff 1973, Gutmann 1978). Saunders and Saunders (1990b) showed that coronal leakage was a significant problem in root-filled molars. They demonstrated that the common practice of packing excess gutta-percha and root canal sealer over the floor of the pulp chamber after completion of lateral condensation, did not provide an adequate coronal seal of the root canals.

Swanson and Madison (1987) have shown *in vitro* that root-filled teeth exposed to artificial saliva for various time periods exhibited extensive coronal leakage.

Madison and Wilcox (1988) confirmed that exposure of root canals to the oral environment allowed coronal leakage to take place, in some cases, along the whole length of the root canal. Torabinejad *et al.* (1990), in an *in vitro* study, found that over 50% of root canals were completely contaminated after 19 days or 42 days exposure to a microbial marker, depending on the micro-organism. Magura *et al.* (1991) recommended that obturated root canals that had been exposed to the oral environment for longer than 3 months should be root canal retreated prior to placement of a permanent coronal restoration.

Recently, Ray and Trope (1995) evaluated the relationship of the quality of the coronal restoration and of the root canal obturation on the radiographic periradicular status of endodontically treated teeth. In 1,010 endodontically treated teeth examined radiographically, it was shown that the technical quality of the coronal restoration was significantly more important than the technical quality of the endodontic treatment for apical periodontal health.

There is little doubt that an adequately sealed root canal is necessary to obtain consistent, long-lasting endodontic success. Because of its importance, the quality of the seal of endodontically treated teeth has been studied extensively. It is generally accepted that evaluation of the leakage between a root filling and the root canal wall is a necessary test to ascertain whether a root canal filling fulfils its purpose.

1.8 ASSESSMENT OF MICROLEAKAGE

Over the last 20 years, an increasing number of leakage studies have been published in the endodontic literature. Leakage along root canal fillings may be expected to occur between sealer and dentine, sealer and root filling materials or through the sealer itself. Many different techniques have been used to detect this leakage using bacteria, fluids, molecules or ions. The test methods used to evaluate the sealing ability of root filling materials *in vitro* are usually based on the assessment of penetration of a tracer along the root canal of an extracted, human tooth.

1.8.1 Dye Tracers

The use of organic dyes as tracers is one of the oldest methods of detecting leakage *in vitro*. It represents a simple and inexpensive technique and is still the most popular. The advantages of dye penetration studies are that:

- it is a simple and inexpensive technique

- may be non-destructive and can demonstrate the leakage without the need for a chemical reaction or exposure to hazardous materials.

Dye penetration studies have utilised various dyes, at differing concentrations and varying the length of immersion time (Table 1.1). Methylene blue dye appears to be the most widely employed tracer. Advantages of aqueous methylene blue dye are:

- it easily penetrates the water compartment of the tooth

- does not stain the hard tissue

Table 1.1 Examples of dye penetration studies at differing concentrations and varying periods of immersion.

Dye	Immersion period	Authors	Year
0.5% Basic fuchsin	24h	Barthel *et al.*	1994
1.0% Crystal violet	3h	Goldman *et al.*	1989
1.0% Eosin	48h	Pitt Ford & Rowe	1989
India ink	90h	Saunders & Saunders	1994
India ink	9d	Lloyd *et al.*	1995
0.25% Methylene blue	1wk	Greene *et al.*	1990
0.5% Methylene blue	2wk	Oguntebi & Shen	1992
2.0% Methylene blue	3h	Peters & Harrison	1992
5.0% Methylene blue	48h	Dummer *et al.*	1993
Pelikan ink	48h	Swanson & Madison	1987
Procion blue	5d	Barkins & Montgomery	1992
5% Prussian blue	1wk	Chohayeb	1992

- is readily detected under visible light (Matloff *et al.* 1982, Limkangwalmongkol *et al.* 1991).

In contrast, an aqueous solution of methylene blue dye is acidic unless buffered and if used in dye studies it may demineralise dental hard tissues leading to an increase in penetration. Comparison of some of the studies on linear measurements of dye penetration showed that there appears to be a high level of variation although the experimental methods used were quite similar (Wu & Wesselink 1993).

Different techniques based upon dye immersion have been used.

1.8.1.1 *Passive dye penetration*

The mechanism of dye penetration is by capillary action and diffusion. When a void along a root filling is dry, capillary action may occur, whilst diffusion of dye can take place when the void is filled with liquid. The depth of dye penetration by capillary action depends upon the diameter of the void and the hydrophobicity of dentine and the filling materials (O'Brien *et al.* 1968). In root canal treatment, the capillary force is modified by the fact that the capillary lumen is closed at one end.

1.8.1.2 *Centrifugation*

Centrifuging works by effectively increasing the hydrostatic pressure of the dye solution and by compressing the air in the voids, thus allowing the dye to penetrate further (Oliver & Abbott 1991). A similar effect may be achieved in passive techniques by immersing specimens to a greater depth in the dye solution. However, in a recent

study no significant difference in penetration depth of India ink was found between obturated teeth that were centrifuged in ink and those that were only immersed in ink (Karagöz-Kücükay *et al.* 1993).

1.8.1.3 *Reduced pressure technique*

Goldman *et al.* (1989) reviewed the erratic and inconsistent findings of dye penetration studies in the endodontic literature and concluded that quantitative evaluation of leakage is meaningless unless entrapped air is totally evacuated from the voids. Dye penetration by capillary force will not occur further at the point where a balance is reached between the collapse pressure and the surface tension of the fluid. A study by Spångberg *et al.* (1989) showed that passive dye penetration resulted in incomplete filling of the voids regardless of size of the voids, whereas vacuum dye delivery resulted in complete filling of the voids.

Oliver and Abbott (1991) compared a vacuum technique, centrifugation and passive dye penetration by using capillary tubes with voids in the lumen. They found that the vacuum technique showed 100 per cent penetration in all cases whilst centrifuging had a mean of 91.7 ± 8.7 per cent penetration and use of the passive dye had a mean of 20.7 ± 5.4 per cent penetration. However, many researchers (Peters & Harrison 1992, Dalat & Spångberg 1994, Masters *et al.* 1995) failed to find any differences. Whether entrapped air can be eliminated by reduced pressure or not remains questionable. In addition, a reduced pressure technique is a severe test of leakage and not relevant clinically.

More recently, Pathomvanich and Edmunds (1996) studied the variation in the microleakage produced by four different techniques: passive dye penetration alone, centrifugation alone, vacuum plus passive dye penetration and increased pressure plus passive dye penetration in simulated root canals. The results showed that there were large variations in the leakage in each individual specimen and between the specimens within each group. There were statistically significant differences between passive dye penetration and each of the other three techniques but there were no significant differences between the centrifugation, vacuum and pressure techniques.

1.8.2 Chemical Tracers

This technique involves the use of two colourless compounds to produce an opaque precipitate. Usually, the *in vitro* specimen is immersed in a 50 per cent silver nitrate solution and then reacted with a photographic developer. A dark area of precipitated silver ions is seen where leakage has occurred (Hovland & Dumsha 1985). The main problem with the interpretation of these results is that a single section of the tooth provides at best a two-dimensional representation of the three-dimensional restoration.

1.8.3 Radioisotope Studies

The leakage studies with radioisotopes as tracers have been used in a manner similar to that of dyes. After removal from the isotope solution, the obturated teeth are sectioned longitudinally through the root canal by grinding and placed on dental x-ray films to produce autoradiographs (Matloff *et al.* 1982). Factors such as type of isotope, distance between radiation source and emulsion, the length of exposure time and the development of the autoradiographs can affect the results obtained with this technique.

The most widely used radioisotope in marginal leakage studies is ^{45}Ca as CaCl$_2$ because it is easily obtained and being a weak beta emitter, it produces sharp autoradiographs (Delivanis & Chapman 1982).

Matloff *et al.* (1982) compared the marginal leakage of root canal fillings using methylene blue dye, ^{45}Ca, ^{14}C-labelled urea and ^{125}I-labelled albumin. They found that methylene blue penetrated further than any isotope tracers with both ^{45}Ca and ^{125}I penetrating approximately half as far as methylene blue. ^{14}C penetrated further than ^{45}Ca and ^{125}I, approximately 65% that of methylene blue. However, a careful technique is necessary for sectioning and sanding the teeth to avoid spreading the tracers to areas where leakage has not occurred.

Canalda-Sahli *et al.* (1992) have described an external radionuclide technique for evaluation of the apical seal of root canal sealers. They submerged 2 mm of root apices in a solution containing the radioisotope ^{99}Tc, which is an emitter of pure gamma radiation. After 5 hours the teeth were removed from the solution and washed thoroughly in running water to remove tracers of the radionuclide from the external surface. A gamma camera was used to measure the radiation from the teeth. The detected image was sent to a computer to quantify the leakage in millimetres.

1.8.4 Scanning Electron Microscopy (SEM)

Scanning electron microscopy provides a direct view of the adaptation of root filling materials to the canal wall. It was used by Lifshitz *et al.* (1983) to assess Schilder's technique of root canal obturation. This method is suitable for studying irregular

characteristics of an internal surface because of the depth of the focal field and the degree of magnification available. However, observation of bacterial penetration using SEM is a qualitative method, since it is very difficult to quantify the penetration of micro-organisms in a longitudinal direction from the apical foramen (Canalda-Sahli *et al.* 1992).

The disadvantages of this technique are that the SEM can be used to evaluate only an extracted tooth in a limited area and artefacts may be produced during specimen preparation which may lead to misinterpretation of the results. Zmener and Gimenes (1991) suggested that the gap found between the filling material and dentinal walls was because of shrinkage of the gutta-percha as a consequence of dehydration of the specimens and exposure to vacuum during SEM examination. Jacobsen and Begole (1992) recommended a freeze-fracture technique to eliminate tearing of the gutta-percha during specimen preparation for SEM.

1.8.5 Electrochemical Studies

Jacobson and von Fraunhofer (1976) reported an electrochemical technique which permitted the rapid quantitative evaluation of periapical leakage. The principle of this technique is that an electric current will flow between two pieces of metal of differing composition when both are immersed in an electrolyte and are connected by an external power source. A piece of stainless steel tape was immersed in a 1 per cent solution of potassium chloride, acting as the cathode in a galvanic cell whilst a copper wire was placed in the coronal portion of a root filled tooth, to act as the anode. When leakage occurred, an electrolytic pathway was established between the two electrodes.

The magnitude of the current flow could be used to measure the degree of microleakage.

This technique offered a quantitative measurement and gave an opportunity to study leakage continuously whilst dye penetration and radioisotope studies could not. Delivanis and Chapman (1982) demonstrated that the electrochemical method was superior to dyes or autoradiographic methods. However, there are some limitations: it is destructive of tooth substance and cannot be used *in vivo*. Furthermore, apical microleakage might occur within the root canal space without establishing contact with the detector electrode resulting in false negative readings.

1.8.6 Liquid Pressure Technique

This technique was developed by Pashley *et al.* (1983) for measuring dentine permeability and permitted quantitative measurements over time in individual samples. This method has been used to evaluate the seal of endodontic retrograde fillings from the both apical and coronal directions by Yoshimura *et al.* in 1990. A stainless steel tube is sealed to either the coronal or the apical root canal orifice. The tubing is connected to a pressure reservoir containing coloured phosphate buffered saline via a micropipette and a microsyringe. If the fillings permit leakage the saline will flow through the micropipette which contains an air bubble. By measuring the movement of the air bubble the flow of saline can be quantified. The microsyringe is used to adjust the position of the air bubble between measurements.

Wu *et al.* (1993) have modified this method for evaluating leakage along obturated root canals. They mounted the obturated root between the pressurised liquid reservoir and the micropipette. Thus, they measured the volume of the fluid that passed out of the apical end of the obturated root canal rather than that which moved into the coronal end. The use of a pressure system to detect differences in sealing quality appears to be effective (Derkson *et al.* 1986) although it is not suitable for simulating clinical circumstances.

1.8.7 Bacteria and Bacterial Metabolites

Since micro-organisms and their end-products are considered the major causes of pulpal and periradicular pathosis (Nair *et al.* 1990) and there are inherent inadequacies associated with dye studies (Mortensen *et al.* 1965, Krakow *et al.* 1977), penetration of micro-organisms has been considered as more relevant than tracer penetration. Goldman *et al.* (1980) described a test method where bacteria were used to test the sealing ability of root canal filling material *in vitro*. They emphasised that using bacteria instead of a small molecular dye reduced the chances of false readings in testing for leakage of hydrophilic materials.

Khayat *et al.* (1993) have shown that all root canal fillings were contaminated apically with bacteria from saliva exposed to the coronal part of the root canal within 30 days of exposure. Magura *et al.* (1991) examined salivary penetration through obturated root canals using histological examination and dye penetration. They reported that saliva penetration at 90 days was significantly greater than that seen after 2, 7, 14 and

28 days. They also found that saliva penetration assessed in histological sections was significantly less than that visualised with dye analysis.

A large number of bacteria species have been used to measure leakage in many endodontic studies shown in Table 1.2. However, the absence of bacteria does not mean that there is no deleterious effect. Marginal gaps smaller than bacterial size (0.5-1 mm) will not allow bacterial penetration but will allow their toxins and end-products to enter. Kersten and Moorer (1989) compared the ability of four obturation methods to prevent leakage of bacteria-sized particles (latex beads) or large protein molecules (endotoxin). Secondly, leakage of the commonly-used dye, methylene blue, was comparable with that of a small bacterial metabolic product of similar molecular size (butyric acid). Their findings showed that microleakage of the small molecules could not be prevented, whereas leakage of bacteria-sized particles and large-sized protein molecules could be prevented only when both sealer and pressure were used in obturation techniques.

Horiba *et al.* (1990) studied the distribution of endotoxin in the dentinal walls of infected root canals. The endotoxin content was significantly higher in samples from the pulpal surface of the root canals to 300 μm in depth than from specimens taken near the cementum. Horiba *et al.* (1991) also found that the endotoxin content from the root canals of 30 teeth with apical periodontitis was higher in symptomatic teeth, teeth with apical rarefactions and teeth with an exudate.

Table 1.2 Examples of micro-organism species that have been used to measure leakage in endodontic studies.

Species	Exposure period	Authors	Year
Proteus mirabilis	42d	Goldman *et al.*	1980
Streptococcus salivarius			
Proteus mirabilis	7d	Kos *et al.*	1982
Streptococcus salivarius			
Proteus vulgaris	38d		
Streptococcus faecium	3wk	Safavi *et al.*	1989
Proteus vulgaris	73d	Torabinejad *et al.*	1990
Staphylococcus epidermidis	51d		
Streptococcus anginosus	21d	Gish *et al.*	1993
Actinomyces viscosus			
Lactobacillus casei			
Streptococcus anginosus	90d	Gish *et al.*	1994
Streptococcus salivarius	45d	Dharananidhikul *et al.*	1995
Escherichia coli	72h	Pileggi *et al.*	1995
Staphylococcus epidermidis	30d	Moshonov *et al.*	1995a
Staphylococcus epidermidis	80d	Moshonov *et al.*	1995b
Staphylococcus epidermidis	90d	Torabinejad *et al.*	1995
Proteus vulgaris	21d	Behrend *et al.*	1996

Trope *et al.* (1995) evaluated the penetration of bacterial endotoxin (Lipopolysaccharide) through obturated root canals. The samples were tested for presence of endotoxin after 24 hours and then every 3 days for a period of 21 days by Limulus lysate assay. They concluded that the obturated root canal was not able to inhibit leakage of endotoxin in approximately 30 per cent of the root canals after 21 days. The importance of the sealer in root canal obturation was also confirmed by the fact that all obturated root canals without sealer allowed the endotoxin to pass through within 24 hours. The difficulty with tests involving endotoxin is the possibility of some extraneous bacterial contamination during processing which cannot be excluded entirely (Kersten & Moorer 1989, Trope *et al.* 1995).

1.8.8 Assessment of Depth of Penetration

1.8.8.1 *Semi-quantitative data*

The most popular method is linear measurement. This method is based on the supposition that linear tracer penetration can indicate the length of the gap appearing between the root filling and the root canal wall (Wu *et al.* 1993). Measurements are made after different methods of sample preparation:

Cross-and longitudinal sectioning of the teeth

Longitudinal sectioning of the roots provides a better view to determine the depth of dye penetration whilst cross-sectioning of the roots allows evaluation of other qualities of root canal filling materials such as the amount of sealer present in the filling, the

presence and diameter of open spaces or how well the root canal filling materials are adapted to the canal wall (Kersten *et al.* 1986, Limkangwalmongkol *et al.* 1991).

However, the use of either of these techniques does not allow a three-dimensional assessment of leakage to be made, therefore the results are difficult to evaluate and extrapolate clinically. The cutting blade may heat and plasticize the gutta-percha during the sectioning procedure and interfere with the detection of the dye. Furthermore, the specimen is irreversibly altered as a result of sectioning.

Clearing technique

The advantage of this technique is that the whole tooth can be examined by direct vision, thereby developing a better understanding of root canal anatomy and the relationship of the root filling materials to the canal walls. This simple and inexpensive technique was first described by Robertson *et al.* (1980). They used nitric acid to decalcify the teeth, followed by dehydration in ascending concentration of ethyl alcohol. The specimens were then cleared and stored in methyl salicylate. Contrast between the canal system and the dentine was obtained with haematoxylin or India ink. The use of nitric acid, however, may affect certain dye tracers, for example methylene blue dye, and interfere with detection of the dye.

1.8.8.2 *Quantitative data*

Douglas and Zakariasen introduced a volumetric analysis technique in 1981. The teeth were immersed in 2 per cent methylene blue dye and the excess dye was removed. Each tooth was then dissolved in dilute nitric acid to return the dye to solution. The

resulting solution was analysed spectrophotometrically and the dye concentration determined. Knowing the dye concentrations and volumes of the test solution, the volume of leakage into the obturated canals was calculated. Kersten *et al.* (1988) developed a leakage model in which the dye under pressure from a coronal reservoir passed through the voids of a root canal filling and was collected in an apical reservoir. This volume was determined by spectrophotometric analysis.

Various methods have been used for assessing apical and coronal leakage experimentally. However experimental studies cannot exactly reproduce clinical conditions and the relationship of *in vitro* leakage measurements to the *in vivo* situation has not yet been established. The most reasonable way of testing the efficacy of leakage is extrapolation of the data obtained from *in vitro* studies to clinical conditions and long-term clinical evaluation of the results.

CHAPTER 2 PRELIMINARY PROCEDURES

2.1 PREPARATION OF THE LEAKAGE MARKERS

This study used: 1. Three different species of micro-organisms:

- *Streptococcus sanguis*

- *Fusobacterium nucleatum*

- *Prevotella intermedia*

2. Mixed micro-organisms:

- *Streptococcus sanguis* and *Prevotella intermedia*

3. Lipopolysaccharide

as markers for the investigation of coronal leakage of root canal filling materials. These markers were selected because they are believed to be involved in the pathogenesis of periradicular lesions (van Winkelhoff *et al.* 1985, Yoshida *et al.* 1987, Iwu *et al.* 1990, Baumgartner & Falkler 1991, Brook *et al.* 1991, Horiba *et al.* 1991, Sundqvist 1992, Wayman *et al.* 1992, Yamasaki *et al.* 1992, Gomes *et al.* 1994a, Tani-Ishii *et al.* 1994).

2.1.1 *Streptococcus sanguis*

Streptococcus sanguis is a Gram-positive coccus, non-motile and non-spore forming facultative anaerobe. This strain was chosen because of the ability of these bacteria to adhere to the hard tissues (Ørstavik *et al.* 1974). It is also associated with dental plaque and is occasionally found in periradicular lesions (Iwu *et al.* 1990, Wayman *et al.* 1992, Tani-Ishii *et al.* 1994). This organism tends

to form chains and, in the event of prolonged incubation, clusters. In size, it usually ranges from 0.4-0.6 µm (Fig. 2.1, 2.2).

2.1.2 *Fusobacterium nucleatum*

Fusobacterium nucleatum is an obligate anaerobic, non-motile and non-sporing Gram-negative fusiform rod (Fig. 2.3). This strain was chosen because they are part of the human normal oral flora and frequently found in mixed infections with other anaerobes and facultative anaerobes. (Haapasalo 1989, Baumgartner & Falkler 1991, Brook *et al.* 1991, Sundqvist 1992, Wayman *et al.* 1992). Cells are usually 5-10 µm long but shorter forms are often seen. Colonies on anaerobe blood agar are 1-2 mm in diameter, which are slightly convex with irregular margins (Fig. 2.4).

2.1.3 *Prevotella intermedia*

Prevotella intermedia is a black pigment-producing, obligate anaerobic, Gram-negative rod and usually 0.3-0.4 µm wide and 0.6-1 µm long (Fig. 2.5, 2.6). It was previously known as *Bacteroides intermedius*. In 1990, Shah and Collins reclassified the saccharolytic black-pigmented *Bacteroides* into a new genus, *Prevotella*. This strain is frequently found in infected root canals associated with clinical symptoms (Yoshida *et al.* 1987, Sundqvist *et al.* 1989, Gomes *et al.* 1994a). Some studies (van Winkelhoff *et al.* 1985, Sundqvist 1992, Haapasalo 1993) have shown that *Prevotella intermedia* is the most commonly found species in root canal infection.
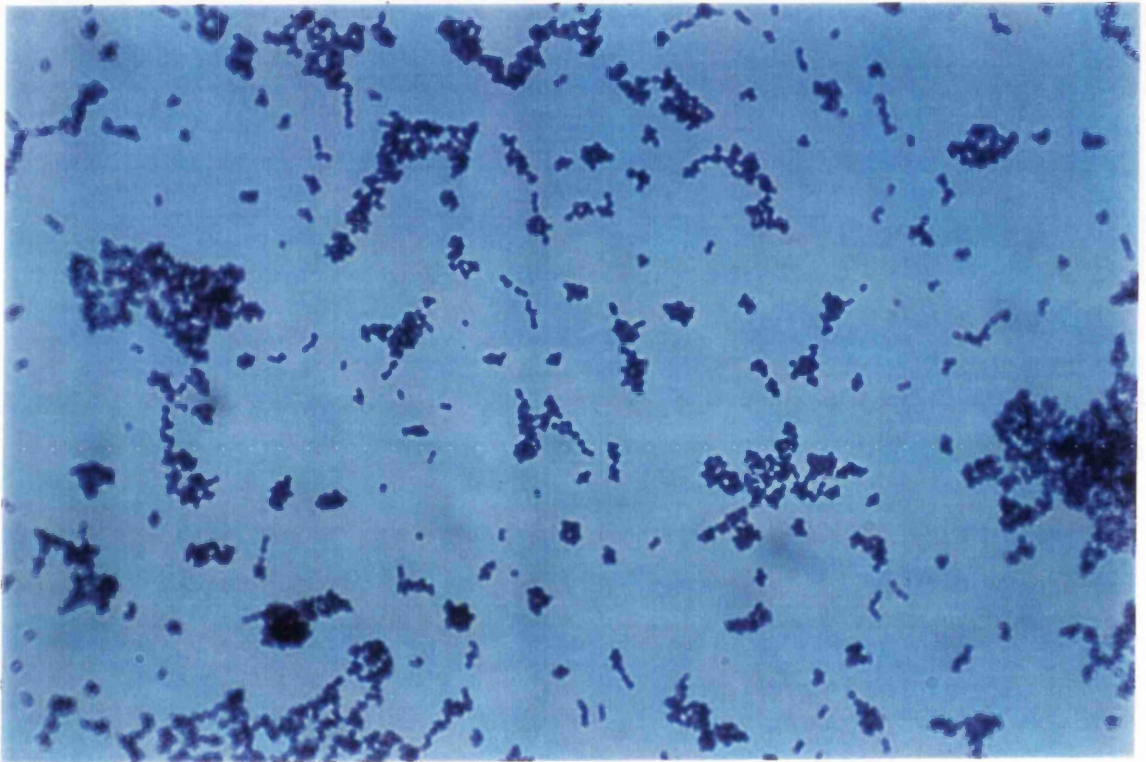
Fig. 2.1 Gram stain of *Streptococcus sanguis* from a 24-h colony on Columbia

blood agar, illustrating Gram-positive spherical or ovoid shapes in pair or chains.
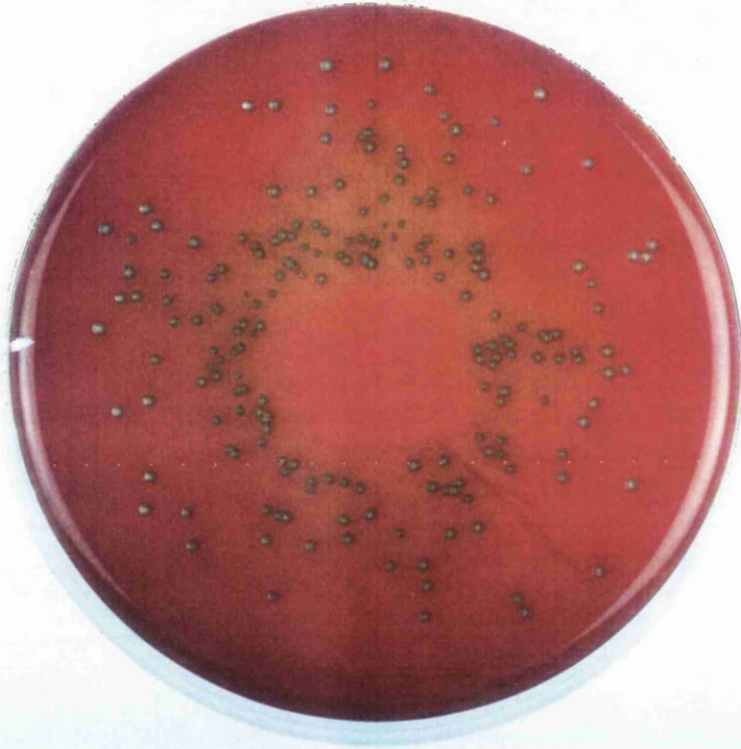
Fig. 2.2 Characteristic colonies of *Streptococcus sanguis* on Columbia blood agar
plate after 48 hours of incubation at 37°C. Note the α hemolysis on the agar.
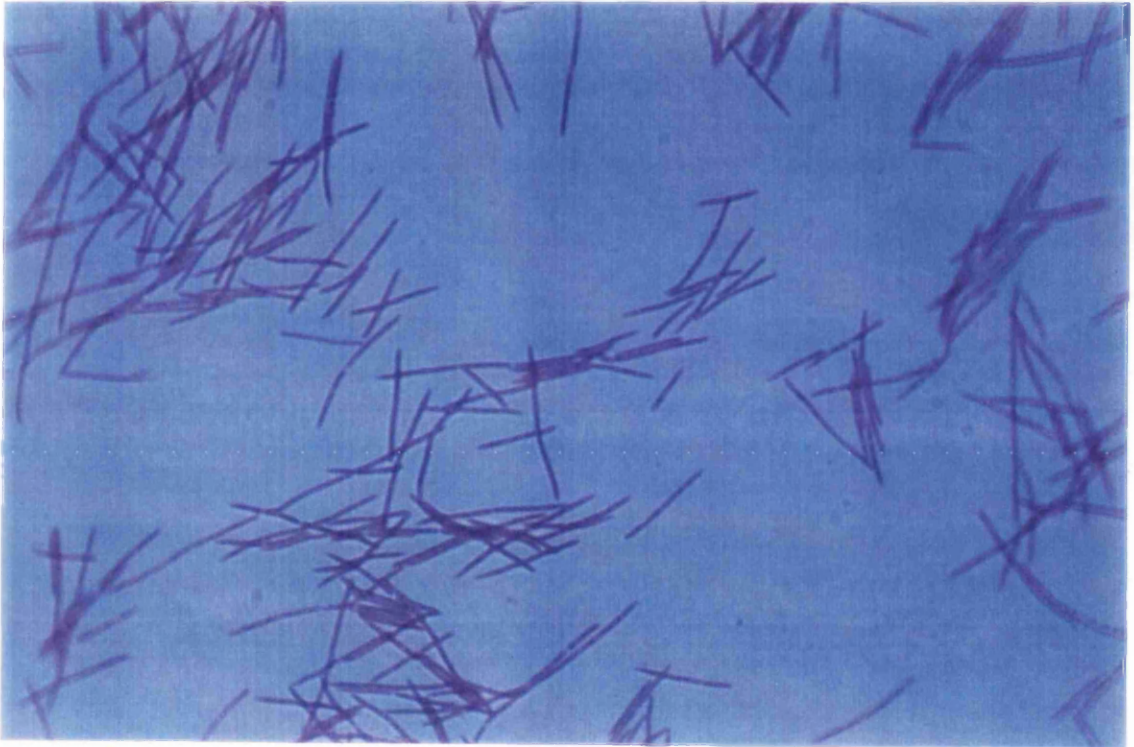
Fig. 2.3 Gram stain of *Fusobacterium nucleatum* from a 48-h colony on Columbia blood agar, illustrating Gram-negative fusiform rods.

Fig. 2.4 Characteristic colonies of *Fusobacterium nucleatum* on Columbia blood agar plate after 48 hours incubation in an anaerobic incubator at 37°C.
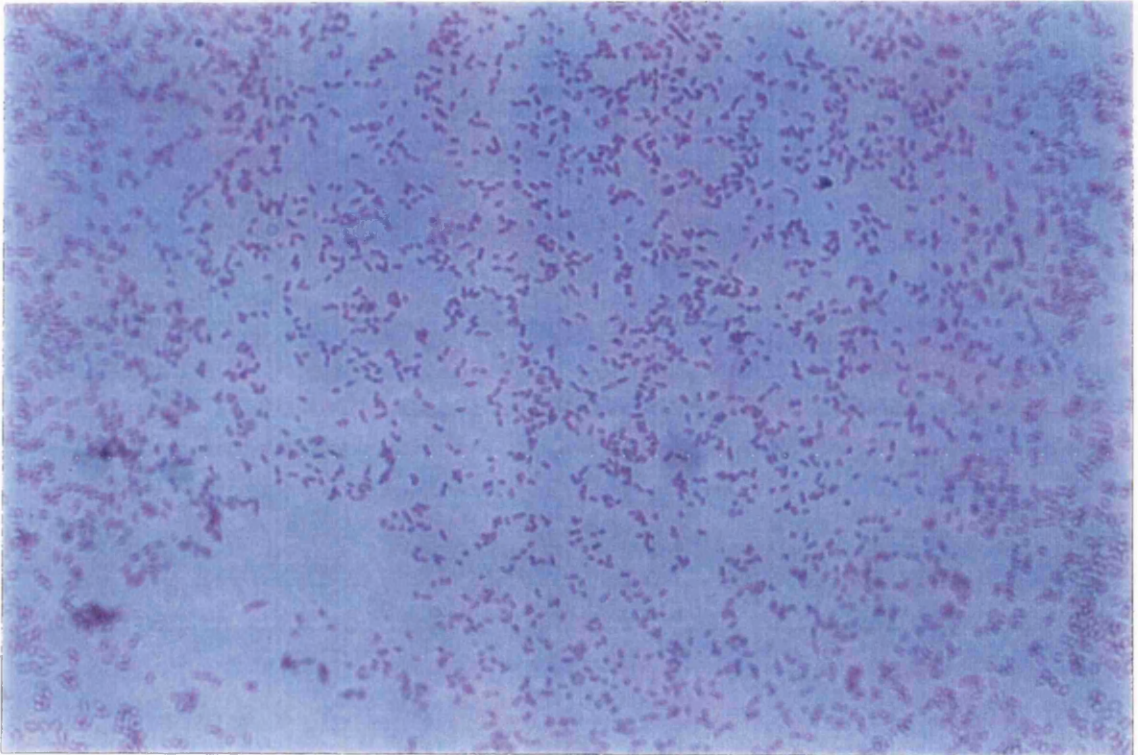
Fig. 2.5 Gram stain of *Prevotella intermedia* from a 48-h colony on enhanced
blood agar, illustrating Gram-negative pleomorphic rods.
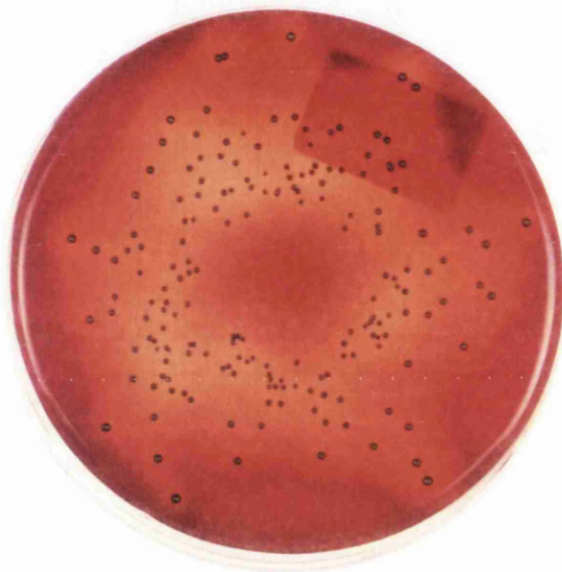
Fig. 2.6 Characteristic colonies of *Prevotella intermedia* on enhanced blood agar plate after 4 days incubation in an anaerobic incubator at 37°C. Note the black pigmentation and zone of hemolysis.

2.1.4 Mixed Micro-organisms

Mixed facultative anaerobic and obligate anaerobic micro-organisms are predominantly found in periradicular lesions dominated by anaerobic flora (Tronstad *et al.* 1987, Haapasalo 1989, Sundqvist *et al.* 1989, Brook *et al.* 1991, Haapasalo 1993). The number of different species in one canal is usually low, approximately 4-7 species (Haapasalo 1993). Several studies (Korzen *et al.* 1974, Fabricius *et al.* 1982a, Sundqvist *et al.* 1989, Baumgartner & Falkler 1992) showed that the degree of inflammation was less severe with a monoinfection than with a mixed infection. Gomes *et al.* (1994b) investigated the positive and negative associations between bacterial species in dental root canals. They found that there were positive and highly significant associations (P<0.01) between *Prevotella* species and *Streptococcus* species. Therefore, in this study, *Streptococcus sanguis and Prevotella intermedia* were chosen to represent the mixed microflora (Fig. 2.7).

2.1.5 Lipopolysaccharide

Lipopolysaccharide (LPS, endotoxin) is a structural component of the outer cell envelope of Gram-negative bacteria. It can be either secreted in vesicles by growing organisms or released after the death of the organisms. These molecules are heat-stable and contain polysaccharides and phospholipids. The lipid component (lipid A) determines the endotoxic properties of LPS. Researchers have shown that there is a significant relationship between the presence of endotoxin in periradicular lesions and the extent of inflammation (Dahlén & Bergenholtz 1980, Dwyer & Torabinejad 1981, Schonfeld *et al.* 1982, Horiba *et al.* 1991). Pinero *et al.*
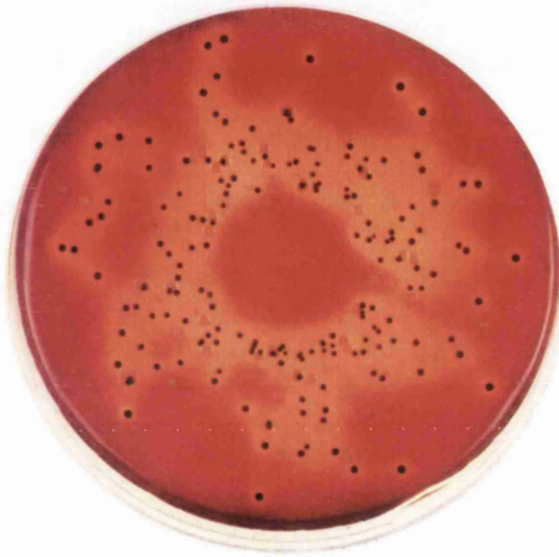
Fig. 2.7 Characteristic colonies of *Streptococcus sanguis* and *Prevotella intermedia* on enhanced blood agar plate after 4 days incubation in an anaerobic incubator at 37°C. Note the black pigmented colonies of *Prevotella intermedia*, the greyish colonies of *Streptococcus sanguis*.

(1983) reported that a high level of LPS was obviously toxic to connective tissue containing fibroblasts and the extracellular matrix and would result in tissue necrosis.

2.1.6 Source of Type Cultures

The *Streptococcus sanguis* and *Fusobacterium nucleatum* cultures were obtained from the National Collection of Type Cultures (NCTC) (Central Public Health Laboratory, London, UK). The *Prevotella intermedia* was obtained from the American Type Culture Collection (ATCC) (Rockville, MA, USA) and endotoxin standard (*Escherichia coli*: 0.55: B5 lipopolysaccharide) was obtained from Sigma Chemical Company (St. Louis, MO, USA).

2.1.7 Preparation for Cultures

2.1.7.1 *Streptococcus sanguis*

Preparation for *Streptococcus sanguis* cultures

Cultures of *Streptococcus sanguis* (NCTC 7864) were grown on Columbia agar plates (supplemented with 7.5% v/v defibrinated horse blood) and incubated in an LTE incubator (Laboratory Thermal Equipment, Oldham, UK) containing 5% CO_2 and 95% air at 37°C for 24 h. Using a sterile wire loop, 2 to 3 colonies of *Streptococcus sanguis* were inoculated into sterile Todd-Hewitt Broth (THB) (Unipath, Basingstoke, Hants, UK) and incubated in the LTE incubator at 37°C for 24 h. Cultures were then centrifuged at 1,500 **g** for 10 min in an MSE Super minor centrifuge (MSE, Crawley, Sussex, UK). The supernatant was poured

off and the suspension was mixed thoroughly with a Vortex mixer (Fisher Scientific, Leicester, UK). Finally, aliquots of 0.1 mL of the suspension were placed into 100 mL of THB. This method was repeated 4 times in order to standardise the inoculum to approximately 10^9 bacteria mL^{-1}.

<u>Viable microbial counts</u>

- Serial dilution method

This method consists of making consecutive dilutions of the culture and adding a known volume of these dilutions to an appropriate culture medium. In this study, serial dilutions were made in THB from neat to 10^{-8}.

The spiral plate method (Spiral Plater Model D, Spiral System Inc. 6740, Clough Pike, Cincinnati, OH, USA), was used to assess microbial counts. The model D spiral plater (Fig. 2.8) is a specialised dispenser which distributes 50 μL of sample onto the surface of a rotating 10-cm culture plate. The inoculating tip moves from the centre of the plate to the edge in an Archimedean spiral thereby creating a dilution effect and facility to count colonies easily. After incubation, colonies appear on the lines of the spiral inoculation and a plate that contains 100 to 300 colonies is chosen for counting. The microbial numbers are determined by counting the colonies on a suitable portion of the plate with the help of a counting grid. The major divisions on this grid are 4 concentric circles and 8 pie-shaped sectors, which result in a number of annular segments. Areas typically counted for 10-cm culture plate are shown in Fig. 2.9. A suitable sector is chosen and counted starting from the outer edge towards the centre. Usually, the same segments in the

Fig. 2.8 Spiral plater, Model D used for inoculating fluid bacterial cultures onto agar plates.
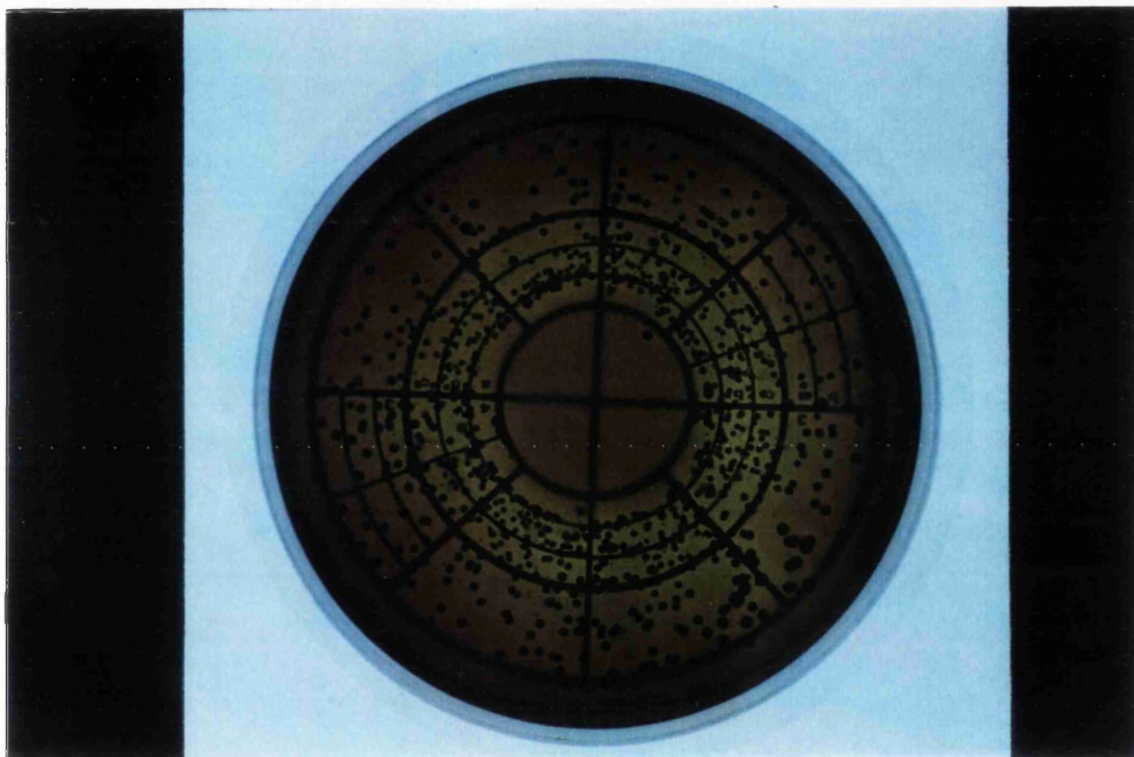
Fig. 2.9 A 10-cm spiral plater of culture plate with a manual grid underneath for counting bacterial colonies.

opposite sector are counted in order to balance any irregularities in the sample deposition. As 50 µL of the dilution is added to the culture plate, multiplying that colony count by the dilution and area counted will give the number of viable micro-organisms per mL in the original culture.

Maintenance of cultures

In order to maintain the viability of the culture of *Streptococcus sanguis*, the sample was incubated in an aerobic incubator (Gallenkamp, Loughborough, Leics, UK) at 37°C without replenishing the medium. The dilution and spiral plate methods were performed with this sample on day 3, 5 and 7. The result showed that the number of *Streptococcus sanguis* decreased from 10^9 mL^{-1} to 10^5 mL^{-1} on day 5, therefore, after this time half of the broth was removed and replaced.

2.1.7.2 *Fusobacterium nucleatum*

Preparation for *Fusobacterium nucleatum* cultures

Cultures of *Fusobacterium nucleatum* (NCTC 10596) were grown on Columbia agar plates (supplemented with 7.5% v/v defibrinated horse blood) and incubated in an anaerobic incubator (Don Whitley Scientific, Shipley, Yorkshire, UK) (Fig. 2.10) containing 85% N_2, 10% H_2 and 5% CO_2 at 37°C for 72 h. A heavy inoculum of *Fusobacterium nucleatum* was inoculated into sterile Fastidious Anaerobe Broth (FAB) (Lab M, Bury, UK) and incubated in the anaerobic incubator at 37°C for 48 h. Cultures were then centrifuged at 1,500 **g** for 10 min in an MSE Super minor centrifuge (MSE, Crawley, Sussex, UK). The supernatant
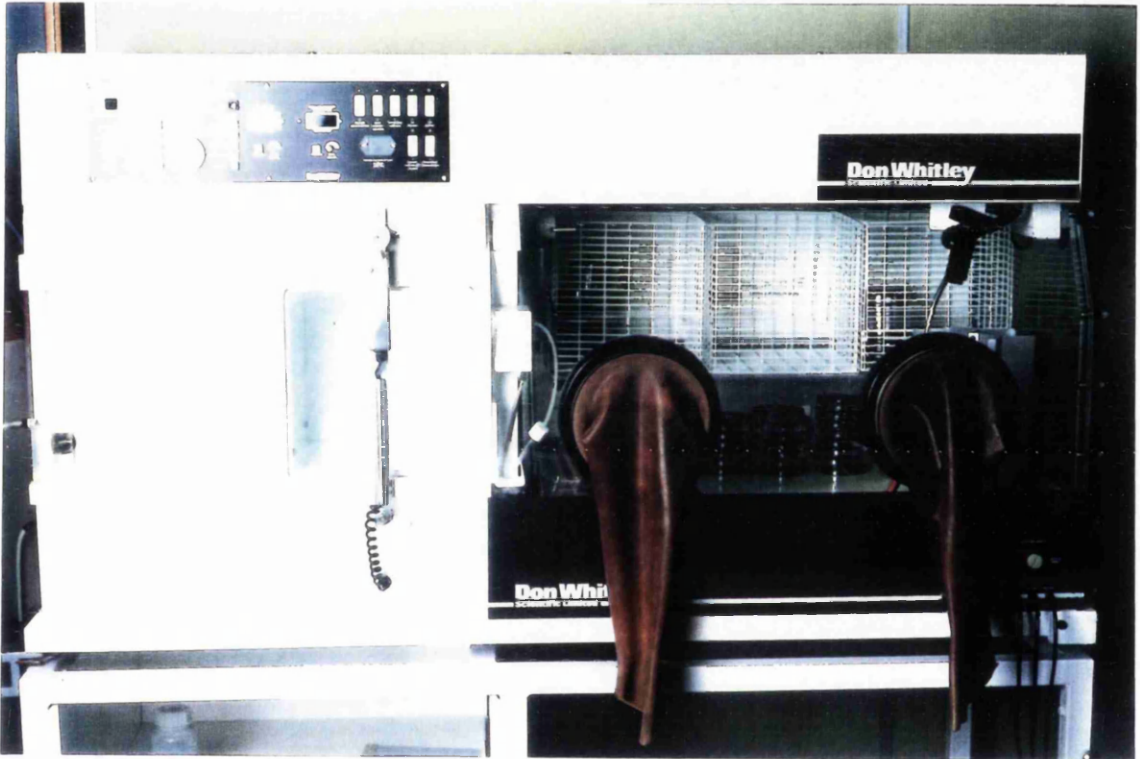
Fig. 2.10 Anaerobic incubator (Don Whitley Scientific). Materials are passed in and out of the chamber through an interchange compartment.

was poured off and the suspension was mixed thoroughly with a Vortex mixer (Fisher Scientific, Leicester, UK). Finally, aliquots of 0.2 mL of the suspension were placed into 100 mL of FAB. This method was repeated 4 times in order to standardise the inoculum to approximately 10^9 bacteria mL^{-1}. The method of counting colonies was the same as for the *Streptococcus sanguis* specimens.

Maintenance of cultures

In order to maintain the viability of the culture of *Fusobacterium nucleatum*, the sample was incubated in an anaerobic incubator at 37°C without replenishing the medium. The dilution and spiral plate methods were performed with this sample on day 3, 5 and 7. The result showed that the number of *Fusobacterium nucleatum* decreased from $10^9 mL^{-1}$ to $10^5 mL^{-1}$ on day 7, therefore, after this time half of the broth was removed and replaced.

2.1.7.3 Prevotella intermedia

Preparation for *Prevotella intermedia* cultures

Cultures of *Prevotella intermedia* (ATCC 25611) were grown on enhanced blood agar plates (supplemented with 7.5% v/v defibrinated horse blood and 1% v/v vitamin K/haemin solution) and incubated in an anaerobic incubator (Don Whitley Scientific, Shipley, Yorkshire, UK) containing 85% N_2, 10% H_2 and 5% CO_2 at 37°C for 72 h. A heavy inoculum of *Prevotella intermedia* was inoculated into sterile Brain Heart Infusion Broth (BHIB) (Pro-Lab Diagnostic, Neston, Cheshire, UK) plus 0.5 gL^{-1} cysteine hydrochloride (BDH Chemicals, Poole, UK) and

incubated in the anaerobic incubator at 37°C for 48 h. Cultures were then centrifuged at 1,500 **g** for 10 min in an MSE Super minor centrifuge (MSE, Crawley, Sussex, UK). The supernatant was poured off and the suspension was mixed thoroughly with a Vortex mixer (Fisher Scientific, Leicester, UK). Aliquots of 0.2 mL of the suspension were placed into 100 mL of BHIB plus cysteine. This method was repeated 4 times in order to standardise the inoculum to approximately 10^9 bacteria mL^{-1}. The method of counting colonies was the same as for the *Streptococcus sanguis* specimens.

Maintenance of cultures

In order to maintain the viability of the culture of *Prevotella intermedia*, the sample was incubated in an anaerobic incubator at 37°C without replenishing the medium. The dilution and spiral plate methods were performed with this sample on day 3, 5 and 7. The result showed that the number of *Prevotella intermedia* decreased from 10^9 mL^{-1} to 10^5 mL^{-1} on day 5, therefore, the broth was removed and replaced with fresh cultures at 5 d-intervals.

2.1.7.4 *Mixed micro-organisms*

Preparation for *Streptococcus sanguis* and *Prevotella intermedia* cultures

Cultures of *Streptococcus sanguis* (NCTC 7864) and *Prevotella intermedia* (ATCC 25611) were prepared as described previously. Aliquots of 0.1 mL of *Streptococcus sanguis* suspension and 0.2 mL of *Prevotella intermedia* suspension were placed into 100 mL of Brain Heart Infusion Broth (BHIB) (Pro-Lab

Diagnostic, Neston, Cheshire, UK) plus 0.5 g L^{-1} cysteine hydrochloride (BDH Chemicals, Poole, UK). This method was repeated 4 times in order to standardise the inoculum to approximately 2×10^9 bacteria mL^{-1}. The method of counting colonies was the same as for the *Streptococcus sanguis* specimens.

Maintenance of cultures

In order to maintain the viability of the culture of *Streptococcus sanguis and Prevotella intermedia*, the sample was incubated in an anaerobic incubator at 37°C without replenishing the medium. The dilution and spiral plate methods were performed with this sample on day 3, 5 and 7. The results showed that *Streptococcus sanguis* overgrew *Prevotella intermedia* on day 5, therefore, half of the broth was removed and replaced with fresh *Prevotella intermedia* cultures at 5 d-intervals.

2.1.7.5 *Lipopolysaccharide*

Preparation for lipopolysaccharide

Endotoxin standard (*Escherichia coli*: 0.55: B5 lipopolysaccharide) was diluted with endotoxin free water (Sigma Chemical Co., Poole, UK) to 800 E.U. mL^{-1}. Since endotoxin standard dilutions containing 400 or more E.U. mL^{-1} are generally stable for at least one week (Bulletin from Sigma Chemical Co.), the solution in each coronal chamber was removed and replaced with the fresh endotoxin standard weekly.

2.2 EXPERIMENTAL DESIGN

Five hundred extracted human teeth, with fully developed apices, straight roots and single root canals were used in this study. No data regarding age, sex or reason for extraction were available. Teeth with large, grossly carious lesions approaching the pulp were excluded to minimise the possibility of pre-operative bacterial contamination of the root canal. Bone, calculus or soft tissue on the root surface were removed with curettes. Teeth were stored in deionized water with a few thymol crystals at room temperature prior to the study.

Teeth were separated into three groups for three major studies.

- Smear layer study 120 teeth.

- Immediate study 140 teeth.

- Long-term storage study 240 teeth.

The experimental design is summarised in Fig. 2.11.
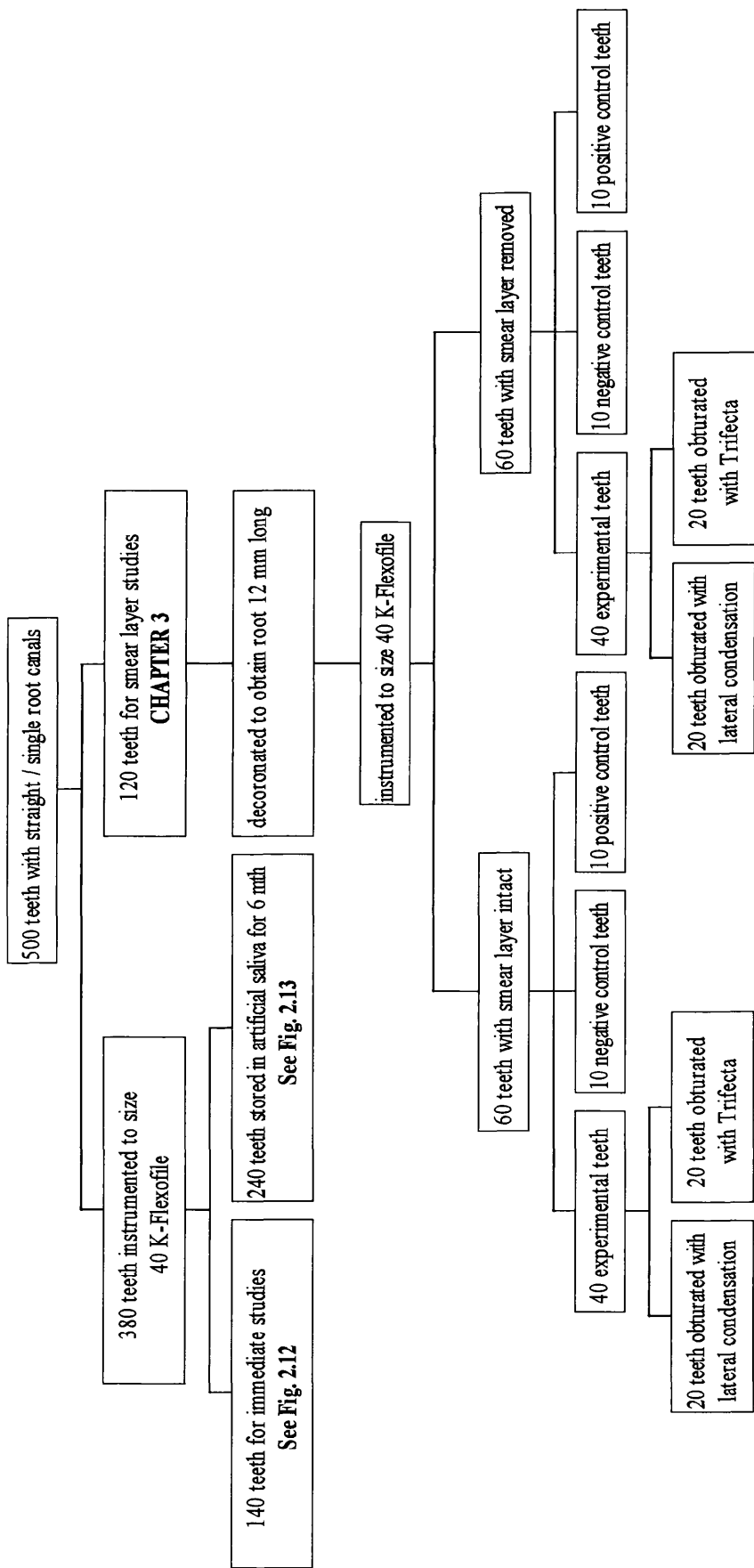
Fig. 2.11 Flow chart of the experimental design.
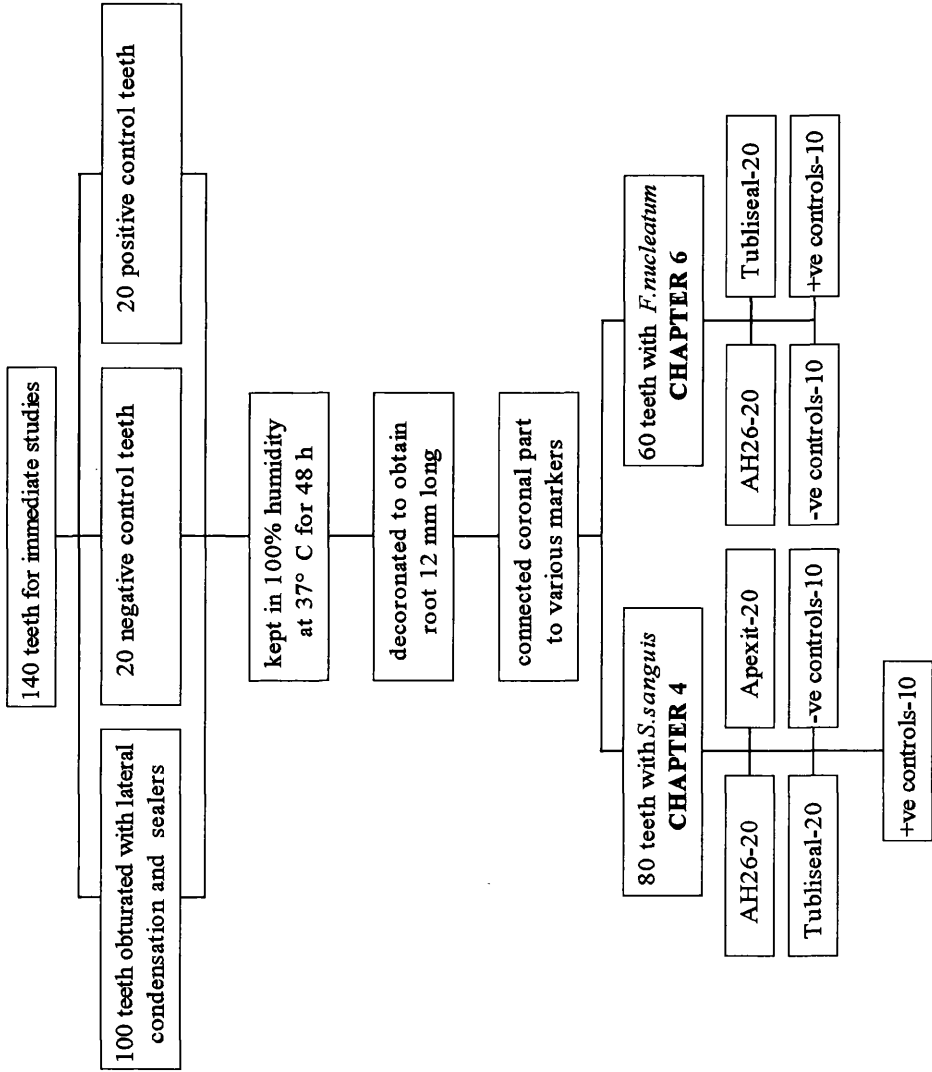
Fig. 2.12 Flow chart of the experimental design for immediate studies.
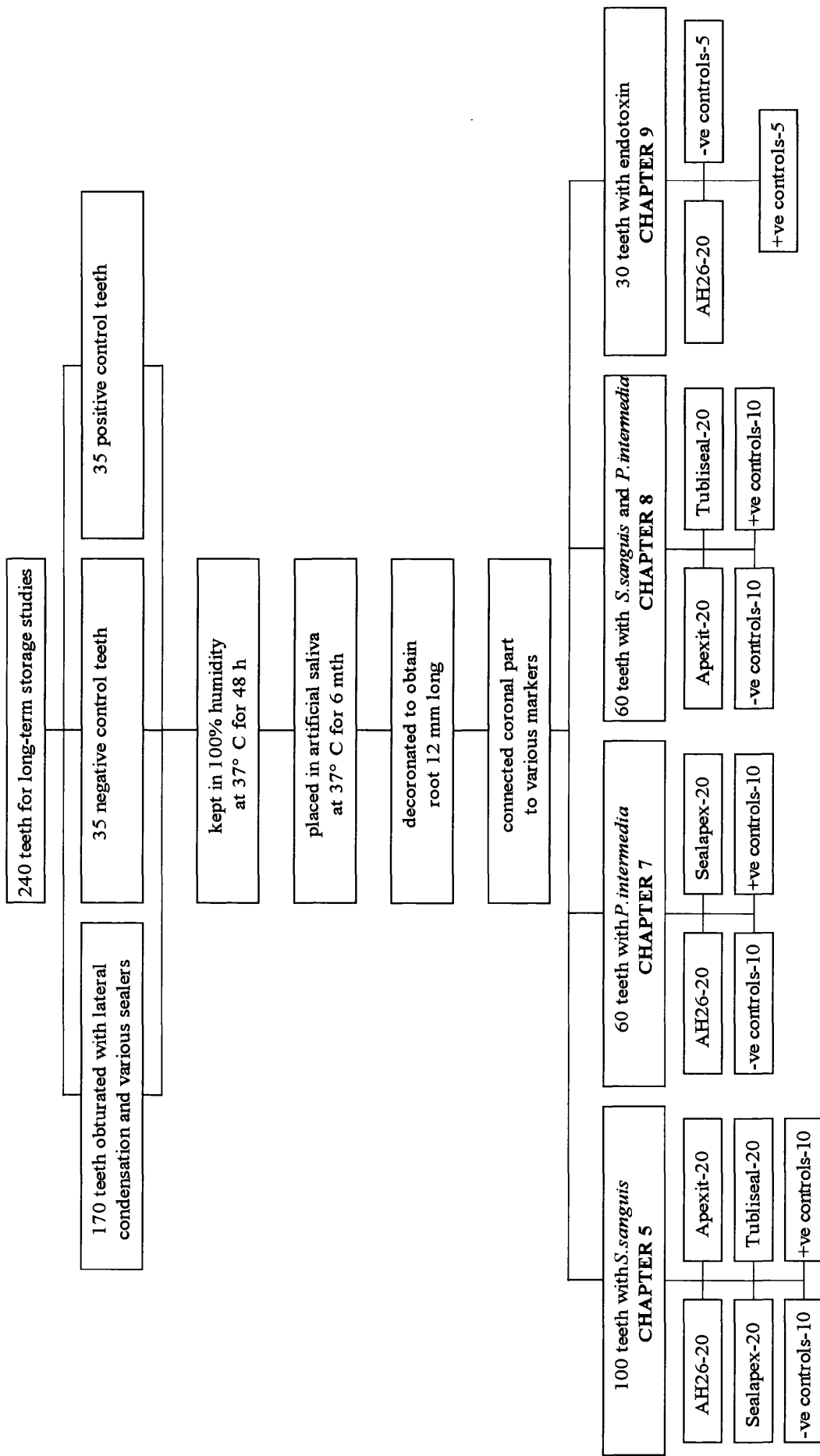
Fig. 2.13 Flow chart of the experimental design for long-term storage studies.

CHAPTER 3 THE EFFECT OF SMEAR LAYER ON MICROBIAL CORONAL LEAKAGE OF GUTTA-PERCHA ROOT FILLINGS

3.1 INTRODUCTION

In root canal treatment, when the root canals are instrumented, a layer of material composed of dentine, remnants of pulp tissue and odontoblastic process and sometimes micro-organisms, is always formed on the canal walls. This layer has been called the smear layer. The advantages and disadvantages of the presence of smear layer and whether it should be removed or not from the root canals remain controversial (Czonstkowsky *et al.* 1990).

In an attempt to elucidate further the influence that the smear layer may have on coronal leakage of root filled teeth, an *in vitro* study was instituted to investigate microbial penetration of obturated root canals in the presence or absence of the smear layer.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of Teeth

One hundred and twenty extracted human teeth were used in this study. Criteria for choosing and methods of storing the teeth were described previously (chapter 2). To maintain uniformity of root canal length, the crowns of all specimens were

cut with a diamond saw (Labcut, Agar Scientific, Stansted, UK) (Fig. 3.1) rotating

at approximately 300 rpm, with water coolant, to obtain a root 12 mm long. A size

15 K-Flexofile (Maillefer SA, Ballaigues, Switzerland) was placed into the root

canal until it just protruded through the apex. The working length was obtained by

subtracting 1 mm from that measurement. The root canal was prepared using the

modified double-flared technique with balanced force (Saunders & Saunders

1992a). New instruments were used after the preparation of 10 canals.

3.2.1.2 *The modified double-flared technique with balanced force*

A size 40 non-cutting tipped file was instrumented in the straight part of the canal,

using the balanced force method. This part of the canal was then enlarged sequentially,

up to a size 090 Gates-Glidden drill. Using the balanced force technique, a size 20 file

was taken to the working length. The apical section was prepared sequentially to a

MAF size 40 file. A stepback technique using balanced force was then used to prepare

the remaining curved portion of the canal. All instrumentation was accompanied by

lubrication with Hibiscrub (ICI Ltd., Macclesfield, UK) and copious irrigation with 2%

sodium hypochlorite (J. Sainsbury plc., London, UK) delivered in a luer lock

syringe (Optident, Skipton, Yorks, UK) fitted with a 27-gauge needle with a blunt

endodontic tip.

Canal preparation was considered to be complete, if the master apical file was able

to reach the working length without excessive force, with no rotation and a B

spreader (Maillefer) could fit passively in the canal to within 2 mm of the working

length. After preparation was complete, a size 15 file was passed 1 mm through

Fig. 3.1 Diamond saw (Labcut) for cutting the crowns of the experimental teeth.

the apex to remove any dentinal plug and to ensure patency of the foramen. The prepared teeth were divided randomly into experimental groups (80 teeth) and control groups (40 teeth). The experimental design is summarised in Fig. 2.11.

The smear layer on the root canal wall of 40 experimental and 20 control teeth was removed by injecting 8 mL of 40% citric acid slowly into each root canal for 2 min using a luer lock syringe (Optident) and a 27-gauge needle with a blunt endodontic tip. Final irrigation of all root canals was carried out with 3 mL of 2% sodium hypochlorite and the canals dried with sterile paper points.

3.2.2 Obturation

Experimental Group

The root canals of 20 teeth with smear layer intact and 20 teeth with smear layer removed were obturated with lateral condensation of cold gutta-percha and Apexit (Vivadent, Schaan, Liechtenstein) root canal sealer. A further 20 teeth with smear layer intact and 20 teeth with smear layer removed were obturated with thermally softened injectable gutta-percha (Trifecta, Hygenic Corp., Akron, OH, USA) with the same sealer.

Control Group

The root canals of 10 teeth with smear layer intact and 10 teeth with smear layer removed were obturated with lateral condensation of cold gutta-percha and Apexit (Vivadent) sealer. These teeth were used as the negative controls. The remaining

20 teeth were subdivided into two groups of 10 teeth each with either smear layer intact or smear layer removed. These teeth were not obturated and served as the positive controls.

3.2.2.1 *Lateral condensation of cold gutta-percha technique*

A master gutta-percha cone (Maillefer) which gave "tug back" at the working length was coated with the freshly-mixed root canal sealer and placed into the canal with a gentle pumping action. Accessory cones size B (Maillefer) were placed until the spreader could not penetrate to a depth of 2 mm in the root canal without excessive force. Excess root filling material was removed coronally with a scalpel, making the gutta-percha as level as possible with the surface of the root face.

3.2.2.2 *Trifecta technique*

The root canal was first coated with the freshly-mixed sealer using a size 35 file. A size 40 file was then coated with the softened gutta-percha Successfil from the syringe and placed into the root canal to the working length. The gutta-percha was deposited onto the wall of the root canal by moving the file anti-clockwise within the canal. This increment of gutta-percha was condensed with pluggers and the remainder of the root canal was obturated with injectable gutta-percha Ultrafil (Hygenic) regular set. Again the gutta-percha was condensed with pluggers until the material had cooled. Excess root filling material coronally was removed with a sharp scalpel. All teeth were kept in 100% humidity at 37°C for 48 h to allow setting of the sealer.

3.2.3 Preparation of Specimens

The coronal part of the root canals of each of the 120 teeth were connected with the cut end of a 2 mL polypropylene tube (Fisons Scientific Equipment, Loughborough, Leics, UK) using 2 layers of cyanoacrylate glue and sticky wax, to prevent leakage at the connection. Each layer of glue was dried at room temperature for 24 h. The sticky wax was applied after these 2 layers of glue. A pilot study had been done to compare several types of glue with and without sticky wax for prevention of bacterial leakage up to 90 d. The results showed that some types of glue including those containing epoxy resin did not adhere to the polypropylene tube. Using 2 layers of cyanoacrylate glue without sticky wax prevented bacterial leakage at the connection up to 60 d only whilst 2 layers of cyanoacrylate glue combined with sticky wax showed a bacterial tight seal throughout the experimental period.

The teeth and tubes at the connection were covered entirely with 2 layers of nail varnish (Maxfactor, Procter & Gamble, Cosmetics & Fragrances, Weybridge, Surrey, UK) except the apical 2 mm of the root. Each layer was dried at room temperature for 24 h. The 20 teeth that were to be used as the negative controls were covered completely with two layers of nail varnish, to include the apical portion of the tooth (Fig. 3.2). All specimens were then sterilised using ethylene oxide gas.

The leakage apparatus for each tooth was set up in a laminar airflow hood (MDH, Andover, UK) using sterile gloves. The polypropylene tubes were attached to a

Fig. 3.2 Negative control tooth was connected to a cut end of polypropylene tube with 2 layers of cyanoacrylate glue, a layer of sticky wax and 2 layers of nail varnish, including the apical portion of the tooth.

screw top (Fig. 3.3) which, in turn, was placed into a 30 mL sterile glass bottle.

Aliquots of 0.5 mL of Todd-Hewitt Broth (THB) (Unipath, Basingstoke, Hants,

UK) were injected into the polypropylene tube (coronal chamber) using a sterile

syringe and a disposable 22 gauge needle and a disposable 25 gauge needle as a

vent. The rubber top of each bottle was sterilised by wiping with alcohol and

flaming for 10 sec, the needles and syringes were changed after every 10

injections. Aliquots of 12 mL of THB were placed in the glass bottle (apical

chamber) to ensure that the apical portion of the root was placed in liquid (Fig.

3.4). In order to ensure that entrapped air within the root canal would not affect

bacterial penetration adversely, the model system was centrifuged (MSE, Crawley,

Sussex, UK) at 168 **g** for 5 min. Sterilisation of each device was checked by

placing it in an aerobic incubator (Gallenkamp, Loughborough, Leics, UK) at 37°C

for 48 h to check for turbidity in the apical chamber.

3.2.4 Bacterial Leakage

If bacterial contamination of the system was not noted the coronal chamber was

inoculated with 0.5 mL of THB containing approximately 10^9 bacteria mL^{-1}

Streptococcus sanguis (NCTC 7864). The method of inoculating *Streptococcus*

sanguis into the coronal chamber was the same as the method of injection THB

into the coronal chamber. A pilot study had indicated that bacteria were viable for

5 d without replenishing the medium and it was after this time that 0.5 mL of the

broth in each coronal chamber was removed and replaced with 0.5 mL sterile

THB. The discarded broth was plated on Columbia agar plates (supplemented with

7.5% v/v defibrinated horse blood) to check for the presence of bacteria and

Fig. 3.3 The polypropylene tube and tooth was attached to a rubber cap which was placed in a screw top.
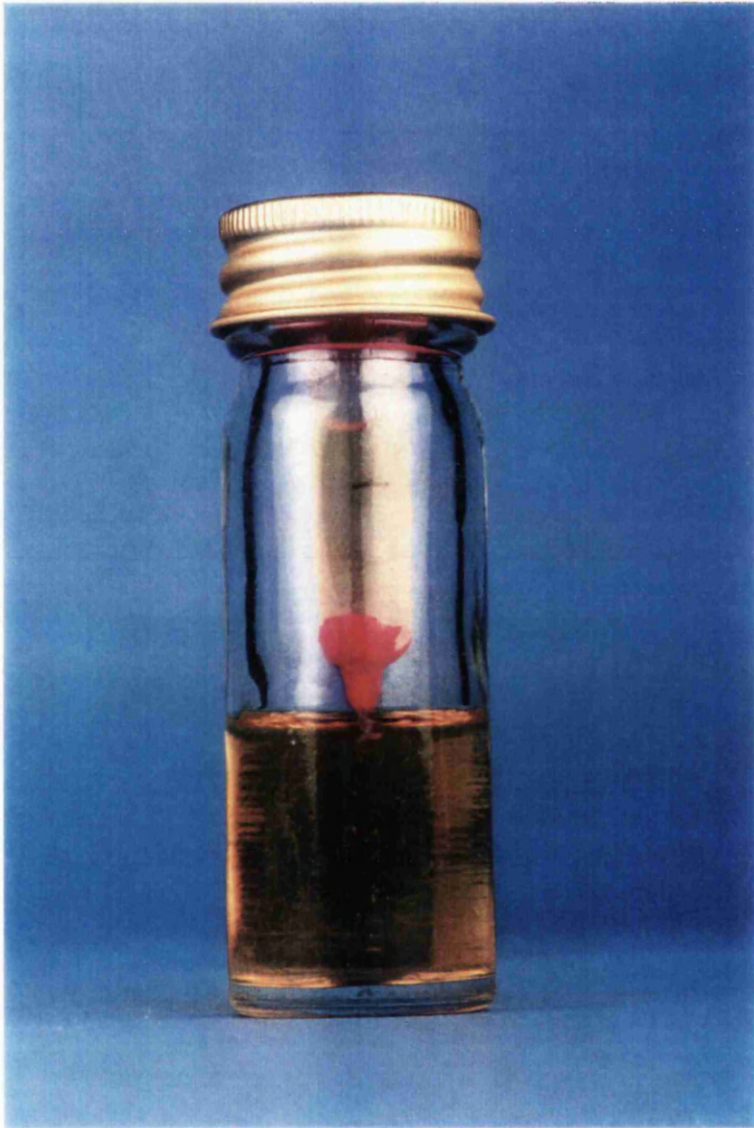
Fig. 3.4 Apparatus set-up with an uncontaminated fresh broth media. Note the apical portion of each root placed into the culture media.

possible coronal contamination. The model system was stored in an aerobic incubator at 37°C and any change in opacity of the broth in the apical chamber checked daily for 90 d.

Contamination by micro-organisms of the apical chamber was detected by the broth becoming turbid (Fig. 3.5). The time taken for this to occur was recorded as an indicator of entire root canal recontamination. Cultures from the apical chamber were streaked onto Columbia agar plates. These were then checked by macroscopic morphological examination and Gram staining. Statistical analysis of the results was undertaken to establish if there were significant differences in leakage between the groups using the Kruskal-Wallis test, corrected for ties.

3.3 RESULTS

All positive control teeth exhibited bacterial leakage after 24 h, while the lower chamber of negative control teeth remained uncontaminated throughout the test period (Fig. 3.6). Leakage through the experimental teeth was variable ranging from 7 to 86 d with leakage ranging from 60% to 80% of the specimens in each group (Table 3.1). In all cases when turbidity occurred, bacteriological testing confirmed that the micro-organism present in the apical chamber was *Streptococcus sanguis*. The Kruskal-Wallis test did not reveal any statistically significant differences in leakage among the 4 obturation groups (P>0.05).

Fig. 3.5 Apparatus set-up with contaminated broth in the apical chamber demonstrated microbial leakage from the coronal chamber.

Fig. 3.6 Comparison of the apparatus set-up, showing uncontaminated broth (left) and contaminated broth (right) in the apical chamber.

Table 3.1 Number of teeth which showed leakage of *S.sanguis* after 90 days.

Group	Leakage		Time (days) for leakage to occur	
	number of specimens	percentage	Mean	Range
Lateral condensation with smear layer intact	12	60	35.4	7-81
Lateral condensation with smear layer removed	16	80	37.0	7-84
Trifecta with smear layer intact	16	80	20.7	7-80
Trifecta with smear layer removed	13	75	31.5	7-86

Total number of specimens in each group = 20.

3.4 DISCUSSION

In this study, the presence or absence of the smear layer did not affect bacterial leakage either with cold lateral condensation of gutta-percha or the Trifecta method of obturation. The result is in agreement with Lloyd *et al.* (1995) who assessed the sealability of the Trifecta technique in the presence or absence of a smear layer. They found that removal of the smear layer did not enhance sealability despite increasing the proportion of specimens with dentinal penetration of sealer and gutta-percha. Evans and Simon (1986) reported that the presence or absence of smear layer had no significant effect on the apical seal of injected thermoplasticized gutta-percha or lateral condensation with and without root canal sealer.

A study by Safavi *et al.* (1989) concluded that removal of the smear layer facilitated passive penetration of *Streptococcus faecium* into the root canal tubules *in vitro*. Some studies (Saunders & Saunders 1992b, Gencoglu *et al.* 1993, Karagöz-Kücükay & Bayirli 1994) have shown reduced levels of dye leakage when the smear layer has been removed. Possible reasons might be the type of tracers and sealers used. It has been shown that removal of smear layer may allow sealer to penetrate into the dentinal tubules thereby giving a greater area of surface contact which may delay the penetration of leakage materials (White *et al.* 1984, Saunders & Saunders 1992b, Saunders *et al.* 1992, Gencoglu *et al.* 1993, Gutmann 1993, Oksan *et al.* 1993).

Whether the smear layer should be removed or not from the instrumented root canals is still controversial. Williams and Goldman (1985) showed that smear layer delayed the penetration of *Proteus vulgaris*, but was not a complete barrier to this bacteria. Meryon and Brook (1990) observed that *Actinomyces viscosus*, *Corynebacterium* species and *Streptococcus sanguis* digested the smear layer and facilitated their penetration. When the smear layer is not removed, the durability of both coronal and apical seal should be evaluated over a long period, since the layer may disintegrate and dissolve, creating a void between the root canal wall and the sealer.

Citric acid was used in this study to remove the smear layer from the canal walls and dentinal tubules. A study by Yamada *et al.* (1983) showed that a final flush with 25% citric acid followed by 5.25% NaOCl produced canal walls free of organic and inorganic debris similar to a final flush with 17% EDTA followed by 5.25% NaOCl. The citric acid group, however, left precipitated crystals in the root canal. The use of citric acid, as in the present study, may support the growth of the micro-organisms in root canals. Recently, Pileggi *et al.* (1995) evaluated apical leakage of ultrasonically cut retro-preparations using *Escherichia coli*. The result showed that treatment with 50% citric acid in retro-preparations restored with adhesive materials significantly increased bacterial penetration.

A reference *Streptococcus sanguis* strain was chosen for this study because of the ability of these bacteria to adhere to the hard tissues (Ørstavik *et al.* 1974). It is also associated with dental plaque and is occasionally found in periradicular lesions

(Iwu *et al.* 1990, Wayman *et al.* 1992, Tani-Ishii *et al.* 1994). This organism is a non-motile and non-spore forming facultative anaerobe. It tends to form chains and, in the event of prolonged incubation, clusters. In size, it usually ranges from 0.4-0.6 μm. Perez *et al.* (1993) have shown that *Streptococcus sanguis* can penetrate deep into the dentinal tubules. The migration of this strain into dentinal tubules is dependent both on incubation time and on the maturation of the tooth. *Streptococcus sanguis* migrates less deeply through the tubules of a mature tooth than an immature one because the diameter of the dentinal tubules is reduced by peritubular dentine formation.

Calcium hydroxide has been widely used in root canal therapy as an intracanal medicament, especially as an antibacterial agent and to promote hard tissue formation (Holland & deSouza 1985, Schroder 1985). Apexit is a calcium hydroxide-based sealer whose manufacturer claims has good sealing ability and is biocompatible with the surrounding periradicular tissues. It is a relatively new product and has not been widely tested, and the effect the smear layer may have on adhesive strength to tooth structure is unknown. A study by Gettleman *et al.* (1991) has shown that smear layer removal allowed greater adhesive strengths for AH26 (epoxy-resin based) but there was no significant difference with Sultan (zinc oxide-eugenol based) and Sealapex (calcium hydroxide-based) whether the smear layer was removed or left intact.

The efficacy of vacuum conditions to ensure reproducible dye penetration has been questioned (Peters & Harrison 1992, Masters *et al.* 1993, Roda & Gutmann

1995). It also may lead to an overestimation of the microleakage that would occur in the clinical setting. In this study, a centrifuge was used to force the broth through any defects in the root canal filling to reduce the likelihood that air bubbles in the obturated tooth may disrupt the passage of micro-organisms. Evans and Simon (1986) and Limkangwalmongkol et al. (1991) used the centrifugation technique in an apical leakage study, with dye penetration. They claimed that with the use of this technique, it was not necessary to leave the specimen in the dye solution for an extended period. Although centrifuging is not clinically relevant, it may provide a more exacting leakage test in vitro.

Sixty to eighty per cent of teeth in the present study demonstrated bacterial penetration within the 90-day period (Table 3.1). The observation time of 90 days was chosen because of the results of a coronal leakage study by Magura et al. (1991). They suggested root canal re-treatment of unsealed obturated root canals that have been exposed to the oral cavity for at least 3 months.

As in the other studies, the speed of penetration by the bacteria varied (Torabinejad et al. 1990, Magura et al. 1991, Gish et al. 1994, Moshonov et al. 1995b). Many factors could have affected bacterial penetration, including the model design, canal preparation, canal obturation technique, the type of bacteria used and the nature of the solution to which the coronal portions of the root canal were exposed. The clinical significance is that, regardless of the preparation and obturation technique or the experimental method of measuring leakage, coronal exposure of the root filling is damaging.

Ethylene oxide [(CH$_2$)$_2$O] is the primary gas used in hospitals to sterilise items that cannot be sterilised by steam. It is a colourless gas with an unpleasant odour. At low temperature ethylene oxide is a liquid, with a boiling point at atmospheric pressure of 10.8°C (Christensen & Kristensen 1992). The vapours of this gas are flammable and explosive, however the fire hazards can be reduced by diluting with carbon dioxide or fluorocarbon. Based on information developed in the 1980s, this gas has now been classified as both a mutagen and carcinogen, resulting in stricter regulations on its use.

Ethylene oxide is an alkylating agents and the inactivating effect on micro-organisms is considered to be a result of the alkylation of sulfhydryl, amino, carboxyl, phenolic and hydroxyl groups in the spore or vegetative cell. Alkylation is the replacement of a hydrogen atom with an alkyl group. In a bacterial cell or spore, this type of substitution can cause injury or death. As a result, it inactivates all types of micro-organisms, including endospores of bacteria and viruses (Phillips 1977). This action is influenced by the concentration of ethylene oxide, temperature, duration of exposure and water content of the micro-organisms.

The apparatus used to examine leakage in the present study, was refined from a model developed by Goldman et al. (1980). The main advantage of the present experimental set-up over previous models used is that the coronal chambers were attached to the cap of the glass bottles. This reduced the chance of accidental overflow of the bacteria-containing solution to the tooth. This arrangement also

provided a contamination-free chamber even though the study period was more than 90 days.

Clinically, it is likely that passage of bacteria or bacterial end-products follows the dissolution or disintegration of the root canal sealer or through voids at the sealer-root canal wall interface. The sealer, therefore, plays an important role in preventing leakage. Thus it is necessary to investigate the effect of various root canal sealers on coronal leakage of obturated root canals by micro-organisms.

CHAPTER 4 AN ASSESSMENT OF MICROBIAL CORONAL LEAKAGE IN TEETH ROOT FILLED WITH GUTTA-PERCHA AND THREE DIFFERENT SEALERS

4.1 INTRODUCTION

The material most commonly used for root canal obturation is gutta-percha, combined with a root canal sealer. Gutta-percha has been shown to have no adhesive qualities to dentine regardless of the obturation technique used. Therefore, sealers are used as a binding agent and to fill in minor discrepancies between the filling and the canal wall. Generally, there are 3 major types of root canal sealers and these include cements based upon organic resin, calcium hydroxide or zinc oxide-eugenol. Several types of sealers have been tested for their ability to produce an adequate seal. However, only a few leakage studies have been done using micro-organisms (Madison *et al.* 1987, Moshonov *et al.* 1995b, Barthel *et al.* 1996).

Thus, the aim of this *in vitro* study was to determine the effect of three root canal sealers: AH26, Apexit and Tubliseal EWT, on the penetration coronally by *Streptococcus sanguis* through obturated root canals.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of Teeth

Eighty extracted human teeth were used in this study. Criteria for choosing and methods of storing the teeth were described previously (chapter 2). The mechanical preparation technique of the root canals was described in chapter 3. The smear layer was left intact after instrumentation. The prepared teeth were divided randomly into experimental groups (60 teeth) and control groups (20 teeth). The experimental design is summarised in Fig. 2.12.

4.2.2 Obturation

Experimental Group

The root canals of 60 teeth were obturated with lateral condensation of cold gutta-percha using different root canal sealers, 20 root canals each with:

- AH26 (De Trey, Zurich, Switzerland)

- Apexit (Vivadent, Schaan, Liechtenstein)

- Tubliseal EWT (Kerr, Romulus, MI, USA)

Control Group

The root canals of 10 teeth were obturated with lateral condensation of cold gutta-percha and different root canal sealers. These teeth were used as the negative controls. The remaining 10 teeth were not obturated and served as the positive controls.

The lateral condensation of cold gutta-percha technique was the same as chapter 3. All sealers were mixed according to manufacturer's instructions. The excess root filling material was removed coronally with a heat transfer instrument and a suitably-sized cold plugger was used to plug the gutta-percha apically. All teeth were kept in 100% humidity at 37°C for 48 h to allow setting of the sealer. The crowns of all specimens were then cut with a diamond saw (Labcut, Agar Scientific, Stansted, UK) rotating at approximately 300 rpm, with water coolant, to obtain a root 12 mm long.

4.2.3 Preparation of Specimens

The leakage apparatus set-up was the same as the apparatus set-up in the previous chapter (chapter 3).

4.2.4 Bacterial Leakage

The protocol for the bacterial leakage study was the same as the previous study (chapter 3) and any change in opacity of the broth in the apical chamber checked daily for 90 d. Statistical analysis of the results was undertaken to establish if there were significant differences in leakage between the groups using the Kruskal-Wallis test, corrected for ties.

4.3 RESULTS

All positive control teeth exhibited bacterial leakage after 24 h, while the lower chamber of the negative control teeth remained uncontaminated throughout the

test period. Leakage through the experimental teeth varied from 7 to 86 d, ranging

from 35 to 60 % of specimens in each group. The criteria for leakage scores and

the number of teeth through which leakage occurred is shown in Table 4.1 and

Table 4.2 respectively. When turbidity occurred, bacteriological testing confirmed

that the micro-organism present in the lower chamber was *Streptococcus sanguis*.

The Kruskal-Wallis test did not reveal any statistically significant differences in

leakage between the 3 root canal sealers ($P>0.05$).

4.4 DISCUSSION

Three major types of root canal sealers were used in this study. AH26 (De Trey) is a

commonly-used epoxy resin-based root canal sealer. Some studies (Goldman *et al.*

1980, Madison *et al.* 1987, Limkangwalmongkol *et al.* 1991, Oguntebi & Shen 1992)

have reported that AH26 provides a better apical seal than other types of sealers.

Apexit (Vivadent) is a new calcium hydroxide-based sealer the manufacturer of which

claims has good sealing ability and is biocompatible with the surrounding tissues.

Recently, Kerr (Romulus, MI, USA) have developed a new formulation of Tubliseal

(zinc oxide-eugenol-based sealer) with an extended working time (Tubliseal EWT). Al-

Khatib *et al.* (1990) have shown that zinc oxide-eugenol-containing sealers have more

antimicrobial activity than other types of sealers.

In this present study, AH26 tended to have a better sealing ability than Apexit and

Tubliseal EWT sealer (Table 4.2), although the differences were not statistically

significant. Moshonov *et al.* (1995b) used *Staphylococcus epidermidis* to detect

Table 4.1 Criteria for leakage scores.

Degree of leakage	Time (days) for leakage to occur
0	no leakage at 90 days
1	69-90
2	46-68
3	23-45
4	1-22

Table 4.2 Leakage scores for the experimental groups exposed to *S.sanguis* immediately following obturation.

Group	Number of specimens	Leakage scores					Percentage of specimens showing leakage after 90 d
		0	1	2	3	4	
AH26	20	13	0	2	0	5	35
Apexit	20	8	3	1	1	7	60
Tubliseal EWT	20	10	1	2	1	6	50

the leakage of 3 root canal sealers: AH26, Roth 801 and Ketac-Endo after incubation for 80 days at 37°C. They found that AH26 was as effective as the Ketac-Endo group but better than the Roth group. Recently, Barthel *et al.* (1996) used the same micro-organisms and sealers as Moshonov *et al.* (1995b) but incubated for 38 days. The results showed that AH26 was significantly more effective than the Ketac-Endo group. However, Madison *et al.* (1987) have shown that AH26 group demonstrated significantly more coronal microleakage than Sealapex and Roth's sealer when exposed to artificial saliva.

The incidence of leakage in this study ranged from 35 to 60% of specimens in each group. Barthel *et al.* (1996) found leakage ranged from 32 to 53%, whilst, Moshonov *et al.* (1995b) showed only 20 to 40% of teeth in each group leaked. When leakage occurred, it was detectable no later than day 20. The reason for the difference may lie with the different model designs, the canal obturation techniques, the incubation periods, the media and the type of bacteria used.

Clinically, if delay in placing a coronal restoration occurs following root canal treatment, saliva may have deleterious effects on both the root canal sealer and the smear layer. The solubility and some other properties of sealers may negatively influence their long-term sealing quality. Therefore it was necessary to investigate the leakage of unrestored obturated root canals by micro-organisms following long-term storage in saliva.

CHAPTER 5 THE EFFECT OF STORAGE TIME ON MICROBIAL CORONAL LEAKAGE OF GUTTA-PERCHA ROOT FILLINGS WITH FOUR DIFFERENT SEALERS

5.1 INTRODUCTION

An ideal root canal sealer should seal the root canal, be dimensionally stable and be insoluble in tissue fluid. These properties of a sealer are of the utmost importance since the sealer component of the root filling gives the root canal the long lasting bacteria-tight seal that is necessary for successful root canal therapy. However, all sealers have some degree of solubility in tissue fluids (Branstetter & von Fraunhofer 1982). The interface between the root canal wall and the sealer is the critical area where most leakage takes place (Hovland & Dumsha 1985). The sealer could, therefore, be regarded as the weak link in the long-term successful obturation of the root canal.

The aim of this *in vitro* study was to investigate the long-term effect of artificial saliva on coronal leakage of obturated root canals with 4 root canal sealers: AH26, Apexit, Sealapex and Tubliseal EWT, using *Streptococcus sanguis* as a marker.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of Teeth

One hundred extracted human teeth were used in this study. Criteria for choosing and methods of storing the teeth were described previously (chapter 2). The mechanical preparation technique of the root canals was as described previously (chapter 3). The smear layer was left intact after instrumentation. The prepared teeth were divided randomly into experimental groups (80 teeth) and control groups (20 teeth). The experimental design is summarised in Fig. 2.13.

5.2.2 Obturation

Experimental Group

The root canals of 80 teeth were obturated with lateral condensation of cold gutta-percha using different root canal sealers, 20 root canals each with:

- AH26 (De Trey, Zurich, Switzerland)
- Apexit (Vivadent, Schaan, Liechtenstein)
- Sealapex (Kerr, Romulus, MI, USA)
- Tubliseal EWT (Kerr, Romulus, MI, USA)

Control Group

The root canals of 10 teeth were obturated with lateral condensation of cold gutta-percha and different root canal sealers. These teeth were used as the negative

controls. The remaining 10 teeth were not obturated and served as the positive controls.

The lateral condensation of cold gutta-percha technique was the same as chapter 3. All sealers were mixed according to manufacturer's instructions. The excess root filling material was removed coronally with a heat transfer instrument and a suitably-sized cold plugger was used to plug the gutta-percha apically. All teeth were kept in 100% humidity at 37°C for 48 h to allow setting of the sealer. The coronal portion of the root filling was left exposed to the storage environment. Each group were then put into separate glass containers filled with 60 mL artificial saliva, kept at 37°C for 6 months and the solution was changed monthly. The artificial saliva was prepared using a modified formula from Gish *et al.* (1994), the composition being:

- 1 mmol L^{-1} Calcium chloride

- 3 mmol L^{-1} Sodium phosphate

- 20 mmol L^{-1} Sodium hydrogen carbonate

- 1% w/v Glucose

- 1% w/v Proteose peptone

- 10 mg mL^{-1} Gentamycin

  Freshly prepared solutions had pH 6.8-7.5

After 6 months, the teeth were removed from the storage medium, rinsed in running water and the crowns of all specimens were cut with a diamond saw

(Labcut, Agar Scientific, Stansted, UK) rotating at approximately 300 rpm, with water coolant, to obtain a root 12 mm long.

5.2.3 Preparation of Specimens

The leakage apparatus set-up was the same as the apparatus set-up in chapter 3.

5.2.4 Bacterial Leakage

The bacterial leakage study was the same as the previous study (chapter 3) and any change in opacity of the broth in the apical chamber checked daily for 90 d. Statistical analysis of the results was undertaken to establish if there were significant differences in leakage between the groups using the Kruskal-Wallis test, corrected for ties.

5.3 RESULTS

All positive control teeth exhibited bacterial leakage after 24 h, while the lower chamber of negative control teeth remained uncontaminated throughout the test period. Leakage through the experimental teeth varied from 14 to 89 d, ranging from 10 to 15 % of specimens in each group. The criteria for leakage scores were the same as the previous study (Table 4.1). The number of teeth through which leakage occurred is shown in Table 5.1. When turbidity occurred, bacteriological testing confirmed that the micro-organism present in the lower chamber was *Streptococcus sanguis*. The Kruskal-Wallis test did not reveal any statistically significant differences in leakage between the 4 root canal sealers ($P > 0.05$).

Table 5.1 Leakage scores for the experimental groups after 6 months storage in artificial saliva and exposed to *S.sanguis*.

Group	Number of specimens	Leakage scores					Percentage of specimens showing leakage after 90 d
		0	1	2	3	4	
AH26	20	18	2	0	0	0	10
Apexit	20	17	0	2	0	1	15
Sealapex	20	18	1	0	1	0	10
Tubliseal EWT	20	18	0	0	2	0	10

5.4 DISCUSSION

There are numerous factors which may affect coronal leakage in root canal treatment, one of which is the solubility of the root canal sealer. When compared with testing for apical microleakage, more area of the obturated material is exposed to leakage in the coronal portion of the root canal. Calcium hydroxide-based sealers have been introduced as they supposedly have biological properties which stimulate a calcific barrier at the root apex. If calcium hydroxide is released by these sealers then solubility must be of concern. Tronstad *et al.* (1988) have shown that the calcium hydroxide-containing sealer, Sealapex is lost when the material is implanted into the mandible of dogs. Wu *et al.* (1995) investigated the influence of storage in water for 1 year of 4 root canal sealers: AH26, Ketac-Endo, Tubliseal and Sealapex at different thicknesses. Sealapex showed significantly more leakage after storing in water for 1 year than other sealers. Conversely, some studies have shown that calcium hydroxide-containing sealers have as good sealing properties as sealers based on zinc oxide-eugenol sealers (Alexander & Gordon 1985, Sleder *et al.* 1991).

In the present study, there was no significant difference in coronal leakage among the 4 root canal sealers. The results are in agreement with many researchers. Saunders and Saunders (1995) showed that there was no significant difference in coronal leakage after 1 year of storage in saline between 2 calcium hydroxide-containing sealers: Apexit and Sealapex, when lateral condensation had been used. Sleder *et al.* (1991) revealed that Sealapex statistically had no greater dissolution than Tubliseal when exposed to saline at both 2 and 32 weeks. Hovland and

Dumsha (1985) also found that all the sealers they studied provided an adequate seal. Using Procosol, Tubliseal and Sealapex, they demonstrated no significant difference in leakage between sealers up to 30 days. Recently, Horning and Kessler (1995) compared sealing ability of three different sealers: Procosol, Sealapex and Ketac-Endo, in moisture contaminated canals. They found that there was no significant difference in the amount of dye penetration after 9 months of storage over that found initially.

However, the result disagrees with the study by Peters (1986), who demonstrated significant loss of zinc oxide-eugenol sealer over a 2-yr period in a fluid environment. Zmener (1987) found that none of the sealers he investigated: Tubliseal, CRCS and Sealapex, provided an adequate seal and increased in leakage after 10 days. Moreover, Swanson and Madison (1987) concluded that all teeth exposed to artificial saliva for varying time periods of 3 to 56 days showed considerable dye penetration. It may be that the difference between this investigation and the present study may be due to differences in surface area of the sealer exposed to the fluid, composition of storage media, storage time and the different markers used.

To determine whether there was any significant difference between the teeth immediately tested following obturation (chapter 4) and the teeth stored in artificial saliva for 6 months (chapter 5), pairs of groups were compared using the Mann-Whitney U test, adjusted for ties. The results showed that AH26 (delay) group showed significantly less leakage (P<0.05) than AH26 (immediate) group.

Both Apexit and Tubliseal (delay) groups also showed significantly less leakage (P<0.01) than Apexit and Tubliseal EWT (immediate) groups (Table 5.2). It may be assumed that, once these sealers reach a full set, they are relatively impervious to fluids and do not undergo a significant dissolution of material whilst subjected to this liquid environment for 6 months.

In this study, Gentamycin was added to the artificial saliva in order to prevent the growth of micro-organisms. It is a broad spectrum antibiotic and might have some effect on the microbial leakage test. Therefore, each tooth was rinsed thoroughly prior to testing and from the results obtained with the positive controls, it seems unlikely that any gentamycin residue was left on the tooth which may have affected the passage of the micro-organisms. The artificial saliva had pH 6.8-7.5 and was changed monthly to allow for continued dissolution of the sealers and to prevent establishment of an equilibrium between the solution and sealers. However, it is possible that the pH and the composition of the artificial saliva may allow equilibrium to be reached sooner than after 1 month. Clinically, the lower pH and the presence of a constant flow of saliva over an open obturated root canal would not allow a chemical equilibrium to develop, which implies that this *in vitro* test may not be as vigorous as clinical conditions.

This study used a facultative anaerobe, *Streptococcus sanguis,* as the biological leakage marker. However, anaerobic bacteria are predominantly found in periradicular lesions (Iwu *et al.* 1990, Wayman *et al.* 1992, Brauner & Conrads 1995). According to Sundqvist (1992) *Fusobacterium nucleatum* is the most

Table 5.2 Leakage scores for the specimens exposed to *S.sanguis* immediately after obturation and after storage in artificial saliva for 6 months.

Group	Leakage scores					Percentage of specimens showing leakage after 90 d
	0	1	2	3	4	
AH26 (immediate)	13	0	2	0	5	35
AH26 (delay)	18	2	0	0	0	10
Apexit (immediate)	8	3	1	1	7	60
Apexit (delay)	17	0	2	0	1	15
Tubliseal EWT (immediate)	10	1	2	1	6	50
Tubliseal EWT (delay)	18	0	0	2	0	10

Total number of specimens in each group = 20.

prevalent species in infected root canals. Thus it is desirable to investigate the coronal leakage of unrestored obturated root canals exposed to an inoculum of *Fusobacterium nucleatum*.

CHAPTER 6 AN *IN VITRO* STUDY OF THE CORONAL LEAKAGE OF TWO ROOT CANAL SEALERS USING *FUSOBACTERIUM NUCLEATUM* AS A MICROBIAL MARKER

6.1 INTRODUCTION

It has become increasingly apparent that the greatest cause of endodontic and periradicular pathosis is microbial infection of the pulp and root canal. Sophisticated culturing techniques have identified infections of endodontic origin as being polymicrobial and predominantly anaerobic. Short-chain fatty acids such as propionic, butyric and isobutyric acid are metabolic end-products of anaerobic bacteria and are released at the infection site. Short-chain fatty acids are virulence factors that effect neutrophil chemotaxis, degranulation, chemiluminescence, phagocytosis and ultrastructural changes (Baumgartner 1991). Butyric acid is one of the major acid end-products produced by *Fusobacterium nucleatum*. Eftimiadi *et al.* (1991) showed that the activity of butyrate in inhibiting T-cell blastogenesis and mixed lymphocyte culture was greater than propionate, which was greater than isobutyrate.

The aim of this *in vitro* study was to investigate the coronal leakage of two root canal sealers: AH26 and Tubliseal EWT, using *Fusobacterium nucleatum* as a marker.

6.2 MATERIALS AND METHODS

6.2.1 Preparation of Teeth

Sixty extracted human teeth were used in this study. Criteria for choosing and methods of storing the teeth were described previously (chapter 2). The mechanical preparation technique of the root canals has been described previously (chapter 3). The smear layer was left intact after instrumentation. The prepared teeth were divided randomly into experimental groups (40 teeth) and control groups (20 teeth). The experimental design is summarised in Fig. 2.12.

6.2.2 Obturation

Experimental Group

The root canals of 40 teeth were obturated with lateral condensation of cold gutta-percha using different root canal sealers, 20 root canals each with:

- AH26 (De Trey, Zurich, Switzerland)

- Tubliseal EWT (Kerr, Romulus, MI, USA)

Control Group

The root canals of 10 teeth were obturated with lateral condensation of cold gutta-percha either with AH26 (De Trey) or Tubliseal EWT (Kerr) sealer. These teeth were used as the negative controls. The remaining 10 teeth were not obturated and served as the positive controls.

The lateral condensation of cold gutta-percha technique was the same as chapter 3. All sealers were mixed according to manufacturer's instructions. The excess root filling material was removed coronally with a heat transfer instrument and a suitably-sized cold plugger was used to plug the gutta-percha apically. After 48 h following obturation to allow setting of the sealer, the crowns of all specimens were cut with a diamond saw (Labcut, Agar Scientific, Stansted, UK) rotating at approximately 300 rpm, with water coolant, to obtain a root 12 mm long.

6.2.3 Preparation of Specimens

The leakage apparatus set-up was the same as the apparatus set-up in the previous study (chapter 3) except that Fastidious Anaerobe Broth (FAB) was used in the coronal and apical chamber instead of Todd Hewitt Broth (THB).

6.2.4 Bacterial Leakage

If bacterial contamination of the system was not noted, the coronal chamber was inoculated with 0.5 mL of FAB containing approximately 10^9 bacteria mL^{-1} *Fusobacterium nucleatum* (NCTC 10596). The method of inoculating *Fusobacterium nucleatum* into the coronal chamber was the same as the method of inoculating *Streptococcus sanguis* into the coronal chamber in chapter 3. A pilot study had indicated that bacteria were viable for 7 d without replenishing the medium and it was after this time that 0.5 mL of the broth in each coronal chamber was removed and replaced with 0.5 mL sterile FAB. The discarded broth was plated on Columbia agar plates (supplemented with 7.5% v/v defibrinated horse blood) to check for the presence of bacteria and possible coronal contamination.

The model system was stored in an anaerobic incubator (Don Whitley Scientific, Shipley, Yorkshire, UK) at 37°C and any change in opacity of the broth in the apical chamber checked daily for 90 d. Samples from the apical chamber were checked weekly using isotachophoresis and then examined for the presence of *Fusobacterium nucleatum* using the polymerase chain reaction (PCR). The time taken for contamination of the apical chamber by micro-organisms, or their acid end-products, was recorded as an indicator of entire root canal recontamination. Statistical analysis of the results was undertaken to establish if there were statistical differences in leakage between the two root canal sealers using the Mann-Whitney U test, adjusted for ties.

6.2.4.1 *Isotachophoresis*

Isotachophoresis is an electrophoretic separation technique that can be utilised for qualitative and quantitative analysis of ionic species. It uses the principle of electrophoresis in a discontinuous electrolyte system and once equilibrium has been reached, the sample ions are separated by differences in their effective mobilities. This technique has been named isotachophoresis (*iso*-same, *tacho*-velocity) because the ions are migrating at the same velocity as the leading electrolyte.

The discontinuous electrolyte system has a leading electrolyte, having an ion with a high net mobility, and a terminating electrolyte, having an ion with a low net mobility. The two electrolytes should be chosen so that the mobilities of the sample ions under observation are lower than that of the leading electrolyte ion but

higher than that of the terminating electrolyte ion. A counter ion is chosen to give a good buffering capacity on the pH interval in which the separation takes place. This technique has been employed in a wide variety of analytical procedures including analysis of bacterial endotoxins (Yotis & Catsimpoolas 1975) and acid end-products of anaerobic bacteria (Shah *et al.* 1985).

Preparation for isotachophoresis

Aliquots of 100 µL of each sample from the apical chambers were taken weekly and replaced with fresh sterile FAB to ensure that the apical 2 mm of the root was placed into the liquid. Isotachophoresis was performed on a LKB 2127 Tachophor (LKB Instruments, Bromma, Sweden) (Fig. 6.1). Separation takes place in a 300 mm capillary tube of 0.5 mm diameter kept at a constant temperature of 12°C. This tube is connected between reservoirs containing the leading and the terminating electrolytes. The detectors are placed at the end of the capillary and the detection current was 50 µA. A conductivity detection system, and its differential signal was used.

The leading electrolyte was 5 mmolL^{-1} hydrochloric acid adjusted to pH 4.2 by the addition of 6-amino-n-hexanoic acid, which acted as the buffering counter ion, and containing 0.2% (w/v) hydroxypropylmethylcellulose, which reduces electro-endosmosis thus sharpening zone boundaries. The terminating electrolyte was 4 mmolL^{-1} octanoic acid adjusted to pH 5.5 by the addition of 2-amino-2-(hydroxymethyl) propane-1, 3-diol (Tris).

Fig. 6.1 LKB 2127 Tachophor (LKB) for the isotachophoresis analysis.

Qualitative data were obtained by reference to standard runs of formic, pyruvic, phosphoric, lactic, succinic, acetic, propionic and butyric acids. Analysis of sample components was performed by estimating the relative step heights of each component in the detector tracing. The components can be identified by referring to the step heights found for the standard solution. Comparison of the acids produced in the control and experimental broth showed that there was an increase in the amount of butyric acid when leakage of bacterial end-products occurred (Fig. 6.2).

All chemicals used in the isotachophoresis were obtained from BDH Chemicals Ltd., Poole, UK, except hydroxypropylmethylcellulose, which was obtained from Sigma Chemical Co., Poole, UK.

6.2.4.2 *Polymerase chain reaction*

Polymerase chain reaction (PCR) is a rapid and sensitive cyclical *in vitro* enzymatic DNA amplification process which results in a 2^n accumulation of target DNA sequences where n equals the number of cycles of amplification (Ehrlich & Sirko 1994). This technique has been used in dentistry for detection of strains of micro-organisms such as *Porphyromonas gingivalis, Bacteroides forsythus* and *Actinobacillus actinomycetemcomitans* (Evans *et al.* 1994, Guillot *et al.* 1996, Tran & Rudney 1996). One of the advantages in using PCR-based diagnostics is that only a very small quantity of the specimen is usually required to permit adequate gene amplification (Greenberg 1994).
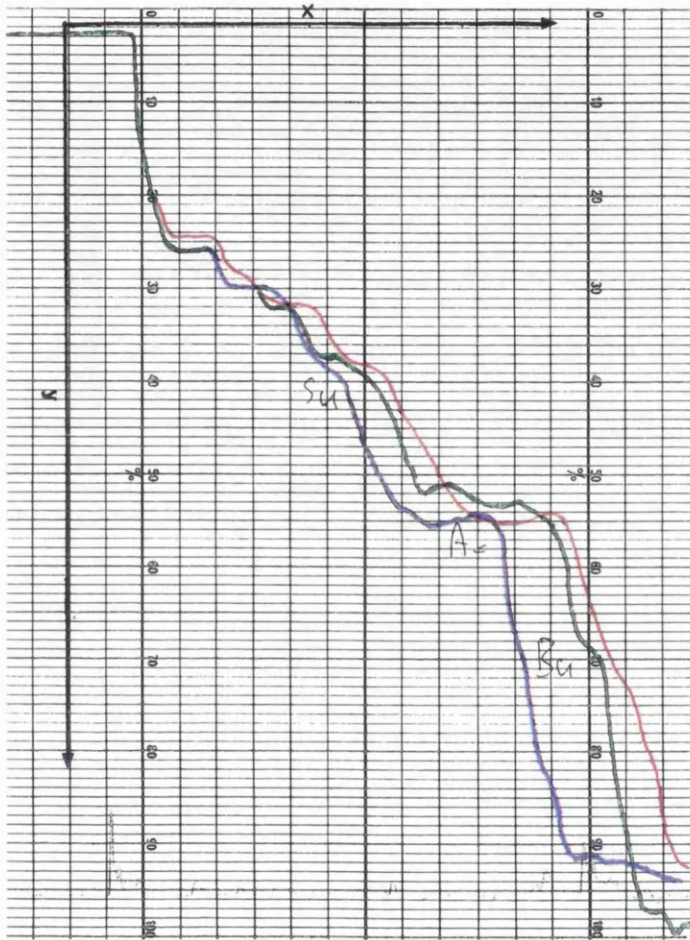
Fig. 6.2 Analysis of sample components showed that an increase in the amount of butyric acid occurred. Note green colour is the sample, red colour is the positive control and blue colour is the negative control.

Preparation of lysates for PCR

After the experimental period, each sample was centrifuged at 1,500 **g** for 10 min in an MSE Super minor centrifuge (MSE, Crawley, Sussex, UK). The supernatant was poured off, the cells suspended in 200 µL of distilled water and boiled for 10 min. Debris was removed by recentrifugation and the supernatant retained for analysis by PCR.

The PCR reaction

A set of universal 16S ribosomal RNA primers was used to amplify bacterial DNA from the lysates. The primer sequences were 5'-TCCTACGGGAGGCAGCAG-3' (16S1) and 5'-CCCGGGAACGTATTCACCG-3' (16S2), which correspond to *Escherichia coli* 16S rRNA positions 340-357 and 1369-1387, respectively.

The PCR reaction mix comprised 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.2 µM of each primer and 2.5 units of Dynazyme DNA polymerase (Flowgen). Aliquots of 2 µL of lysate was added to each reaction, which was carried out in a total volume of 100 µL. Primers and sample were separated from the other reaction components by a layer of wax, in order to delay commencement of the reaction until the wax had melted at a higher temperature. This "hot start" PCR prevents primer-dimer formation and increases the specificity of the assay. Samples were placed in a Hybaid Omnigene thermal cycler and the following cycling conditions carried out: 94°C/5 min (1 cycle), 94°C/1 min, 55°C/1 min, 72°C/1.5 min (30 cycles) and 72°C/10 min (1 cycle).

Agarose gel electrophoresis of PCR reaction products

Aliquots of 20 μL of each product was electrophoresed on a 2% agarose gel containing ethidium bromide (0.5 μgmL^{-1}) and visualisation was done under UV light. A 100 base pair DNA ladder was used as a size marker. The presence of bacterial DNA was indicated by a PCR product of 1,056 base pairs.

RFLP analysis of PCR products

The PCR products were concentrated by ethanol precipitation and an aliquot digested with the restriction enzyme. *Hha*I Fusobacterial DNA would give a ladder of bands of the following sizes: 279-, 257-, 216-, 185-, 89-bp.

6.3 RESULTS

It was not possible to observe the turbidity in the apical chamber because some crystallisation of the media took place (Fig. 6.3). All positive control teeth exhibited leakage of bacterial end-products, which was butyric acid, within a week, whilst the lower chamber of the negative control teeth remained uncontaminated throughout the test period. All the experimental teeth exhibited leakage of butyric acid within 12 weeks (Table 6.1). The mean leakage in the AH26 and the Tubliseal EWT groups was 8.4 and 8.2 weeks respectively and leakage was variable ranging from 1 to 12 weeks (Table 6.2). The Mann-Whitney U test did not reveal any statistically significant difference in leakage between the AH26 and the Tubliseal EWT groups (P>0.05).

Fig. 6.3 Apparatus set-up for *Fusobacterium nucleatum*, illustrating some crystallisation of the broth making it impossible to observe the turbidity in the apical chamber. In this illustration, bottle number 6 was a negative control and number 12 was an experimental specimen.

Table 6.1 Cumulative numbers of teeth with leakage of butyric acid in the 12 week-period (number of specimens in each group = 20).

Group	Number of teeth with leakage		
	4 wk	8 wk	12 wk
Lateral condensation with AH26	1 (5%)	8 (40%)	20 (100%)
Lateral condensation with TubliSeal EWT	2 (10%)	6 (30%)	20 (100%)

Cumulative percentage of specimens with leakage is shown in brackets.

Table 6.2 Mean time for leakage of butyric acid (with range).

Group	Leakage (weeks)	
	mean	range
Lateral condensation with AH26	8.4	2-12
Lateral condensation with Tubliseal EWT	8.2	1-11

Fusobacterium nucleatum was detected in the apical chamber in all positive control teeth but was not detected in the apical chamber in all negative control teeth. Ten samples from the experimental group gave a PCR product of the expected size (Fig. 6.4) Digestion of these 10 products with *Hae*III revealed that none shared the DNA fingerprint pattern that would be expected from Fusobacterial DNA (Fig. 6.5). It is concluded that *Fusobacterium* species were not present in any of the samples from the apical chamber.

6.4 DISCUSSION

This study showed that acid end-products of Gram-negative bacteria could penetrate a seemingly well obturated root canal within the 12-week period (Table 6.1). The result is in agreement with a study by Kersten and Moorer (1989). Their finding showed that microleakage of the small molecules (butyric acid or methylene blue dye) could not be prevented, whereas leakage of bacteria-sized particles (latex beads) and large sized protein molecules (endotoxin) could be prevented only when both sealer and vertical pressure were used in obturation techniques. Recently, Moshonov *et al.* (1995a) used *Staphylococcus epidermidis* to detect the leakage of 3 root canal sealers: AH26, Roth 801 and Ketac-Endo by monitoring the turbidity of the broth and colour change of a phenol red dye in the apical chamber. They found that colour change occurred significantly earlier than the turbidity of the broth and the Roth 801 group allowed bacterial penetration significantly less than the other 2 groups (P<0.05). Bacterial metabolites may induce media changes through root filled teeth prior to or in the absence of
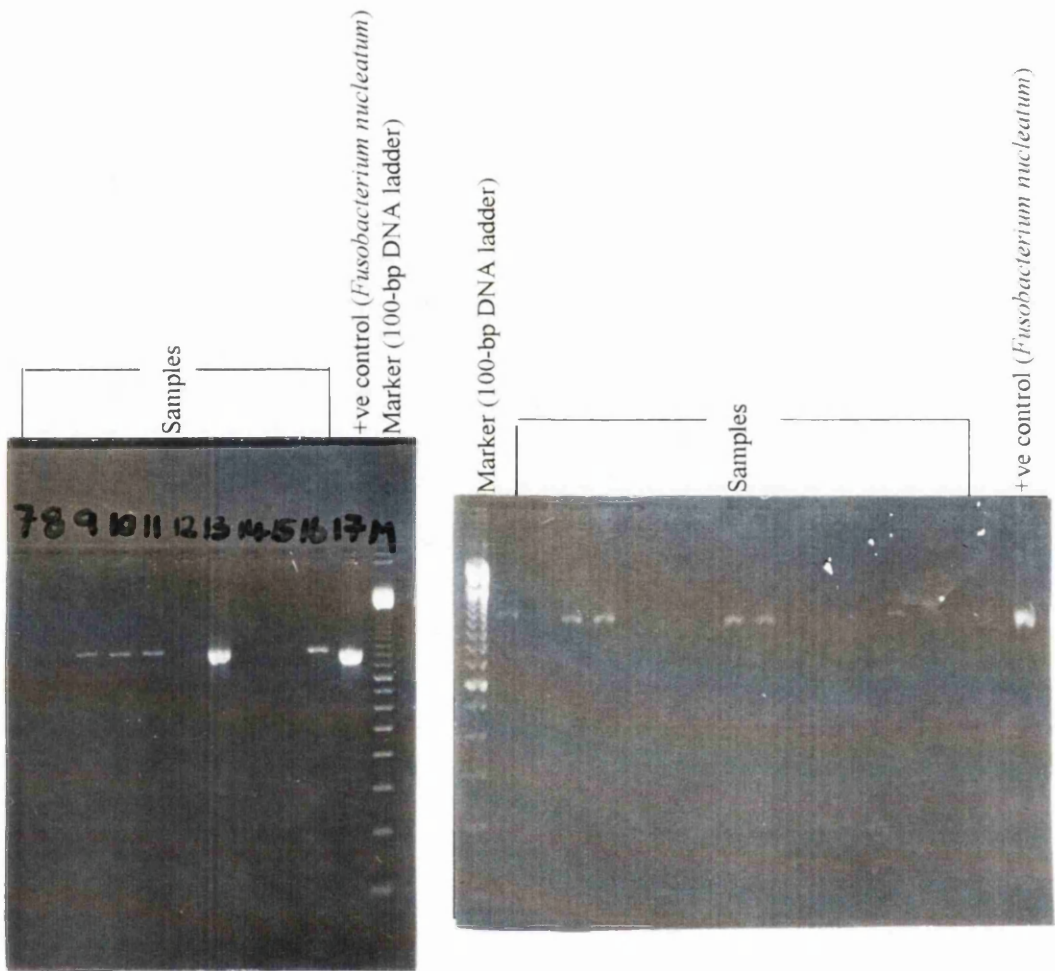
Fig. 6.4 2% Agarose gel electrophorosis of PCR products obtained from samples
from the experimental group, using a 100-bp DNA ladder as a size marker.
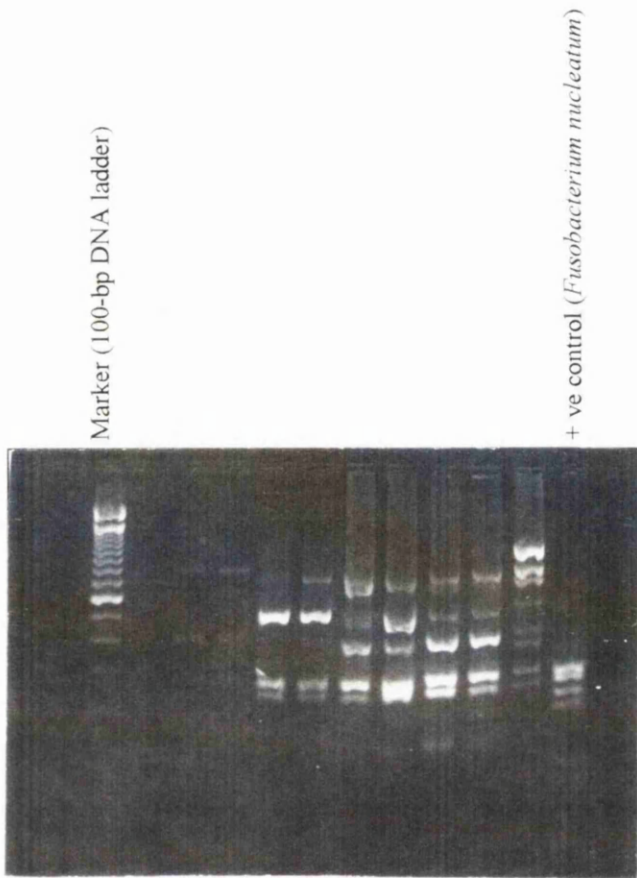
Fig. 6.5 Digestion of samples from Fig. 6.4 with *Hae*III revealed that none shared
the DNA fingerprint pattern that would be expected from Fusobacterial DNA.

bacterial penetration and the sealers used may affect metabolite and bacterial penetration differently.

Acid end-product analysis is regarded as one of the essential biochemical tests for the characterisation of anaerobic bacteria. The types of acids produced by a particular species are reasonably constant under standardised conditions (Shah *et al.* 1985). Various techniques have been used to identify acid end-products, however, the most sensitive method of analysis is by isotachophoresis where nanomole levels of acid end-products may be detected (Shah *et al.* 1985). This technique can be utilised for both qualitative and quantitative analysis of ionic species.

A reference strain of *Fusobacterium nucleatum* was chosen because they are part of the human normal oral flora and frequently found in mixed infections with other anaerobes and facultative anaerobes (Baumgartner & Falkler 1991, Brook *et al.* 1991, Sundqvist 1992, Wayman *et al.* 1992). Oguntebi *et al.* (1982) found *Fusobacterium* species and *Streptococcus mitis,* in combination, in 50% of the examined periradicular abscesses. A study by Wayman *et al.* (1992) showed that in the 29 periradicular lesions with no detectable communication with the oral cavity, *Fusobacterium nucleatum* was one of the most commonly isolated microbes. Baumgartner *et al.* (1992) have developed a mouse model to study the abscessogenic potential of pure and mixed cultures of oral anaerobes associated with infections of endodontic origin. They found that strains of *Fusobacterium nucleatum, Peptostreptococcus anaerobius* and *Veillonella parvula* were

pathogenic in pure culture. A mixed culture of *Fusobacterium nucleatum* with either *Porphyromonas gingivalis* or *Prevotella intermedia* was significantly more pathogenic than *Fusobacterium nucleatum* in pure culture.

In this present study, it was not possible to observe the turbidity in the apical chamber because some crystallisation of the broth in the apical chamber took place which masked any turbidity. The reason for this is unknown but it may be that the agar in FAB coagulated or there was a reaction between the nail varnish and FAB. Furthermore, by using Gram stain or plating on Columbia agar plates, it still was not possible to confirm that *Fusobacterium nucleatum* was in the apical chamber. The results from PCR confirmed that there was no *Fusobacterium nucleatum* in the apical chamber. It may be that the environment was not suitable for this obligate anaerobic strain to grow and eventually caused them to die away. Moreover, the size of *Fusobacterium nucleatum* is large, usually 5-10 μm long, which may mean that the organisms may take a considerable period to pass through the root canal and contaminate the apical chamber.

Ten samples from the experimental group gave a PCR product of the expected size but no *Fusobacterium* species was detected. The reasons for this may be there were some contamination with other micro-organisms in these samples, but the nature of the non-Fusobacterial PCR products was unknown. It is likely that this contamination was from the PCR enzyme itself, commercial preparations of which are invariably contaminated with small traces of bacterial DNA. In addition, a study by Sato *et al.* (1996) has shown that there is substantial variation in

sequence among the genes of *Fusobacterium nucleatum* with variation among genes from the same strain (up to 2.5%) less than that seen between strains (up to 6%).

When compared to chapter 4 where the teeth were tested with *Streptococcus sanguis* immediately after obturation, 35% of teeth in AH26 group and 50% of teeth in Tubliseal EWT group were contaminated with *Streptococcus sanguis* whilst there was no teeth contaminated with *Fusobacterium nucleatum* but 100% of teeth exhibited leakage of butyric acids within 12 weeks (Table 6.3). The reasons for this may be that the environment was more suitable for *Streptococcus sanguis,* a facultative anaerobe, to grow and the size of *Streptococcus sanguis* is smaller compared with *Fusobacterium nucleatum* which may make penetration of the former easier to achieve. Furthermore, FAB is composed of a small concentration of agar which may block or delay the penetration of *Fusobacterium nucleatum.*

Anaerobic bacteria are predominantly found in periradicular lesions (Iwu *et al.* 1990, Wayman *et al.* 1992). Clinically, it is possible that Gram-negative bacteria could be present coronally in a defective coronal restoration, and their acid end-products could move through the obturated canal to cause an inflammatory response apically. It is important, therefore, to develop a model to allow obligate anaerobes to grow and thereby investigate leakage through root-filled teeth with these organisms.

Table 6.3 Leakage scores compared for the experimental groups exposed to either *S.sanguis* or *F.nucleatum* immediately after obturation.

Group	Leakage scores					Percentage of specimens showing leakage after 90 d
	0	1	2	3	4	
AH26 exposed to *S.sanguis*	13	0	2	0	5	35
AH26 exposed to *F.nucleatum*	20	0	0	0	0	0
Tubliseal EWT exposed to *S.sanguis*	10	1	2	1	6	50
Tubliseal EWT exposed to *F.nucleatum*	20	0	0	0	0	0

Total number of specimens in each group = 20.

CHAPTER 7 AN *IN VITRO* STUDY OF THE CORONAL LEAKAGE OF TWO ROOT CANAL SEALERS USING *PREVOTELLA INTERMEDIA* AS A MARKER

7.1 INTRODUCTION

During the past twenty years, obligate anaerobic bacteria in infected root canal systems are identified as playing a decisive role in periradicular inflammation. One of the most commonly found species in root canal infection is *Prevotella intermedia* (van Winkelhoff *et al.* 1985, Sundqvist 1992, Haapasalo 1993). It is also frequently found in infected root canals associated with clinical symptoms (Yoshida *et al.* 1987, Sundqvist *et al.* 1989, Gomes *et al.* 1994a).

The purpose of this *in vitro* study was to investigate the long-term effect on the coronal leakage of *Prevotella intermedia* into root canals obturated with lateral condensation of cold gutta-percha with two root canal sealers: AH26 and Sealapex.

7.2 MATERIALS AND METHODS

7.2.1 Preparation of Teeth

Sixty extracted human teeth were used in this study. Criteria for choosing and methods of storing the teeth were described previously (chapter 2). The mechanical preparation technique of the root canals was described previously in chapter 3 and the smear layer was left intact. The prepared teeth were divided

randomly into experimental groups (40 teeth) and control groups (20 teeth). The experimental design is summarised in Fig. 2.13.

7.2.2 Obturation

Experimental Group

The root canals of 40 teeth were obturated with lateral condensation of cold gutta-percha using different root canal sealers, 20 root canals each with:

- AH26 (De Trey, Zurich, Switzerland)

- Sealapex (Kerr, Romulus, MI, USA)

Control Group

The root canals of 10 teeth were obturated with lateral condensation of cold gutta-percha using either AH26 (De Trey) or Sealapex (Kerr) sealers. These teeth were used as the negative controls. The remaining 10 teeth were not obturated and served as the positive controls.

The lateral condensation of cold gutta-percha technique was the same as chapter 3. All sealers were mixed according to manufacturer's instructions. The excess root filling material was removed coronally with a heat transfer instrument and a suitably-sized cold plugger was used to plug the gutta-percha apically. Following obturation, all teeth were kept in 100% humidity at 37°C for 48 h to allow setting of the sealer. The coronal portion of the root filling was left exposed to the storage environment. Each group of specimens were put into separate glass containers

filled with 60 mL artificial saliva, kept at 37°C for 6 months and the solution

changed monthly. The formula of the artificial saliva was the same as that

described in chapter 5. After 6 months, the teeth were removed from the storage

medium, rinsed in running water and the crowns of all specimens were cut with a

diamond saw (Labcut, Agar Scientific, Stansted, UK) rotating at approximately

300 rpm, with water coolant, to obtain a root 12 mm long.

7.2.3 Preparation of Specimens

The leakage apparatus set-up was the same as the apparatus set-up in chapter 3

except Brain Heart Infusion Broth (BHIB) (Pro-Lab Diagnostic, Neston, Cheshire,

UK) plus 0.5 gL^{-1} cysteine hydrochloride (BDH Chemicals, Poole, UK) was used

in the coronal and apical chamber instead of Todd-Hewitt Broth (THB).

7.2.4 Bacterial Leakage

The bacterial leakage study was the sama as chapter 3 except the cultures of

Prevotella intermedia in BHIB plus 0.5 gL^{-1} cysteine hydrochloride (approximately

10^9 bacteria mL^{-1}) were used as the leakage marker. Any change in opacity of the

broth in the apical chamber was checked daily for 90 d. Aliquots of 0.5 mL of the

broth in each coronal chamber were removed and replaced with 0.5 mL fresh

cultures of *Prevotella intermedia* at 5 d-intervals. The discarded broth was plated

on enhanced blood agar plates (supplemented with 7.5% v/v defibrinated horse

blood and 1% v/v vitamin K/haemin solution) to check for the presence of bacteria

and possible coronal contamination. Statistical analysis of the results was

undertaken to establish if there were significant differences in leakage between the groups using the Mann-Whitney U test, adjusted for ties.

7.3 RESULTS

All positive control teeth exhibited bacterial leakage within 48 h, while the lower chamber of the negative control teeth remained uncontaminated throughout the test period. Leakage through the experimental teeth varied from 7 to 86 d, ranging from 30% in the Sealapex group to 50 % in the AH26 group. The criteria for leakage scores and the number of teeth through which leakage occurred is shown in Table 4.1 and Table 7.1 respectively. When turbidity occurred, bacteriological testing confirmed that the micro-organism present in the lower chamber was only *Prevotella intermedia*. Five samples had contamination of the coronal chambers on day 60 and three of these had the same micro-organisms passed through to the apical chamber. Macroscopic morphological examination, Gram staining and an identification kit, API 20A (Bio-Merieux, Basingstoke, Hants, UK) established that the contaminating micro-organism present in both the coronal and apical chamber was *Propionibacterium acnes*. The Mann-Whitney U test did not reveal any statistically significant difference in leakage between the 2 root canal sealers (P>0.05).

Table 7.1 Leakage scores for the experimental groups after 6 months storage in artificial saliva and subsequent exposure to *P. intermedia*.

Group	Number of specimens	Leakage scores					Percentage of specimens showing leakage after 90 d
		0	1	2	3	4	
AH26	20	10	6	1	3	0	50
Sealapex	20	14	2	0	1	3	30

7.4 DISCUSSION

The results of this study showed that there was no statistically significant difference in microbial leakage between Apexit and Tubliseal EWT groups. This is similar to the results obtained by Madison and Wilcox (1988). They evaluated the coronal leakage of posterior teeth in monkeys by exposing the access openings to the oral environment for 1 week. Following removal, the teeth were placed in dye and cleared to allow visualisation of dye penetration. The results showed that there was no statistically significant difference ($P>0.05$) among the three root canal sealers tested: AH26, Sealapex and Roth's 801.

When compared to chapter 5 using *Streptococcus sanguis* as a marker (Table 7.2): in the AH26 groups, 50% of teeth exposed to *Prevotella intermedia* leaked whilst only 10% of teeth exposed to *Streptococcus sanguis* leaked. In the Sealapex groups, 30% of teeth exposed to *Prevotella intermedia* leaked whilst only 10% exposed to *Streptococcus sanguis* leaked. Statistical analysis of the results was undertaken to establish if there were significant differences in leakage between the groups using the Mann-Whitney U test, adjusted for ties. The results revealed that the AH26 group exposed to *Prevotella intermedia* showed significantly more leakage ($P<0.01$) than AH26 exposed to *Streptococcus sanguis*. This implies that the strain of *Prevotella intermedia* may be more virulent than *Streptococcus sanguis*.

Table 7.2 Leakage scores for the experimental groups after 6 months storage in artificial saliva and subsequent exposure to either *S.sanguis* or *P.intermedia*.

Group	Leakage scores					Percentage of specimens showing leakage after 90 d
	0	1	2	3	4	
AH26 exposed to *S.sanguis*	18	2	0	0	0	10
AH26 exposed to *P.intermedia*	10	6	1	3	0	50
Sealapex exposed to *S.sanguis*	18	1	0	1	0	10
Sealapex exposed to *P.intermedia*	14	0	2	1	3	30

Total number of specimens in each group = 20.

However, in the Sealapex group there was no statistically significant difference in leakage (P>0.05) between those exposed to *Prevotella intermedia* or those exposed to *Streptococcus sanguis*. The reason for this may be Sealapex has more antimicrobial activity after setting than AH26. Sealapex contains calcium oxide which, after contact with fluids, will change to calcium hydroxide. The delayed antimicrobial effect may be caused by this formation of calcium hydroxide from calcium oxide. Recently, Shalhav *et al.* (1996) studied the antimicrobial activity of calcium hydroxide-containing sealers on *Streptococcus faecalis in vitro*. The results showed that the antimicrobial activity of the 3 sealers: Sealapex, CRCS and Roth's, changed according to the time interval between mixing and testing. In 1-hour-old mixtures, CRCS and Roth's had a significantly better antimicrobial effect (P<0.01) than Sealapex. In contrast, in 7-day-old mixtures, Sealapex showed significantly better antimicrobial effect (P<0.01) than both CRCS and Roth's sealers.

In this study, cysteine hydrochloride, a reducing agent, was added to the broth media in order to keep oxygen level low, providing a strictly anaerobic environment. Fresh cultures of *Prevotella intermedia* were added to each coronal chamber at 5 d-intervals to maintain the viability of the organism.

An anaerobic Gram-positive rod, *Propionibacterium acnes* was detected from five of the coronal chambers at day 60. Three apical chambers from these five specimens were contaminated by this strain between day 60 and 90. This

contamination may have taken place from the skin of the operator to the needle tip.

Clinically, mixed facultative anaerobic and obligate anaerobic micro-organisms are found predominantly in periradicular lesions (Tronstad *et al.* 1987, Haapasalo 1989, Sundqvist *et al.* 1989, Brook *et al.* 1991, Haapasalo 1993). Several studies (Korzen *et al.* 1974, Fabricius *et al.* 1982a, Sundqvist *et al.* 1989, Baumgartner & Falkler 1992) showed that the degree of inflammation was less severe with monoinfection than with mixed infection. It was decided, therefore, that the coronal leakge of obturated root canals using a polymicrobial marker following long-term storage in saliva should also be investigated.

CHAPTER 8 CORONAL LEAKAGE OF OBTURATED ROOT CANALS AFTER LONG-TERM STORAGE USING A POLYMICROBIAL MARKER

8.1 INTRODUCTION

When strict anaerobic techniques are applied to endodontic samples, many infections that were previously considered to be caused by aerobic or facultative bacteria are now known to be polymicrobial infections dominated by anaerobic bacteria (Tronstad *et al.* 1987, Haapasalo 1989, Sundqvist *et al.* 1989, Brook *et al.* 1991, Haapasalo 1993). Several studies (Drucker *et al.* 1992, Sundqvist 1992, Gomes *et al.* 1994a, Gomes *et al.* 1994b) have shown that there is a correlation between the presence of specific bacteria and some endodontic symptoms and signs.

The purpose of this study was to investigate the leakage of a mixed flora of micro-organisms into root canals obturated with lateral condensation of cold gutta-percha with two root canal sealers, Apexit and Tubliseal EWT, after long-term storage in artificial saliva.

8.2 MATERIALS AND METHODS

8.2.1 Preparation of Teeth

Sixty extracted human teeth were used in this study. Criteria for choosing and methods of storing the teeth were described previously (chapter 2). The

mechanical preparation technique of the root canals was described previously (chapter 3) and the smear layer was left intact. The prepared teeth were divided randomly into experimental groups (40 teeth) and control groups (20 teeth). The experimental design is summarised in Fig. 2.13.

8.2.2 Obturation

<u>Experimental Group</u>

The root canals of 40 teeth were obturated with lateral condensation of cold gutta-percha using different root canal sealers, 20 root canals each with:

- Apexit (Vivadent, Schaan, Liechtenstein)

- Tubliseal EWT (Kerr, Romulus, MI, USA)

<u>Control Group</u>

The root canals of 10 teeth were obturated with lateral condensation of cold gutta-percha using either Apexit (Vivadent) or Tubliseal EWT (Kerr) sealers. These teeth were used as the negative controls. The remaining 10 teeth were not obturated and served as the positive controls.

The lateral condensation of cold gutta-percha technique was the same as chapter 3. All sealers were mixed according to manufacturer's instructions. The excess root filling material was removed coronally with a heat transfer instrument and a suitably-sized cold plugger was used to plug the gutta-percha apically. Following obturation, all teeth were kept in 100% humidity at 37°C for 48 h to allow setting

of the sealer. The coronal portion of the root filling was left exposed to the storage environment. Each group of specimens were put into separate glass containers filled with 60 mL artificial saliva, kept at 37°C for 6 months and the solution changed monthly. The formula of the artificial saliva was the same as that used in chapter 5. After 6 months, the teeth were removed from the storage medium, rinsed in running water and the crowns of all specimens were cut with a diamond saw (Labcut, Agar Scientific, Stansted, UK) rotating at approximately 300 rpm, with water coolant, to obtain a root 12 mm long.

8.2.3 Preparation of Specimens

The leakage apparatus set-up was the same as the apparatus set-up in chapter 3 except the Todd-Hewitt Broth (THB) was replaced in both the coronal and apical chamber by Brain Heart Infusion Broth (BHIB) plus 0.5 gL^{-1} cysteine hydrochloride.

8.2.4 Bacterial Leakage

The bacterial leakage study was again the same as that described in chapter 3 except a mixture of *Streptococcus sanguis* and *Prevotella intermedia* in BHIB plus 0.5 gL^{-1} cysteine hydrochloride (approximately $2x10^9$ bacteria mL^{-1}) was used as the leakage marker. Any change in opacity of the broth in the apical chamber was checked daily for 90 d. Aliquots of 0.5 mL of the broth in each coronal chamber were removed and replaced with 0.5 mL fresh cultures of *Prevotella intermedia* at 5 d-intervals. The discarded broth was plated on enhanced blood agar plates to check for the presence of bacteria and possible coronal contamination. Statistical

analysis of the results was undertaken to establish if there were significant differences in leakage between the groups using the Mann-Whitney U test, adjusted for ties.

8.3 RESULTS

All positive control teeth exhibited bacterial leakage after 24 h, while the lower chamber of negative control teeth remained uncontaminated throughout the test period. Cultures from the positive control teeth confirmed that there were both micro-organisms on enhanced blood agar plates. Leakage through the experimental teeth varied from 17 to 88 d, ranging from 50% in the Apexit group to 70 % in the Tubliseal EWT group. The criteria for leakage scores and the number of teeth through which leakage occurred is shown in Table 4.1 and Table 8.1 respectively. When turbidity occurred, bacteriological testing confirmed that the micro-organism present in the apical chamber was only *Streptococcus sanguis*. The Mann-Whitney U test did not reveal any statistically significant differences in leakage between the 2 root canal sealers (P>0.05).

8.4 DISCUSSION

The results of this study showed that lateral condensation of cold gutta-percha with two different sealers was unable to prevent coronal ingress of a mixed microbial flora used as a leakage marker. Gish *et al.* (1993) used mixed bacterial inocula (*Streptococcus anginosus, Actinomyces viscosus* and *Lactobacillus casei*)

Table 8.1 Leakage scores for the experimental groups after 6 months storage in artificial saliva and subsequent exposure to mixed microbial marker.

Group	Number of specimens	Leakage scores					Percentage of specimens showing leakage after 90 d
		0	1	2	3	4	
Apexit	20	10	1	0	8	1	50
Tubliseal EWT	20	6	2	0	11	1	70

to detect the coronal leakage of root fillings occupying the apical 5 mm of the root canal. They found that at 21 days, the experimental teeth showed no bacterial penetration. In the present study leakage first occurred at day 17 with the Apexit group whilst the Tubliseal EWT group did not show leakage until 21 days. An important property of a root canal sealer is that it should be insoluble and provide a good long-term barrier to microbial penetration of the root canal system. This was not the case with the sealers used in this study with 50% of the Apexit group and 70% of the Tubliseal EWT group showing leakage through the root canals at 90 days.

It is interesting to note from this study, that only *Streptococcus sanguis* penetrated through the experimental teeth into the apical chamber although the coronal part of teeth was exposed to both *Streptococcus sanguis* and *Prevotella intermedia* species. The reasons may be that *Streptococcus sanguis* grows faster than *Prevotella intermedia* and overgrows *Prevotella intermedia*. Therefore, fresh cultures of *Prevotella intermedia* were added to the coronal chamber at 5 d-intervals. Secondly, the environment in this study may not provide strictly anaerobic conditions and may inhibit growth of *Prevotella intermedia*. Finally, *Streptococcus sanguis* is smaller than *Prevotella intermedia* and may penetrate through the root canal faster.

When compared to chapter 5, using *Streptococcus sanguis* (Table 8.2), the results revealed that the Apexit group exposed to the *Streptococcus sanguis* and *Prevotella intermedia* combination showed significantly more leakage (P<0.05,

Table 8.2 Compare leakage scores for the experimental groups after 6 months storage in artificial saliva and subsequent exposure to *S.sanguis* or mixed microbial marker.

Group	Leakage scores					Percentage of specimens showing leakage after 90 d
	0	1	2	3	4	
Apexit exposed to *S.sanguis*	17	0	2	0	1	15
Apexit exposed to *S.sanguis* and *P.intermedia*	10	1	0	8	1	50
Tubliseal EWT exposed to *S.sanguis*	18	0	0	2	0	10
Tubliseal EWT exposed to *S.sanguis* and *P.intermedia*	6	2	0	11	1	70

Total number of specimens in each group = 20.

Mann-Whitney U test, adjusted for ties) than the Apexit group exposed to *Streptococcus sanguis* only. The Tubliseal EWT group exposed to both *Streptococcus sanguis* and *Prevotella intermedia* also showed significantly more leakage (P<0.01, Mann-Whitney U test, adjusted for ties) than the Tubliseal EWT group exposed to *Streptococcus sanguis* only. The reasons may be that the synergistic relationships between these two micro-organisms may increase their virulence.

Polymicrobial infections involving black-pigmented Gram-negative anaerobes are likely to have a greater pathogenic potential than infections with a single species. As a result, the strain of *Prevotella intermedia* and *Streptococcus sanguis* were chosen to represent the mixed infections. *Streptococcus* and *Prevotella* species are often found in infected root canals (Baumgartner & Falkler 1991, Wayman *et al.* 1992, Haapasalo 1993, Gomes *et al.* 1994b). Several studies (Sundqvist 1992, Wayman *et al.* 1992, Gomes *et al.* 1994a, Gomes *et al.* 1994b) have shown that *Prevotella* species frequently occur with other bacteria. Therefore other species such as *Streptococcus* may be necessary to provide the suitable environment required by *Prevotella* and without these micro-organisms, it may not grow in the root canal environment.

Numerous studies have been undertaken to reveal an aetiological connection between certain species of bacteria and the occurrence of clinical symptoms or synergistic-antagonistic relationships among different strains. There is a positive correlation between *Fusobacterium nucleatum* and *Peptostreptococcus micros*,

between *Peptostreptococcus micros* and *Prevotella intermedia* and between *Peptostreptococcus anaerobius* and *Eubacterium* species (Sundqvist 1992). Drucker *et al.* (1992) investigating the microflora of 35 root canals, found highly significant associations between *Prevotella melaninogenica* and *Peptostreptococcus micros*, between *Prevotella melaninogenica* and *Prevotella oralis* and between *Prevotella corpolis* and *Streptococcus morbillorum*.

Gomes *et al.* (1994a) examined 30 infected root canals microbiologically and concluded that a significant association existed between pain and the presence of *Prevotella* and *Peptostreptococcus* species in dental root canals. Furthermore, Gomes *et al.* (1994b) investigated the positive and negative associations between bacterial species in 65 different species isolated. Positive and significant associations occurred between 31 species pairs. The highly significant associations (P<0.01) were found in 20 pairs e.g. between *Peptostreptococcus* and *Prevotella* species, between *Peptostreptococcus micros* and *Prevotella* species and between *Prevotella* and *Streptococcus* species.

Anaerobic and facultative anaerobic species can act together, synergistically, in order to initiate or maintain pulpal or periradicular pathosis (Grenier & Mayrand 1986). Saccharolytic bacteria which primarily obtain their energy by fermenting carbohydrates, e.g. *Streptococcus mutans*, may have less chance to grow in the closed environment of the root canal, which is normally deficient in these nutrients. On the other hand, asaccharolytic bacteria capable of fermenting amino acid and peptides will be more likely to grow. Haemolytic species of the genus

Streptococcus can release protohaem from red blood cells to support the growth of co-colonising black-pigmented species, mainly *Prevotella intermedia*, *Prevotella melaninogenica* and *Porphyromonas endodontalis*, which lack the proteolytic activity to hydrolyse haemoglobin (Gharbia & Shah 1993). Moreover, bacteria can also utilise end-products of other micro-organisms as nutrients in a process called syntropy (Grenier & Mayrand 1986). In addition to nutrient availability, the presence of aerobic bacteria lowers the local oxygen concentration resulting in the creation of a physical environment appropriate for the proliferation of anaerobes, especially in the apical portions of the root canal system.

Clinically, it is likely that the passage of bacteria or bacterial end-products follows the dissolution or disintegration of root canal sealer and smear layer. Inflammatory pulp lesions more often result from permeation of bacterial products than from actual invasion of the dentine and pulp by living micro-organisms (Bergenholtz 1977, Warfvinge 1985). Therefore, the investigation of leakage of obturated root canals by endotoxin following long-term storage in saliva was undertaken.

CHAPTER 9 CORONAL LEAKAGE OF OBTURATED ROOT CANALS AFTER LONG-TERM STORAGE USING AN ENDOTOXIN MARKER

9.1 INTRODUCTION

Gram-negative micro-organisms contain endotoxin, a lipopolysaccharide (LPS), which can be either secreted in vesicles by growing organisms or released after the death of the organisms. Endotoxin has many biological activities including fever induction, adjuvant activity, Schwartzman reaction, cytotoxicity, blood clotting and fibrinolysis. Researchers have shown that there is a significant relationship between the presence of endotoxin in periradicular lesions and the extent of inflammation (Schein & Schilder 1975, Bergenholtz 1977, Dahlén & Bergenholtz 1980, Dwyer & Torabinejad 1981, Schonfeld *et al.* 1982).

Thus, the purpose of this *in vitro* study was to investigate the leakage of endotoxin into root canals obturated with lateral condensation of cold gutta-percha and AH26 after long-term storage in artificial saliva.

9.2 MATERIALS AND METHODS

9.2.1 Preparation of Teeth

Thirty extracted human teeth were used in this study. Criteria for choosing and methods of storing the teeth were described previously (chapter 2). The

mechanical preparation technique of the root canals was described previously in chapter 3 and the smear layer was left intact. The prepared teeth were divided randomly into experimental group (20 teeth) and control groups (10 teeth). The experimental design is summarised in Fig. 2.13.

9.2.2 Obturation

Experimental Group

The root canals of 20 teeth were obturated with lateral condensation of cold gutta-percha and AH26 (De Trey, Zurich, Switzerland) sealer.

Control Group

The root canals of 5 teeth were obturated with lateral condensation of cold gutta-percha with AH26 (De Trey) sealer. These teeth were used as the negative controls. The remaining 5 teeth were not obturated and served as the positive controls.

The lateral condensation of cold gutta-percha technique was the same as chapter 3. All sealers were mixed according to manufacturer's instructions. The excess root filling material was removed coronally with a heat transfer instrument and a suitably-sized cold plugger was used to plug the gutta-percha apically. Following obturation, all teeth were kept in 100% humidity at 37°C for 48 h to allow setting of the sealer. The coronal portion of the root filling was left exposed to the storage environment. Each group of specimens were put into separate glass containers

filled with 60 mL artificial saliva, kept at 37°C for 6 months and the solution changed monthly. The formula of the artificial saliva was the same as that used in chapter 5. After 6 months, the teeth were removed from the storage medium, rinsed in running water and the crowns of all specimens were cut with a diamond saw (Labcut, Agar Scientific, Stansted, UK) rotating at approximately 300 rpm, with water coolant, to obtain a root 12 mm long.

9.2.3 Preparation of Specimens

The coronal part of the root canals of each of the 30 teeth were connected with the cut end of a 1.9 mL polyethylene microcentrifuge tube (Life Sciences International, Basingstoke, Hampshire, UK) using sticky wax, to prevent leakage at the connection. The teeth and tubes at the connection were covered entirely with sticky wax except the apical 2 mm of the root. The 5 teeth that were to be used as the negative controls were covered completely with sticky wax, to include the apical portion of the tooth. All specimens were then sterilised using ethylene oxide gas. Four teeth from the experimental group were discarded because of cracks in the tubes.

The leakage apparatus set up was the same as the previous experiment (chapter 3) except the Todd-Hewitt Broth (THB) was replaced in both the coronal and apical chamber by sterile water (Fig. 9.1).

Fig. 9.1 Apparatus set-up for endotoxin leakage study.

9.2.4 Endotoxin Leakage

If bacterial contamination of the system was not noted, a sample from each apical chamber was checked for endotoxin levels with spectrophotometry before starting the leakage experiment. The coronal chamber was injected with 0.5 mL of endotoxin standards (Sigma Chemical Co., Poole, UK) 800 E.U. mL^{-1} using a sterile syringe and a disposable 22 gauge needle. Since endotoxin standard dilutions containing 400 or more E.U. mL^{-1} are generally stable for at least one week (Bulletin from Sigma Chemical Co.), it was after this time that 0.5 mL of the solution in each coronal chamber was removed and replaced with 0.5 mL fresh endotoxin. The model system was stored in an aerobic incubator at 37°C and samples from the apical chambers were checked after 24 h and then weekly for 4 weeks.

The endotoxin from coronal chamber and the endotoxin levels in apical chamber was checked using spectrophotometric analysis modified from the method by Dische *et al.* (1949). Samples from the apical chamber were streaked onto Columbia agar plates (supplemented with 7.5% v/v defribinated horse blood) weekly to check for the possible bacterial contamination.

9.2.4.1 *Spectrophotometric analysis*

Endotoxin standard solutions containing 0-200 E.U. mL^{-1} were prepared. Sulphuric acid (86%, 25 mL) (BDH Chemicals Ltd., Poole, UK) was added to cysteine hydrochloride (3% w/v, 0.5 mL) (Sigma Chemical Co., Poole, UK) in an ice-water bath. Aliquots of 0.5 mL of this solution was added to each sample and

0.1 mL of endotoxin standards in an ice-water bath. The tubes were shaken and then heated at 100°C for 3 min. These tubes were plunged into ice-water. The absorbance was measured, using a Shimadzu UV-1601 spectrophotometer (Shimadzu Corp., Japan) (Fig.9.2), at 429 nm after 30 min.

9.3 RESULTS

The test for endotoxin in the coronal chambers were positive for all specimens at all experimental periods. All positive control teeth exhibited endotoxin leakage after 24 h and remained positive throughout the experimental period, whilst the lower chamber of negative control teeth remained uncontaminated throughout the test period (Table 9.1). There was no bacterial contamination in the apical chambers in all specimens. In the 16 experimental teeth, 4 teeth became positive after 21 d (25%) and 8 teeth became positive after 28 d (50%). The criteria for leakage scores and the number of teeth through which leakage occurred is shown in Table 9.2 and Table 9.3 respectively.

9.4 DISCUSSION

This study showed that endotoxin could penetrate a seemingly well obturated root canal within the 4-week period (Table 9.3). Some studies (Bergenholtz 1977, Warfvinge 1985) have suggested that inflammatory pulp lesions more often result from permeation of bacterial products than from actual invasion of the dentine and pulp by living micro-organisms. Horiba *et al.* (1991) revealed that teeth with
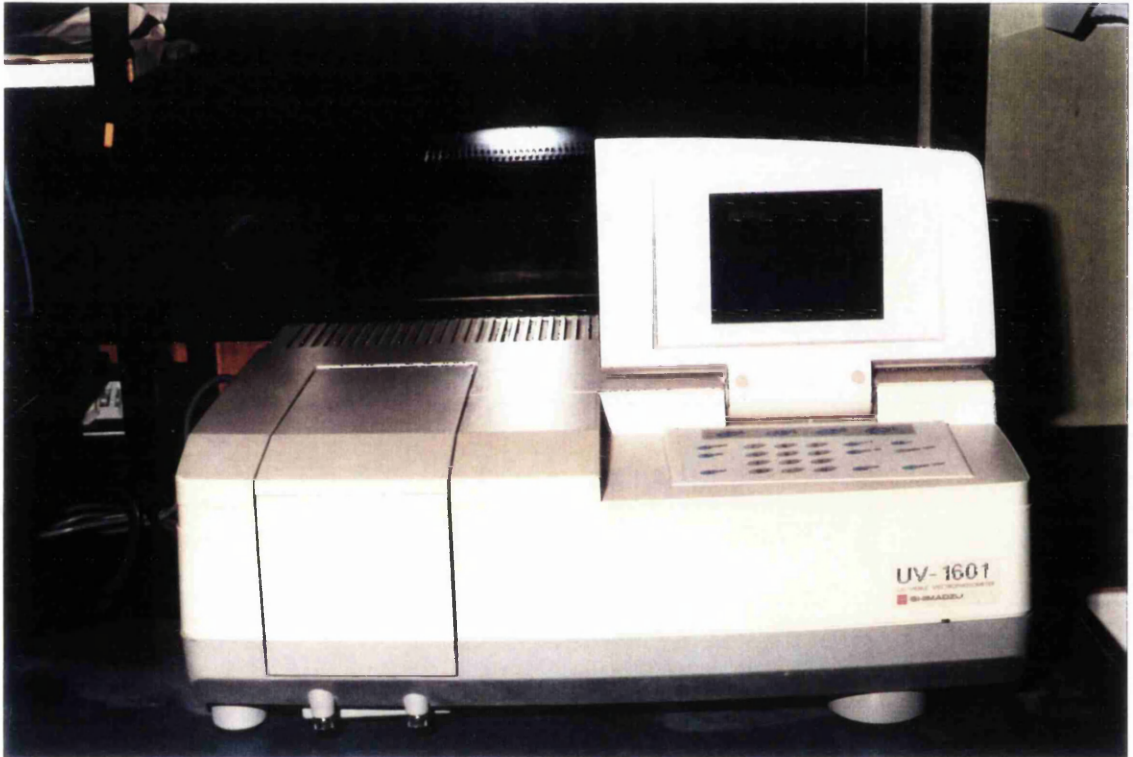
Fig. 9.2 Shimadzu UV-1601 spectrophotometer (Shimadzu).

Table 9.1 Positive (PC) and negative (NC) control teeth with presence of endotoxin in the apical chamber at different experimental periods.

Leakage levels	Experimental periods											
	0 d		24 h		1 wk		2 wk		3 wk		4 wk	
	PC	NC	PC	NC	PC	NC	PC	NC	PC	NC	PC	NC
0	5	5	3	5	2	5	1	5	-	5	1	5
1			1		2		1		1		1	
2			1		1		-		1		1	
3							1		1		1	
4							2		2		1	

Total number of specimens in each group = 5.

Table 9.2 Criteria of endotoxin leakage levels.

Leakage levels	Concentration of endotoxin (E.U.) in apical chambers
0	0-20
1	21-30
2	31-40
3	41-50
4	more than 51

Table 9.3 Number of teeth with presence of endotoxin in the apical chamber at different experimental periods (total 16 specimens).

	Experimental periods					
Leakage levels	0 d	24 h	1 wk	2 wk	3 wk	4 wk
0	16	14	16	16	12	8
1		1			3	2
2		1			1	3
3						2
4						1

clinical symptoms contained higher levels of endotoxin than those that were asymptomatic. Segal *et al.* (1990) found that endotoxin readily diffused through dentine disks ± 0.2 mm in thickness when aided by fluid filtration. However, Nissan *et al.* (1995) have shown that bacterial endotoxin is capable of diffusing through a 0.5-mm layer of smear free dentine without the use of filtration pressure in a relatively short period. Consequently, the fact that endotoxin passes through dentine suggests that all bacterial products are potentially capable of reaching the root canal system.

In this study 25% of the teeth demonstrated endotoxin in the apical chamber after 21 d and 50% after 28 d (Table 9.3). These results can be compared with a study by Trope *et al.* (1995) who showed that 31.5% of root canals were contaminated by endotoxin at 21 d. It is interesting that at 21 d the teeth in this study showed less leakage than the study by Trope *et al.* (1995) although the teeth in this present study were kept in the artificial saliva for 6 months. The reasons are difficult to establish but they may be differences in the preparation and obturation techniques, the experimental design and the detection method. Trope *et al.* (1995) used a commercially available Limulus lysate assay. Furthermore, the artificial saliva used in this study may have some effects on endotoxin penetration.

The experimental set up in this study was the same as chapter 3 except the coronal chamber and the separating media used (Fig. 9.1). The coronal chamber was a polyethylene tube because endotoxin is known to absorb onto untreated glass and polypropylene surfaces (Bulletin from Sigma Chemical Co.). Trope *et al.* (1995)

found that neither nail varnish nor cyanoacrylate glue provided an obstruction to the diffusion of endotoxin but sticky wax was a successful barrier. Therefore, in the present study the root was connected to the coronal chamber using sticky wax and the external root surface was painted with the sticky wax to separate the endotoxin in the apical chamber from the coronal chamber. Four teeth from the experimental group were discarded because of a crack in the tubes. This probably occurred because the polyethylene tube was more fragile compared with the polypropylene tube used in the previous studies.

Inevitably, all the apical chambers in this study showed some contamination by endotoxin ranging from 0-20 E.U. since it is so plentiful in the environment. As a result, the leakage levels was set at level 0 if the concentration of endotoxin in the apical chamber was between 0-20 E.U. (Table 9.2). It could be argued that the test endotoxin in the apical chamber may be a false positive by contamination. However, the endotoxin levels in the negative control teeth stayed at level 0 throughout the experimental period (Table 9.1), therefore, it can be concluded that the endotoxin detected in the apical chamber originated from the coronal chamber and was not because of contamination.

In this study, the endotoxin levels were checked using a spectrophotometric method modified from that described by Dische *et al.* (1949). Generally, a spectrophotometer has 2 components in one cabinet: a spectrometer and a photometer. A spectrometer is a device for producing coloured light of any selected wavelength. A photometer is a device for measuring the intensity of the

beam produced by a spectrometer. This is a simple quantitation method. A calibration curve was obtained by measuring the absorbance of known concentrations of endotoxin. These calibration curves produced straight line graphs (Fig. 9.3). Using linear regression, the values of the intercepts and slope of the lines were determined. The equation for a straight line is:

$$y = mx + c$$

Where y = the absorbance

x = the concentration

m = the slope of the line

c = the intercept

Thus the concentration of the unknown sample could be determined.

This method was used for detection and determination of hexoses. Since endotoxin is a lipopolysaccharide, this method was used indirectly to measure endotoxin levels by measuring hexoses after denaturation of endotoxin by acids. Although this method is less sensitive than the Limulus lysate assay, it was much cheaper and sensitive enough to detect the endotoxin levels in this *in vitro* study.

When these results are compared to the previous studies using *Streptococcus sanguis* (chapter 5) or *Prevotella intermedia* (chapter 7) to detect leakage of obturated canals, it was found that, at 21 d no teeth leaked in both these groups and only 1 tooth leaked when *Prevotella intermedia* was used as a marker at 28 d (Table 9.4). With the present study, the results showed that endotoxin penetrated obturated root canals more rapidly than bacteria. This has been corroborated by

ENDOTOXIN CALIBRATION CURVE
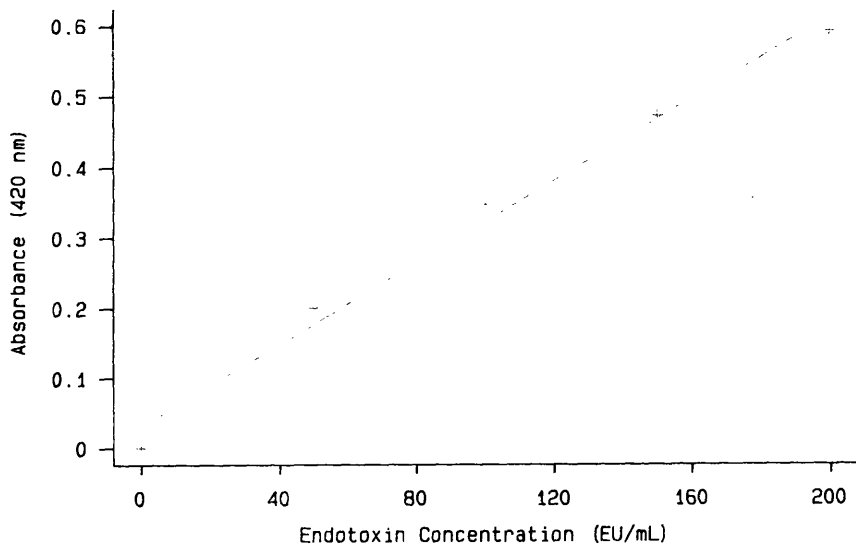
Fig. 9.3 Endotoxin calibration curve.

Table 9.4 Experimental teeth showing leakage when exposed to different markers.

Group	Number of specimens	Experimental periods	
		3 wk	4 wk
S.sanguis	20	-	-
P.intermedia	20	-	1 (5%)
Endotoxin	16	4 (25%)	8 (50%)

Cumulative percentage of specimens with leakage is shown in brackets.

Alves *et al.* (1996) who compared penetration of endotoxin and bacterial cells through post-prepared root canals which had 5 mm of gutta-percha remaining at the apex. Every three days, two samples were taken from the lower chamber: one was assessed for endotoxin using QCL-1000 Chromogenic Quantitative LAL Assay System, the other was spiral-plated to check for bacteria. In the experimental teeth, bacterial leakage occurred in 20 d, while endotoxin leakage began after 11 d.

Clinically, it would be possible that with a defective coronal restoration, Gram-negative bacteria could be present coronally and release endotoxin which, after passage through an obturated canal, could induce periradicular inflammation and failure of treatment.

CHAPTER 10 GENERAL DISCUSSION

10.1 USE OF NATURAL TEETH

Some research workers have used simulated root canals, usually in transparent plastic blocks, to study the effects of various root canal preparation procedures, to test new instruments or to determine microleakage in endodontics. The advantages of simulated root canals include the transparent nature of the plastic block which allows direct visual inspection of the simulated canal and standardised morphology such as diameter, length and shape. However, it must be recognised that simulated root canals do have drawbacks such as the simple shape of the simulated root canals with no attempt being possible to simulate the complexity of the situation found in root canals *in vivo*. Moreover, the physical properties of the resin used in the construction of the canals are not comparable with dentine.

Natural teeth were used in this study because leakage assessment requires a reliable and clinically realistic test, although the morphology of root canals of extracted teeth are more difficult to control because of the variation in shape and size. In an attempt to reduce variation, only straight single root canals which are considered to be round or oval in transverse section were used. These roots were randomly divided into the experimental groups. The access and the instrumentation was completed with the crown intact (except chapter 3 in which the crown was removed before instrumentation), which makes preparation of the root canal more relevant clinically. The size of the canal after instrumentation was controlled by

preparing each canal to the same size of master apical file and using the same technique. All roots investigated were sectioned to the same length after preparation. Furthermore, the patency of the foramen was confirmed after instrumentation using a size 15 file.

10.2 EFFECT OF SMEAR LAYER

At present, the two major irrigating solutions that can be used to remove smear layer efficiently are ethylene diamine tetra-acetic acid (EDTA) and citric acid. The latter was used to remove dentinal smear layer in this study because it occurs naturally in the body thus it may be more acceptable biologically than other acids (Wayman et al. 1979). A study by Yamada et al. (1983) showed that a final flush with 25% citric acid followed by 5.25% NaOCl produced canal walls free of organic and inorganic debris similar to a final flush with 17% EDTA followed by 5.25% NaOCl. Saunders et al. (1992) examined root canals which had been treated with 8 mL of 40% solution of citric acid for 2 min using SEM. They found that the coronal and middle parts of the root canal showed open tubules with no smear layer present and with only small amounts of debris. There was more debris in the apical third of the root canal but, in general, this section too was free from smear layer.

10.3 DESIGN OF THE MODEL

The model used in this study was refined from a model developed by Goldman *et al.* (1980). The main advantage of the present experimental set-up over previous models used is that the coronal chambers were attached to the cap of the glass bottles. This reduced the chance of accidental overflow of the bacteria-containing solution to the experimental tooth. This arrangement also provided a contamination-free chamber even though the study period was 90 days. It is presumed that the model would be satisfactory for studies of longer duration.

A pilot study had been done to compare several types of glue with and without sticky wax for prevention of bacterial leakage. The results showed that some types of glue including those containing epoxy resin did not adhere to the polypropylene tube. Using 2 layers of cyanoacrylate glue without sticky wax prevented bacterial leakage at the connection only up to 60 d. Application of two layers of cyanoacrylate glue, a layer of sticky wax and another two layers of nail varnish on the wax surface prevented bacterial leakage and provided a negative control group which did not show signs of bacterial leakage throughout the experimental period. An absence of turbidity in the apical chambers in the negative control group indicated that this set-up did provide a contamination-free chamber.

This model has proved to be a reliable *in vitro* method to test coronal microleakage since the results obtained in this study were reproducible. However, compared with clinical conditions, this model had some disadvantages. Firstly, it

was static and the media used for bacterial growth was not similar to saliva. Secondly, the micro-organisms used as leakage markers in this study were non-motile bacteria. These micro-organisms move by diffusion and Brownian movement from the coronal chamber to the apical chamber. In addition, gravity may have had some effect on the bacterial penetration. However, a study by Torabinejad *et al.* (1990) showed that the motility of micro-organisms may not be an important factor for the recontamination of an exposed obturated root canal system. Finally, this model may not provide a strictly anaerobic condition sufficient for the growth of obligate anaerobic micro-organisms because there is an air space in the upper part of the apical chamber. To solve this problem the screw top was loosened during storage of the model in the anaerobic incubator and a reducing agent such as cysteine hydrochloride was added to the media to reduce oxygen tension.

This model was also used for the endotoxin study (chapter 9). Inevitably, all the apical chambers in this study showed a variation in contamination by endotoxin ranging from 0-20 E.U. The contamination of endotoxin may arise from the tooth itself, the apparatus used such as the glass bottle, pipette or polyethylene tube, and from the surrounding environment. Schein and Schilder (1975) found that significant amounts of endotoxin were recovered from endodontically involved teeth, especially painful teeth and teeth that have radiolucent areas at their apex. In addition, Buttler and Crawford (1982) found that 1 mL of either 0.58% or 2.7% NaOCl could not detoxify the endotoxin produced by *Escherichia coli* in an *in vivo* study.

In the present study, the endotoxin levels were checked using a spectrophotometric method modified from that described by Dische *et al.* (1949). In a pilot study, many techniques have been performed to detect the endotoxin levels including isotachophoresis and Limulus lysate assay. It was found that isotachophoresis can only detect ionic substances whilst the latter technique was too expensive to be used in this study.

10.4 CENTRIFUGATION

The efficacy of vacuum conditions to ensure reproducible dye penetration has been questioned (Peters & Harrison 1992, Masters *et al.* 1995, Roda & Gutmann 1995). It also may lead to an overestimation of the microleakage that would occur in the clinical setting. As a result, centrifuging was used as an alternative technique in this study to force the broth through any defects in the root canal filling and to reduce the likelihood that air bubbles in the obturated tooth may disrupt the passage of micro-organisms. Although centrifuging is not clinically relevant, it may provide a more exacting leakage test *in vitro*.

10.5 EFFECT OF ETHYLENE OXIDE GAS

Ethylene oxide is an alkylating agent and the inactivating effect on micro-organisms is considered to be a result of the alkylation of sulfhydryl, amino, carboxyl, phenolic and hydroxyl groups in the spore or vegetative cell of micro-organisms (Phillips 1977). The effect of ethylene oxide gas on the root filling

materials was unknown. In a pilot study, sterilisation of the experimental teeth before obturation and setting up the model was attempted but it was very difficult and impractical to maintain the sterility of the teeth during instrumentation and obturation.

10.6 BACTERIA AND BACTERIAL METABOLITES LEAKAGE

All scientific evidence points towards micro-organisms as the dominating or sole cause of periradicular disease of endodontic origin. Therefore, it is important to concentrate on understanding the importance of quality and quantity of micro-organisms and / or their metabolites in the pathogenesis of periradicular disease.

Micro-organisms, as well as dye, radioisotope and other substances have been employed as tracers for leakage evaluation. Mortensen *et al.* (1965) and Krakow *et al.* (1977) showed that micro-organism penetration might be more appropriate than dye or radioisotope penetration for studying endodontic leakage *in vitro.* Since small molecules (e.g. water) may leak 100-fold more than larger ones (albumin) (Pashley & Livingstone 1978), the question arises as to which particle size can best meet the requirements of a leakage study. Goldman *et al.* (1980) have pointed out that bacteria gives a better indication than dye in testing for leakage of hydrophilic materials and that dyes can give a false positive reading if their molecules are small enough.

In this study, *Streptococcus sanguis*, *Fusobacterium nucleatum* and *Prevotella intermedia* were used as markers for the investigation of coronal leakage of root canal filling materials. These markers were selected because they are believed to be involved in the pathogenesis of periradicular lesions (van Winkelhoff *et al.* 1985, Yoshida *et al.* 1987, Iwu *et al.* 1990, Baumgartner & Falkler 1991, Brook *et al.* 1991, Sundqvist 1992, Wayman *et al.* 1992).

In chapter 8, mixed micro-organisms of *Streptococcus sanguis* and *Prevotella intermedia* were studied because several researchers (Korzen *et al.* 1974, Fabricius *et al.* 1982a, Sundqvist *et al.* 1989, Baumgartner & Falkler 1992) have shown that the degree of inflammation is less severe with a monoinfection than with a mixed infection. The result obtained in this study supports the study reported in chapter 8 in which Apexit group and Tubliseal EWT group exposed to both *Streptococcus sanguis* and *Prevotella intermedia* showed significantly more leakage (P<0.05) than teeth exposed to only *Streptococcus sanguis*.

Clinically, it is possible that Gram negative bacteria could be present coronally in a defective coronal restoration, and their acid end-products could move through the obturated canal. On the other hand, endotoxin may be released into the environment during Gram-negative bacterial cell growth or as a large fragment of membrane after cell death and move through the obturated canal to cause an inflammatory response apically.

Thus, the leakage of butyric acid and endotoxin were also investigated in chapter 6 and 9 respectively. In chapter 6, the result is in agreement with Kersten *et al.* (1987) who concluded that leakage of butyric acid could not be prevented by either the root canal sealer used or vertical pressure applied in the obturation techniques. In chapter 9, 50% of teeth were contaminated with endotoxin after 28 d. In this study, the indirect technique was applied to detect endotoxin levels by measuring hexoses after denaturation of endotoxin by acids. The result collaborates with studies by Trope *et al.* (1995) and Alves *et al.* (1996) in which endotoxin penetrates an obturated root canal faster than bacteria.

These bacterial studies have been qualitative rather than quantitative. If only one bacterium passes through the obturated root canal, it may multiply in the enriched broth and cause turbidity. Large numbers of previous leakage studies have reported considerable variation in results (Wu & Wesselink 1993). In this study therefore, an all or nothing, qualitative approach was employed, i.e. leakage was registered as positive only when turbidity in the apical reservoir indicated complete penetration of the obturated canal system. Importantly, however, it is unknown how many micro-organisms are required to cause periradicular inflammation and lesions.

Variables not included in this investigation which could change the overall coronal leakage patterns are the cyclic nature of masticatory forces, thermocycling, pH change and variations in salivary viscosity. It is not known what effect these factors may have on coronal leakage.

A relatively high frequency of radiographically healthy periradicular bone has also been reported in association with root canal fillings of low technical standard (Ödesjö *et al.* 1990), indicating that only leakage of larger substances like micro-organisms or bacterial end-products of high molecular weight, is necessary for periradicular pathosis, and that such gross microleakage can be prevented by root canal therapy in most cases. Nevertheless, the development and maintenance of a hermetic seal is considered to be a major prerequisite for success in root canal treatment. Thus, the evaluation of the quality of a root canal filling using leakage tests is still relevant.

CHAPTER 11 CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDY

11.1 CONCLUSIONS

Based on the results reported in this study, it can be concluded that:

- a reliable model for the detection of leakage using microbial markers has been developed

- the presence or absence of the smear layer from the root canal wall had no significant effect on the leakage of *Streptococcus sanguis* along obturated root canals immediately after obturation

- there were no significant differences in leakage of *Streptococcus sanguis* in teeth root-filled with gutta-percha and AH26, Apexit or Tubliseal EWT sealers tested immediately after obturation

- both AH26 and Tubliseal EWT sealers in combination with lateral condensation of cold gutta-percha could not prevent leakage of butyric acid produced by *Fusobacterium nucleatum* along obturated canals

- there was no significant difference in coronal leakage of *Streptococcus sanguis* along obturated canals among: AH26, Apexit, Sealapex or Tubliseal EWT sealers after 6 months storage in artificial saliva but AH26 (delay) group showed significantly less leakage ($P<0.05$) than AH26 (immediate) group. Both Apexit and Tubliseal EWT (delay) groups showed significantly less leakage ($P<0.01$) than Apexit and Tubliseal EWT (immediate) groups.

- after 6 months storage in artificial saliva, AH26 and Sealapex sealers had no significant effect on the leakage of *Prevotella intermedia* along obturated canals but AH26 group exposed to *Prevotella intermedia* showed significantly more leakage ($P<0.01$) than AH26 group exposed to *Streptococcus sanguis*. However, both Sealapex groups did not show any statistically significant differences in leakage ($P>0.05$).

- there were no significant differences in leakage when Apexit or Tubliseal EWT sealers combined with lateral condensation of cold gutta-percha were tested with *Streptococcus sanguis* and *Prevotella intermedia* in combination but the Apexit group exposed to both *Streptococcus sanguis* and *Prevotella intermedia* showed significantly more leakage ($P<0.05$) than the Apexit group exposed to only *Streptococcus sanguis*. The Tubliseal EWT group exposed to both *Streptococcus sanguis* and *Prevotella intermedia* showed highly significantly more leakage ($P<0.01$) than the Tubliseal EWT group exposed to only *Streptococcus sanguis*.

- endotoxin penetrated obturated root canals more rapidly than bacteria. Fifty per cent of the obturated root canals allowed the penetration of endotoxin after 28 d.

11.2 SUGGESTIONS FOR FURTHER STUDY

Although Hovland and Dumsha (1985) have shown that most leakage occurs at the interface of dentine and sealer, it may occur at the interface of root filling materials and sealer or through the sealer itself. In this study, no attempt was made to observe the route of micro-organisms. Magura *et al.* (1991) have shown that histological examination of teeth with obturated root canals, exposed for varying periods to saliva, demonstrated unexpected staining characteristics in the H&E-stained sections. It would be interesting to investigate the distribution of micro-organisms within the tooth following microbial leakage studies.

At present, a direct correlation between microleakage and smear layer clinically is still controversial. Pashley and Depew (1986) found that microleakage decreased after the removal of smear layer but dentine permeability increased. When the smear layer is not removed, the durability of the seal should be evaluated over a long period. Since this layer is a nonhomogeneous and weakly adherent structure (Mader *et al.* 1984), it may slowly disintegrate, dissolving around a leaking filling material, thus creating a void between the root canal wall and the sealer.

The experimental teeth used in this study had closed apices but there was no history of the age of the teeth. All teeth were randomly divided into the experimental groups. The diameter of dentinal tubules may have some effect on the bacterial penetration. Perez *et al.* (1993) have shown that *Streptococcus sanguis* migrates less deeply through the tubules of a mature tooth than an immature one because the diameter of the dentinal tubules is reduced by peritubular dentine formation. Manogue *et al.* (1994) related the measurements of the total area of voids within the root canal system to the age group of the patient from whom the tooth was extracted. A significant relationship (P<0.05) was found between the area of voids found in the youngest group when compared with all other age groups.

Therefore, further studies are needed to:

- develop a reproducible microleakage model which can provide strictly anaerobic conditions

- develop other sensitive, reproducible and straight-forward techniques to detect micro-organisms, metabolic end-products and endotoxin

- examine histologically the passage of micro-organisms within the root canal system using staining techniques

- further establish the correlation between dentinal smear layer and microleakage using both *in vivo* and *in vitro* methods

- determine the long-term effect of tissue fluids or saliva on microleakage through root filled teeth

- investigate the microleakage of other micro-organisms, metabolic end-products or more complicated biological markers which may cause periradicular inflammation

- determine the effect of age changes within the tooth on subsequent microleakage of micro-organisms through the root filling.

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