THE ABILITY OF THIOUREA TO SCAVENGE HYDROGEN PEROXIDE AND HYDROXYL RADICALS DURING THE INTRA-CORONAL BLEACHING OF BLOOD-STAINED ROOT-FILLED TEETH.

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DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

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DEDICATION

To Annie, Jem and Charlotte who have made it all worth while.

SUMMARY

The intra-coronal bleaching of root-filled teeth has been described in the literature for over 100 years. Currently the two most common bleaching techniques utilise hydrogen peroxide. The method described in the literature in 1963 by Nutting and Poe relied on the inherent instability of the hydrogen peroxide to decompose into its oxidising components which were capable of penetrating into the stained tooth structure and bleaching the stained dentine. This method has become known as the "walking bleach" technique. The application of heat to activate the hydrogen peroxide has been used since the nineteenth century. In 1965, Stewart termed this method involving the application of heat the "thermocatalytic bleaching" technique.

Harrington and Natkin (1979) first reported the association between the use of hydrogen peroxide in the bleaching of traumatised teeth which had been root-filled and a form of external root resorption. Since that report there have been numerous case reports of a similar association and several theories have been postulated as to the cause of this resorptive process.

Marin *et al.* (1997) demonstrated that the staining of the traumatised tooth with blood was the result of the rupture of red blood cells within the pulp tissue and the release of haemoglobin and haem moieties which pass into the dentinal tubules and cause the resultant discolouration. Hydrogen peroxide is capable of reacting with available iron found associated with haemoglobin within the dentine tubules to produce hydroxyl radicals. Both hydrogen peroxide and hydroxyl radicals are capable of damaging cells and tissues of the periodontal ligament. This destruction may result in an inflammatory response which could be responsible for the initiation of the resorption

process. Dahlstrom *et al.* (1997) reported on the ability of hydroxyl radicals to be generated on the outer surface of the tooth that had been stained with blood and thermocatalytically bleached. Dahlstrom *et al.,* in the same study, detected hydrogen peroxide on the outer surface of the tooth in a small percentage of his samples. This latter finding was in agreement with the findings of Rotstein *et al.* (1991) who had previously reported the detection of hydrogen peroxide on the outer surface of teeth which had been stained with blood and intra-coronally bleached.

Thiourea has been used in the textile industry for many years as a reductive bleaching agent to bleach wool and paper. It has also been used in the medical profession for a number of years to scavenge hydroxyl radicals and a number of other oxygen derived free radicals during the reperfusion of various organs and in the treatment of various disease states. Maroulis (1994) showed that the introduction of thiourea into the intra-coronal bleaching of root-filled teeth had no detrimental effect on the bleaching efficacy of hydrogen peroxide.

The aim of the present research was to (1) Compare the quantity of hydrogen peroxide and the quantity of hydroxyl radicals present on the outer root surface of blood stained teeth which have been intra-coronally bleached with either hydrogen peroxide sealed into the pulp chamber ("walking" bleach), or with hydrogen peroxide which has been activated with heat ("thermocatalytic" bleach) and (2) Determine the ability of thiourea to scavenge hydrogen peroxide and hydroxyl radicals within radicular dentine structure during the intra-coronal bleaching of blood-stained, root-filled teeth.

Thirty-five extracted, single rooted premolar teeth were stripped of their periodontal ligaments and the pulp tissue extirpated. Thirty of these teeth were stained with blood using a method devised by Marin *et al.* (1997). The teeth were obturated with AH26 and gutta percha and bleached with hydrogen peroxide, with or without the application of heat. Thiourea was introduced into the bleaching protocol in various groups. The teeth were suspended in a bath of salicylate and the products of the reaction between hydroxyl radicals and the salicylate were detected using high performance liquid chromatography (HPLC) and electro-chemical detection (ECD).

Twenty-two of the 35 teeth sampled generated reaction products of the hydroxylation of salicylate. There was no statistically significant difference between the quantity of hydroxyl radicals generated between the two methods of intra-coronal bleaching. Greater yields of hydrogen peroxide were detected on the outer surface of the teeth which had been bleached with the "walking" bleach technique. The presence of thiourea in the bleaching protocol resulted in a statistically significant decrease in the amount of hydrogen peroxide and hydroxyl radicals detectable on the outer surface of the tooth. There was a statistically significant decrease in the amount of thiourea that was detected on the outer surface of the tooth when hydrogen peroxide was combined with thiourea, compared to the introduction of thiourea alone into the pulp chamber.

It was concluded from this research that both methods of intra-coronal bleaching of root-filled teeth are capable of generating hydroxyl radicals which are detectable on the outer surface of the tooth. Hydrogen peroxide is capable of diffusing from the pulp chamber to the outer surface of the tooth, although the activation of the hydrogen peroxide with heat resulted in lesser quantities being detected on the outer surface of

the teeth. There was a positive association between the presence of thiourea and a decrease in the detection of hydrogen peroxide and hydroxyl radicals. If hydroxyl radicals and hydrogen peroxide are, in part, responsible for the initial activation of clastic cells involved in invasive cervical resorption, then the introduction of thiourea into the bleaching protocol may provide a method of preventing the initiation of the resorption process.

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1.0 Introduction

The intra-coronal bleaching of internally non-vital teeth has been reported in the literature for many years and has been described as safe and effective. The use of hydrogen peroxide for intra-coronal bleaching of root-filled teeth has been used for over 100 years. The description by Nutting and Poe (1963) who recommended the sealing of hydrogen peroxide into the pulp chamber of a tooth was the first in which hydrogen peroxide was sealed into the tooth between appointments. The authors later modified this to include sodium perborate. Spasser (1961) recommended the use of sodium perborate and water sealed into the pulp chamber as an effective and reliable bleaching agent. The only significant change to these bleaching protocols has been the introduction of heat to "activate" the hydrogen peroxide. This method of intra-coronal bleaching is referred to as thermocatalytic bleaching (Stewart, 1965). Heat is applied to the hydrogen peroxide via a heated instrument or a light source.

In 1979 Harrington and Natkin raised concerns about the safety of intra-coronal bleaching of root-filled teeth. They reported an association between a form of external resorption and the internal bleaching of root-filled teeth. Since this report the use of hydrogen peroxide and the different techniques which are employed have been queried. Many reports of the association between this form of external resorption – termed invasive cervical resorption (ICR), and intra-coronal bleaching have been published since Harrington and Natkin reported their theory on the cause – effect relationship between the two entities.

Considerable research into the association between the use of hydrogen peroxide during intra-coronal bleaching and the resorption process, ICR, has resulted in conflicting views about which bleaching protocol to adopt. Several authors caution against the use of heat to activate hydrogen peroxide (Friedman *et al.* 1988, Madison and Walton 1990). Others have recommended that hydrogen peroxide not be used, advocating the use of a mixture of sodium perborate and water as an effective bleaching agent (Smith *et al.*1992).

The use of hydrogen peroxide in the bleaching protocol has been suspected as the causative agent in the initiation of invasive cervical resorption. Harrington and Natkin (1979) hypothesised that hydrogen peroxide was capable of diffusing through the radicular dentine from the pulp chamber to the outer surface of the root. Here it was able to initiate an inflammatory response in the periodontal ligament sufficient to activate clastic cells that are capable of resorbing both dental hard tissue and the adjacent bony hard tissue. Rotstein *et al.* (1991b) reported the ability of hydrogen peroxide to pass through to the outer surface of the tooth. Dahlstrom *et al.* (1997) found that a significant percentage of the samples in their study allowed the passage of hydrogen peroxide through to the outer surface of the tooth when hydrogen peroxide in the pulp chamber was activated with heat. Hydrogen peroxide is known to be toxic to gingival tissues. It is capable of causing such cellular changes as damage to DNA, membrane lipid peroxidation and cell death.

In 1997, Dahlstrom *et al.* reported on the presence of hydroxyl radicals on the outer

surface of the tooth following the intra-coronal bleaching of blood stained teeth. The hydroxyl radical is an extremely reactive oxygen radical which has been well researched. It is highly toxic to an array of cell types including fibroblasts, the major cell type in the periodontal ligament. Its role in the destruction of connective tissue components, collagen and hyaluronic acid is well documented (Greenwald, 1981).

In biological systems the hydroxyl radical is produced by the Haber –Weiss reaction. This reaction involves the homolytic fission of the O-O bond in hydrogen peroxide resulting in the production of two hydroxyl radicals ($^{\cdot}OH$). Heat or ionising radiation can achieve this homolysis. However, this reaction occurs at a rate in biological systems which is not deemed significant. Fenton in 1894 demonstrated the ability of hydrogen peroxide to react with ferrous (Fe^{2+}) ions to produce hydroxyl radicals. This reaction has become known as the "Fenton" reaction and is able to occur within the dentinal tubules of blood stained teeth.

Marin *et al.* (1997) studied the mechanism by which blood stained the dentine following a traumatic episode to the tooth. This histochemical analysis of blood stained teeth revealed that it is haemoglobin or other forms of haematin molecules which were responsible for the discolouration seen in such teeth. Therefore, the presence of ferrous ions within the dentinal tubules is sufficient to allow the "Fenton" reaction to occur following the introduction of hydrogen peroxide into the pulp canal. The presence of these hydroxyl radicals on the outer surface of the tooth may be in part, responsible for the initiation of the resorptive process seen in ICR.

Alternative bleaching agents have been investigated although the literature on these bleaching agents is scarce. Marin *et al.* (1998) reported on the ability of Desferal (Ciba-Geigy Pharmaceuticals) and Enzymatic Cleaner (Dr Thilo & Co.) to bleach intra-coronal tooth structure. The author concluded that neither was effective in the intra-coronal bleaching of blood stained teeth.

The textile industry has recognised the ability of a combined oxidation-reduction bleaching protocol using hydrogen peroxide – thiourea to produce superior bleaching results to hydrogen peroxide alone. Maroulis (1994) reported on the ability of thiourea in the presence of hydrogen peroxide to bleach blood stained teeth by an oxidation – reduction reaction. The author found that the reaction product of thiourea and hydrogen peroxide – thiourea dioxide, was as effective as hydrogen peroxide in the intra-coronal bleaching of blood stained teeth.

Thiourea has been used for many years in the medical field as a scavenger of oxygen radicals and reactive oxygen species (Detterbeck *et al.* 1990). Thiourea is also capable of scavenging hydrogen peroxide. The ability of thiourea to scavenge both hydrogen peroxide and hydroxyl radicals within tooth structure following the intra-coronal bleaching of non-vital teeth is unknown.

The purpose of this study is two-fold:

(1) Compare the quantity of hydrogen peroxide and the quantity of hydroxyl radicals present on the outer root surface of blood stained teeth which have been

intra-coronally bleached with either hydrogen peroxide sealed into the pulp chamber ("walking" bleach), or with hydrogen peroxide which has been activated with heat ("thermocatalytic" bleach).

(2) Determine the ability of thiourea to scavenge hydrogen peroxide and hydroxyl radicals within radicular dentine structure during the intra-coronal bleaching of blood-stained, root-filled teeth.

2.0 Invasive cervical resorption

This unusual form of resorption of tooth structure is a type of progressive external resorption. It may occur in vital and internally non-vital teeth and in permanent and primary teeth. It appears to follow injury to the cervical attachment apparatus just below the epithelial attachment, after which the damaged root surface is colonised by hard tissue resorbing cells.

There have been many aetiologies proposed for this resorptive process. Southam (1967) and Vincentelli (1973) suggested that the cause of ICR lay in the developmental defect at the cemento-enamel junction (CEJ) where cementum and enamel do not meet thereby leaving dentine in direct contact with the periodontium. However, Langeland (1967) was unable to find that the presence of a protective layer e.g. cementoid, prevented resorption from occurring *in vitro*. Kerr (1961) believed that periodontal inflammation is the cause of this form of resorption. A local chronic inflammatory process resulting, for example, from gingivitis or periodontal disease stimulates clastic cell activity. Tronstad (1988) who described this type of resorption as transient with cemental repair occurring within 2-3 weeks supported such a theory. He suggested that stimulation of the resorbing cells for longer periods might be due to the presence of bacteria and their products via dentinal tubules in the cervical region, not from the pulpal tissues but from the gingival tissues and the surface of the root. However, histopathological studies of this form of resorption (Heithersay, 1999a) have failed to detect the presence of bacteria, or the inflammatory cell infiltrate their presence would evoke.

Clastic cells are activated by a number of factors, as reviewed by Heithersay (1994):

(1) Biochemical forces e.g. orthodontic treatment; an erupting crown or its crypt impinging on the root surface of an adjacent tooth.

(2) Mechanical or surgical trauma which results in damage to the CEJ

(3) Chemical trauma e.g. intra-coronal bleaching of root-filled teeth

(4) Bacteria and their toxins - chronic inflammatory responses in the periodontal ligament space can activate clastic cell which attack tooth structure.

(5) Developmental factors e.g. Hypoplasia of cementum

(6) Neoplasia - clastic cells are activated by some neoplasms

(7) Hormonal - clastic cells directed at the teeth have been associated with hormonal imbalances like hyperthyroidism.

The activation of osteoclasts and osteoclast precursors is probably mediated through osteoblasts (Hammarström and Lindskog, 1992). Following activation by parathyroid hormone, 1,25 dihydroxycholicalciferol and by cytokines (IL-1α, TNFβ), osteoblasts contract to make the bone surface available for clastic cell activity. Contact of the osteoclast with the bone surface stimulates activation of the osteoclast and resorption begins. Whether these findings are capable of being applied to the surface of the tooth is unknown, however, the similarity in structure of the osteoclast and dentinoclast, and the similarity between activating factors for different clastic cell populations suggests that blastic cells may influence the activation of the dentinoclast.

Normally the bone surfaces have a layer of non-mineralised organic material

between the osteoblasts and the mineralised bone and osteoclasts are unable to resorb this layer. Similarly, immediately beneath the cementum there lies a layer of intermediate cementum and cementoid (Hammarström and Lindskog, 1985) which plays an important role in the prevention of inflammatory resorption of replanted teeth by acting as a barrier between the pulp and the periodontal ligament. Trauma, either mechanical or chemical, may damage this layer of intermediate cementum rendering the dentinal surface of the tooth more vulnerable to the resorptive activity of the dentinoclast.

Cytokines have received attention in the literature with respect to bone resorption and much of this research is applicable to the ICR lesion. Macrophages and osteoclasts are thought to play an important role in the pathogenesis of ICR. Macrophages stimulate osteoclastic bone resorption, and dentine and cementum destruction by the activation of lytic processes in the periodontal tissues (Jimenez-Rubio and Segura, 1998). Moreover, particular cytokines produced and released by macrophages (IL1β and TNF) have been found to be associated with periodontal destruction and cervical root resorption (Stashenko *et al.* 1991). It is thought that the effects of these macrophage derived cytokines are mediated by osteoblasts as only osteoblast cells possess receptors for these cytokines (Akamine *et al.* 1994).

The aetiology of ICR is unknown and the theories briefly reviewed above, regarding the initiation of this resorption process are only speculative. No single theory, as yet, has been proven to initiate the resorption process. Harrington and Natkin (1979) found several similarities between bleached root-filled teeth affected by this

resorptive process

(i) All teeth involved sustained pulp death after traumatic injury.

(ii) Bleaching occurred a number of years after the completion of the endodontic therapy in six of the seven cases.

(iii) A caustic bleaching agent (superoxol with or without sodium perborate) and a heat source were used.

(iv) Resorptive defects occurred in the cervical region of the tooth in all cases.

This report by Harrington and Natkin (1979) was the first to find an association between intra-coronal bleaching of root-filled teeth and ICR and has resulted in an abundance of subsequent research attempting to find a link between the two. There are several theories as to the actual mechanism of action of hydrogen peroxide and the hydroxyl radical in the initiation of the resorptive process. A general consensus is that the bleaching agent is capable of diffusing through to the outer surface of the tooth. Friedman (1987) suggested that a decrease in pH to approximately 6.5 on the outer surface of the tooth using the walking bleach technique will produce an acidic environment sufficient to stimulate osteoclast activity. It has been proposed that during the thermocatalytic bleaching the active bleaching agent is forced through the dentinal tubules by the application of heat. Lado *et al.* (1983) suggested that hydrogen peroxide denatures dentine rendering it a foreign body which stimulates an inflammatory response on the outer surface of the tooth. Similarly Madison and Walton (1990) suggested hydrogen peroxide chemically alters the cementum stimulating a foreign body reaction with an associated inflammatory response. Several other authors, (Lewinstein *et al.* 1994; Rotstein *et al.* 1992) who found

changes in the ratio of organic to inorganic components of the dentine and cementum and denaturation of their protein content, support these theories. Webber (1983) believed that teeth with a history of trauma have a resorptive potential, which remains prevalent throughout life. The traumatised tooth will lose cementum by resorption leaving exposed patent dentinal tubules. He suggested that application of a caustic bleaching agent can reactivate an inflammatory resorption process.

Dahlstrom *et al.* (1997) demonstrated the presence of hydroxyl radicals on the outer surface of the tooth following intra-coronal thermocatalytic bleaching of root-filled teeth. The highly reactive nature of the hydroxyl radical and the ease with which it is capable of forming from hydrogen peroxide in the presence of iron, indicates it may be this oxygen derived free radical which is responsible for localised destruction of hard and soft tissue on the outer surface of the tooth (within the periodontal ligament). This localised destruction of cementum and periodontium has the potential of activating clastic cells.

Trauma to the affected tooth has likewise been associated as a potential cause of the initiation of the resorptive process. Heithersay (1999b) in a study of 257 teeth affected by invasive cervical resorption reported that in 15.1% the sole predisposing factor attributable to the onset of the resorptive process was trauma. When other potential predisposing factors were included, 66 teeth or 25.7% of teeth, had a history of trauma. Of these teeth the maxillary central incisors were the most frequently affected teeth, followed by mandibular lateral incisors, mandibular incisors and maxillary canines. These anterior teeth are naturally more prone to

suffering trauma. Other potential predisposing factors include orthodontics, dento-alveolar surgery, bruxism, delayed eruption, developmental defects intra-coronal restorations and periodontal therapy (Heithersay, 1999b). Chivian (1991) added to this list of possible associated factors for the initiation of this resorption process a history of orthognathic surgery. According to Hammarström and Lindskog (1992) damage to the cementum is necessary for ICR to be initiated. All of the outlined predisposing factors result in potential damage to, or alteration of, the cementum lining at the cervical margin of the root surface thereby allowing the potential ingrowth of clastic cells from the periodontal ligament into dentine.

Heithersay (1999b) in the same study of 257 teeth in 222 individuals with a history of ICR found that 24.1% of the affected teeth had a history of orthodontic treatment as the sole associated predisposing factor. During orthodontic treatment some degree of surface resorption may occur as pressure is placed on the periodontal ligament. If this resorption is sufficient to expose the underlying radicular dentine surface then the potential exists for activation of mononuclear precursor cells from the periodontal ligament. Heithersay suggested that excessive pressure on the periodontal ligament may result in localised tissue necrosis adjacent to denuded cementum with the resultant tissue metabolites stimulating mononuclear precursor cells to differentiate into specific clastic cells which may cause tooth resorption.

ICR is a process of resorption of cementum, dentine and enamel originating most often below the gingival crest at the level of the CEJ. It is capable of extending deep into the dentine and will involve the pulp, characteristically, only during the

later stages. Prior to this the pulp appears to be protected by a layer of predentine, rendering the condition asymptomatic until the latter stages. Likewise enamel is usually only involved in the late stages of this resorptive process.

Makkes and Thoden van Velzen (1975) described the lesion as being composed of extensive tunnel shaped ramifications with bone tissue or tissue resembling bone often deposited in the lesion.

The rate at which this resorptive process occurs varies but can occur rapidly to completely undermine the crown of a tooth resulting in the eventual fracture of the coronal segment of the tooth. Another feature of the advanced case is the presence of a "pink spot" or "pink tooth" which occurs when sufficient dentine and enamel have been resorbed to allow the highly vascular granulation tissue of the resorptive lacunae to become visible through the crown of the tooth. This may be the first clinical sign of the underlying resorptive activity. Heithersay (1999b) classified the stages of this form of resorption into four classes:

Class 1 - a small invasive resorption lesion near the cervical area with shallow penetration of the dentine

Class 2 - a well defined invasive resorption lesion which has penetrated close to the coronal pulp chamber but which shows little or no extension into the radicular dentine

Class 3 - a deeper penetration of dentine by the resorbing tissue which not only involves the coronal dentine but also extends at least to the coronal third of the root

Class 4 - a large invasive resorption process that has extended beyond the coronal third of the root.

Tronstad (1988) reported that the origin of ICR occurs at the cervical attachment apparatus below the level of the epithelial attachment. The point of origin at which the resorbing or clastic cells will penetrate the tooth and cause a spreading of the resorption through the radicular dentine may be very small. Heller *et al.* (1992) looked at the association of intra-coronal bleaching and ICR in dogs and found that 2 of 16 teeth bleached using the thermocatalytic method using 30% hydrogen peroxide had signs of ICR after 3 months. Both the lesions were found below the epithelial attachment.

These resorptive lesions contain numerous communicating tunnels from which the dentine has been resorbed and which contain a loosely organised, highly vascular tissue with few cells. These tunnels burrow deep into the surrounding dentine and may communicate with the periodontal ligament providing the potential for further resorption. The blood vessels are large, and there are small deposits of adipose tissue. Active fibroblasts are also present in these tunnels along with extensive resorption lacunae containing numerous multinucleated giant cells and deposits of bone tissue. A lack of other clastic cells in these lesions suggests the giant cells are responsible for the resorption process. Heithersay (1999a) reported that the histopathologic features seen in invasive cervical resorption are dependent on the stage of development of the resorption. In the early stages, the predominant tissue is fibrous tissue, well vascularised, with the presence of resorptive lacunae filled with

clastic cells. This resorption defect is normally devoid of inflammatory tissue and inflammatory cells unless there is communication between the defect and the oral environment thereby allowing invasion of the defect by bacteria. As the lesion progresses the fibro-vascular tissue becomes fibro-osseus by the deposition of bone-like calcifications. These depositions appear within the fibrous tissue and on the dentine surface.

As the lesions become more extensive there is increased potential for the lesion to communicate with the oral cavity which will result in invasion of the lesion by microorganisms, resulting in an inflammatory response characterised initially by the presence of acute inflammatory cells, followed by a chronic inflammatory cell population. At this point the histological appearance of ICR may resemble that of an external inflammatory resorption. Only in the latter stages of this resorptive process will invasion of the pulpal tissue be seen. Prior to this the pulp canal is clearly recognisable through the resorptive defect on a radiograph and histologically the canal can be observed to be separated from the fibrovascular tissue of the lesion by a layer of predentine. The pulpal tissue remains non-inflamed even in the presence of an inflammatory response within the resorptive defect until the latter stages when the pulp canal becomes involved.

3.0 Intra-coronal bleaching of root-filled teeth

3.1 Intrinsic discolouration of teeth

Intrinsic discolouration of teeth results from the changes in colour of the internal calcified tissues of the teeth, which may be local or systemic in origin (Vogel, 1975). This discolouration may be endogenous or exogenous in origin. Endogenous tooth discolourations are rare but will occur in association with such disease processes of the teeth as dentinogenesis and amelogenesis imperfecta, phenyl ketonuria, porphyria, and haemolytic anaemias. Tooth discolouration associated with exogenous agents are, however, more common and are derived from a number of sources:

(a) Systemic intake of drugs - e.g tetracyclines are capable crossing the placenta if taken whilst pregnant. These drugs are capable of binding to calcium during the mineralisation of primary and permanent teeth and form a calcium phosphate - tetracycline complex that has its greatest concentrations in the dentine (Hayes *et al.* 1986). The severity of the discolouration will depend on the dose of the antibiotic and the stage of the mineralisation process at which the drug was administered.

(b) Dental caries - Bacterial degradation of foodstuffs may cause a deep brown/black discolouration (Goldstein and Feinman, 1995).

(c) Dental medicaments - there are a number of dental restorative materials which are capable of discolouring teeth. Corrosion products of amalgam are responsible for a grey discolouration of dentine. Endodontic materials are recognised for their ability to stain dentine and therefore should be confined to the root canal to avoid

discolouration of coronal dentine. Ledermix paste and root canal sealers will penetrate dentine and stain the tooth a yellow colour (Kim *et al.* 2000).

(d) Pulpal necrosis - the necrotic pulp is capable of staining the tooth via pulpal tissue breakdown products, which are able to penetrate the dentine and stain dentine. Prinz (1920) first suggested the role of iron sulphide and concluded that iron was the most important component causing the discolouration. Bacteria which nearly always invade the pulp space following the death of the pulpal tissue will release hydrogen sulphide as a bi-product of their metabolism. This hydrogen sulphide is capable of reacting with ferrous (Fe^{2+}) ions, released from haemoglobin, to produce iron sulphide, a black compound, which stains the coronal dentine grey/black. It has been widely accepted that iron sulphide is largely responsible for the discolouration of dentine in teeth suffering pulp necrosis and therefore the discolouration of such teeth. This theory, which has little supporting evidence, has until recently been used to account for the majority of traumatically induced discolourations of teeth and is often cited in the literature (Grossman, 1988; Frank, 1980; Howell, 1980; Freccia and Peters, 1982; Rotstein *et al.* 1991a). Marin *et al.* (1997) challenged this theory with a histochemical analysis of traumatised and non-infected teeth, which revealed that it is haemoglobin or other forms of haematin molecules which were responsible for the discolouration witnessed in the teeth. The theory originally put forward by Prinz may hold true for teeth that have subsequently become necrotic and invaded by microorganisms.

(e) Extravasion of blood - following trauma to the dentition the rupture of blood vessels in the pulp tissue will result in the release of red blood cells which in turn are capable of rupturing, releasing haemoglobin. The haematin molecule or the

haemoglobin molecule is capable of staining dentine and therefore discolouring the tooth by penetrating into the dentinal tubules (Marin *et al.* 1997). Andreasen (1986) reported that the discolouration first detected following trauma of moderate injury (e.g. luxation injury) can gradually reverse to the original colour within 2-3 months with the revascularisation of the pulp tissue. The author's findings are supported by those of Arwill *et al.* (1967), who suggested that a change in tooth colour implied pulpal haemorrhage and reflected the time which had elapsed since injury. This would correspond to colour changes seen in haematomas in other soft tissue sites. Regions of the pulp in which there was haemorrhage would result in fibrin deposition that would result in the trapping of the cells in the area.

3.2 Oxygen species and intra-coronal bleaching of root-filled teeth

3.2.1 Chemistry of intra-coronal bleaching

Bleaching is the process by which something is made white or whiter. This will occur by the splitting of the chromophore structure in organic molecules resulting in the loss of colour. This loss of colour makes the substance appear white.

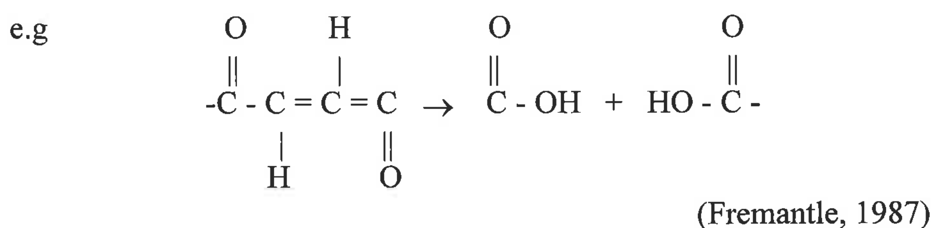
Bleaching can occur by an oxidation or a reduction process:

Oxidation bleaching - an oxidation reaction occurs when oxygen combines with a metal or non-metal to form an oxide. In the process electrons are lost. Thus an

oxidising agent removes electrons from another substance.

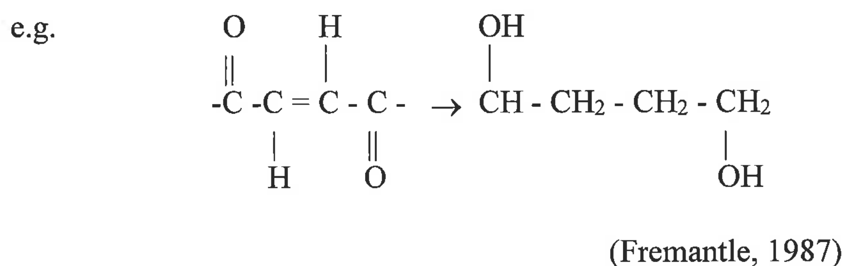
Reduction bleaching - a reduction reaction occurs when an oxygen atom is removed from an oxygen compound. In the process electrons are gained. Thus a reducing agent surrenders electrons to another substance.

The mechanism involved in oxidative bleaching e.g. the use of hydrogen peroxide on fabrics, hair, paper pulp and tooth structure, involves the breaking of a double bond.



(Fremantle, 1987)

Reduction bleaching involves the conversion of a double bond to a single bond:



(Fremantle, 1987)

The end result of both processes is the breakage of the conjugated double bond which results in a loss of colour by producing a hypochromic blue shift i.e. the displacement of a band to a shorter wavelength - towards the blue end of the spectrum. This process, whether it be by oxidation or by reduction, is referred to as chemical bleaching. An oxidation reaction occurs whereby the substance to be

bleached donates electrons to the bleaching agent. Hydrogen peroxide is widely used as the bleaching agent because it is unstable and will readily decompose to oxygen and water.

The rate of the reaction is dependent on the concentration of the hydrogen peroxide. The time required to clear the hydrogen peroxide is directly proportional to the concentration of the hydrogen peroxide: the longer the clearing time, the greater the exposure time of the hydrogen peroxide to the stain which is to be bleached.

3.3 Oxidising bleaching agents

These bleaching agents remove an electron from another substance.

Halogens: All halogens are potential bleaching agents. They have a strong tendency to complete their outer shell octet by forming the halide ion (X^-) by reacting with metals, or with a single covalent bond of another non-metal (Fremantle, 1987).

Oxo - acids: These compounds are effective bleaching agents. In solution they form oxo-acid salts which are not as effective a bleaching agent as the acids. Most domestic liquid bleaches contain sodium hypochlorite (NaOCl) which is an effective bleaching agent (Fremantle, 1987).

Hydrogen Peroxide: This is an acidic, colourless liquid. It is formed as an intermediate by the reaction of water with a wide variety of substances, including

hydrogen gas, metals and various organic compounds. The hydrogen peroxide molecules are H-bonded but the O – O bond is weak and unstable. It will decompose spontaneously into water and oxygen at room temperature.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

It is capable of acting as an oxidising or reducing agent depending on the conditions.

Perborates: e.g. Sodium perborate - used in washing powders it is formed by the reaction of hydrogen peroxide and sodium metaborate or pyroborate. It is stable in the dry state and virtually insoluble in water. During the bleaching process it releases hydrogen peroxide and oxygen (Weiger *et al.* 1994).

3.4 Methods of chemical bleaching

Two bleaching techniques are commonly used in clinical practice for intra-coronal bleaching of root-filled teeth:

(1) Thermocatalytic bleaching most commonly uses 30% hydrogen peroxide solution as the bleaching agent. The solution is placed into the pulp chamber of a root-filled tooth and activated with heat (either a heated instrument or by using a photolamp). The heat source is applied for periods of up to 30 minutes at intervals of 2-5 minutes. Tronstad (1988) reported that the effect of this heat on the hydrogen peroxide is two fold: catalysis of the breakdown of the hydrogen peroxide into its unstable oxidising components and secondly imparting energy to the bleaching solution which may cause it to expand and diffuse more effectively into the dentine

tubules of the stained tooth structure.

Of interest are the findings of Dahlstrom (1992) who found that the activation of hydrogen peroxide using a photolamp that employed ultraviolet light (U.V.) resulted in an increase in production of hydroxyl radicals. In his research, the U.V. light was found to significantly increase the production of hydroxyl radicals from a solution of sodium salicylate, the yield of which was significantly increased again with the introduction of hydrogen peroxide into the solution. The production of hydroxyl radicals is the end result of the homolytic fission of the O - O bond in hydrogen peroxide using electrons derived from the U.V. radiation.

(2) The "walking bleach" technique was first described by Nutting and Poe (1963). The technique outlined by the authors employed a cotton pellet saturated in 30% hydrogen peroxide placed into and sealed into the pulp chamber of a tooth. The bleaching agent would then "walk" into the tooth between appointments. The authors later modified their technique to include sodium perborate hoping that the synergistic effects of the two bleaching agents would more effectively bleach the stained dentine. This latter procedure is generally accepted as the "walking bleach procedure" used today.

3.5 Penetration of oxygen species through dentine

The aim of intra-coronal bleaching of root-filled teeth is the penetration of the bleaching agent into the dentinal tubules where the bleaching process will occur. The longer the bleaching agent is present in the dentinal tubules and the more advanced its penetration into these tubules the more effective the bleaching action. However, there is little bleaching effect after a period of three days at which time the hydrogen peroxide has almost fully decomposed. Fuss *et al.* (1989) examined the diffusion capacity of bleaching materials through human dentine. The change in pH of the medium surrounding the tooth was measured following the introduction of the bleaching agent. From their results the authors were able to conclude that bleaching materials could diffuse through dentine. Kehoe (1987) found significant changes in the pH of cementum following intra-coronal bleaching and supported these results. Rotstein *et al.* (1991) demonstrated *in vitro* the passage of hydrogen peroxide through dentine to the outer surface of the tooth.

The generation and passage of hydroxyl radicals through dentine during intra-coronal bleaching has been demonstrated by Dahlstrom *et al.* (1997). Of interest in these findings was the apparent lack of influence that the presence of intact cementum had at the level of the CEJ. The author suggested that the cementum has little influence on the protection of the surrounding periodontal tissues against the hydroxyl radical.

4.0 Derivatives of oxygen

Slater (1984) described a free radical as a molecule or molecular fragment with an unpaired electron. Each unpaired electron is in its own orbit. These unpaired electrons tend to create instability within the chemical structure of the radical thereby making them reactive.

The oxygen molecule contains two unpaired electrons that have the same spin state. This electronic structure conveniently forms a barrier to the insertion of a pair of electrons i.e. the incoming pair of electrons would be trying to join the parallel spinning unpaired electron of oxygen and one of them would have the same spin state as its partner to be. This situation is energetically unfavourable but there is a way around this barrier. By adding the electrons to oxygen one at a time sufficient time is provided for the inversion of one of the electron spins. As a result, the reduction of oxygen to water occurs by a series of univalent electron transfers. Four electrons in total are required for the complete reduction of oxygen to two water molecules. A consequence of this series of reduction reactions is the creation of intermediates, namely the:

- Superoxide radical ($O_2^{\cdot -}$)
- Hydrogen Peroxide (H_2O_2)
- Hydroxyl radical ($^{\cdot}OH$)

It is these intermediates which are responsible for the toxicity of oxygen. Most of the oxygen consumed by respiring cells is reduced by cytochrome C oxidase, the structure of which allows the four-electron reduction of oxygen to occur without the

formation of intermediates. However, there are enzymes present in cells which catalyse the reduction of oxygen to hydrogen peroxide and the production of superoxide radicals. The amount produced by each reduction is very small, however, the vast number of reactions that occur during respiration by cells results in significant numbers of these species forming.

4.1 Superoxide radical

The superoxide radical is formed following the univalent reduction of oxygen both enzymatically and non-enzymatically.

4.1.1 Enzymatic production of the superoxide radical

The distribution and activity of the enzymatic pathways varies with different cell types depending on their specialised formation (Fisher, 1987).

NADPH oxidase - this enzyme is found only in phagocytes and is used by these cells in the "respiratory burst" when in contact with foreign particles or immune complexes. Because this reaction only occurs in phagocytic cells it does not contribute to the intracellular $O_2^{\cdot-}$ load in most cells.

Xanthine oxidase and aldehyde oxidase - found in all cells and oxidise oxygen to produce both $O_2^{\cdot-}$ and H_2O_2. Whilst their production is of biological interest their role in superoxide - induced injury at elevated oxygen concentrations is minor due to

the low reactivity of the superoxide radical.

4.1.2 Non enzymatic production of the superoxide radical

A minor source of superoxide radical production in the intact cell is related to the auto-oxidation of chemically reactive components produced during reductive processes associated with the mitochondrial electron transport chain. The superoxide radical is capable of acting as a ligand, base, and as an oxidant or a reductant. Thus only reactions having rates comparable with this spontaneous dismutation will occur. As a result the superoxide radical is selective in its reactivity and is capable of diffusing before it encounters a suitable target.

4.2 Hydrogen peroxide

Hydrogen peroxide has been used in dentistry for more than 100 years. Hydrogen peroxide can exhibit strong anti-microbial activity through the release of oxygen. The efficacy of its anti-microbial effects are enhanced by the presence of trace metals such as iron and copper, which accelerate the decomposition of hydrogen peroxide to hydroxyl radicals. Although a strong oxidant, hydrogen peroxide reacts very slowly with most organic substrates. The mechanism of hydrogen peroxide induced cytotoxicity in mammalian cells has not yet been determined. Hydrogen peroxide is capable of cellular damage causing such changes as damage to cellular DNA, membrane lipid peroxidation, and cell death. Cytotoxicity may occur through direct effects on cell membranes that can lead to cell death through loss of

membrane integrity and by inactivation of critical cell components once the cell membrane has been breached and cellular defence mechanisms have been exhausted.

4.3 Hydroxyl radicals

The hydroxyl radical is an extremely powerful oxidant. It is indiscriminately reactive with virtually all biological substrates. Because of this high level of reactivity the hydroxyl radical will only have direct effects close to its site of generation.

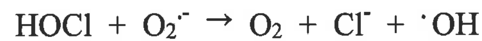
4.4 Hypochlorous acid (HOCl)

Hypochlorous acid is commonly produced by the myeloperoxidase system in activated neutrophils. It is a weak acid that is about 50% ionised at physiological pH.

$$HOCl \rightarrow H^{+} + OCl^{-}$$

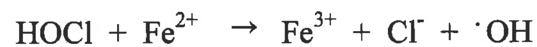
HOCl has attracted much attention due its high reactivity and its ability to damage bio-molecules (Halliwell and Gutteridge, 1999). HOCl is also a powerful oxidising agent, capable of donating two electrons to target sites resulting in damage to range of tissues in sites like the endothelial cell membrane where its target site is the glycoprotein thrombomodulin. This glycoprotein is responsible for the regulation

of the blood coagulation pathway by modifying the action of thrombin. Likewise, HOCl is capable of oxidising thiols, ascorbate, NAD(P)H and can lead to the chlorination of DNA bases in the genome. It is capable of passing through membranes, damaging membrane proteins (especially to –SH groups and methione residues) as it passes through (Halliwell and Gutteridge, 1999). In the presence of superoxide radicals, HOCl is capable of generating hydroxyl radicals.



$$HOCl + O_2^{\cdot -} \rightarrow O_2 + Cl^- + {}^{\cdot}OH$$

Generation of hydroxyl radicals will also occur in the presence of iron ions.



$$HOCl + Fe^{2+} \rightarrow Fe^{3+} + Cl^- + {}^{\cdot}OH$$

5.0 Production of the hydroxyl radical

5.1 The Haber-Weiss reaction

The hydroxyl radical was first encountered during studies of the effects of ionising radiation on H_2O_2. The homolytic fission of the O-O bond in H_2O_2 produces two hydroxyl radicals. Heat or ionising radiation can achieve this homolysis. Beauchamp and Fridovich (1970) when researching the production of ethylene by Xanthine oxidase in endothelial cells found that although the Xanthine-Xanthine oxidase system can generate both superoxide radicals and hydrogen peroxide, an additional product - the hydroxyl radical, was also generated by the system in biological systems. They referred to this process as the Haber-Weiss reaction as it had been these authors (Haber and Weiss, 1934), who had first proposed this reaction as part of their catalytic reduction of hydrogen peroxide by iron (Fe) salts.

This reaction is:

$$H_2O_2 + O_2^{\cdot -} \rightarrow O_2 + OH^- + {}^{\cdot}OH$$

This reaction has been proposed as the mechanism for hydroxyl radical production in biological systems and is thermodynamically favourable. However, it does not proceed at a sufficient rate in biological systems to be significant (Di Guisseppi and Fridovich 1984; Halliwell 1978). This is supported by the work of Kappenol *et al.* (1978) who reported that superoxide did not react with hydrogen peroxide at a rate that could compete with the spontaneous dismutation of the superoxide radical.

5.2 The Fenton Reaction

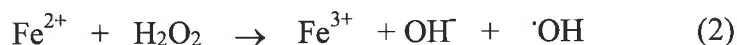
In 1934 Haber and Weiss proposed that the superoxide radical and hydrogen peroxide react to produce hydroxyl radicals. As a result of this it was assumed that the Haber-Weiss reaction, as it became known, was the source of hydroxyl radical production in biochemical systems.

Fenton in 1894 demonstrated that a simple mixture of an iron salt and hydrogen peroxide produced the hydroxyl radical. Much work has been performed based on Fenton's work and the so-called "Fenton reaction" has been well researched. This reduction of hydrogen peroxide occurs by a metal cation. The most commonly used metal cation is the ferrous (Fe^{2+}) ion.

Initially the superoxide radical reduces the ferric form of iron (Fe^{3+}) to the ferrous form (Fe^{2+}).

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2 \quad (1)$$

This is immediately followed by the Fenton reaction in which the ferrous ion (Fe^{2+}) reduces hydrogen peroxide to the hydroxyl radical.

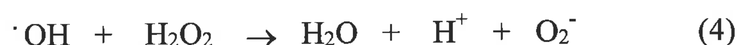


$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH \quad (2)$$

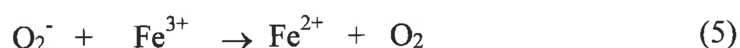
The products of this reaction are the ferric ion (Fe^{3+}), the hydroxyl ion (OH^-) and the hydroxyl radical (${}^{\cdot}OH$). The ferric ions produced can then further react with hydrogen peroxide (Halliwell and Gutteridge, 1984).

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^- + H^+ \quad (3)$$

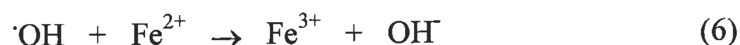
and more reactions are possible:



$$\cdot OH + H_2O_2 \rightarrow H_2O + H^+ + O_2^- \quad (4)$$



$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2 \quad (5)$$



$$\cdot OH + Fe^{2+} \rightarrow Fe^{3+} + OH^- \quad (6)$$

Reaction (5) is referred to as Fenton cycling where the ferric product is reduced back to its ferrous form by the superoxide radical or other one-electron reductants. Thus the presence of transition metal ions of which the ferrous form of iron is the most abundant are capable of catalysing the production of hydroxyl radicals from hydrogen peroxide. The presence of hydrogen peroxide regardless of the method by which it has been introduced into the body tissues requires a transition metal ion for its reduction. The availability of these transition metal ions, especially the ferrous form of iron takes on an important role.

5.3 The availability of iron

Cyclical Fenton-like reactions with hydrogen peroxide producing extremely reactive hydroxyl radicals are now recognised as the driving force initiating cascades of cytotoxic free radical reactions (Borg and Schaich, 1987).

Two-thirds of the body's iron store is bound to haemoglobin. A further 10% is bound to myoglobin and smaller amounts are bound to iron containing enzymes and the transport protein transferrin. The remainder of the body's stored iron is present in intracellular storage proteins - ferritin and haemosiderin (Halliwell and Gutteridge, 1984). These stores are found mainly in the liver, spleen and the bone marrow with limited amounts of ferritin found in blood plasma.

5.3.1 Haemoglobin

Haemoglobin contains approximately 0.34% iron per weight i.e. 1ml of packed red blood cells contains approximately 1mg of iron. In living tissues, the digestion of red blood cells proceeds at a rate by which 20% of haemoglobin is released within a few hours. Plasma iron derived by the action of macrophages is bound to transferrin. The remainder of the iron derived from haemoglobin is stored as ferritin or haemosiderin and turns over very slowly.

Gutteridge (1986) disputed the theory that iron bound to haemoglobin can act as a catalyst for Fenton-like reactions. Such a theory would require the highly reactive hydroxyl radical which is formed at the iron centre to travel through the protein core into free solution to react with a detector molecule which does not appear to be compatible with the known chemistry of the hydroxyl radical. His research found that it is unlikely to be iron bound to haemoglobin which catalyses the production of hydroxyl radicals in free solution. Rather hydrogen peroxide can stimulate the release of iron from haemoglobin that is immediately bound by transferrin – a naturally occurring iron transport protein, or desferrioxamine – an end product of haemoglobin breakdown, which then promotes the hydroxyl radical generation by the Fenton reaction. However, other reports (Sadrzaheh *et al.* 1986) are conflicting and suggest haemoglobin and cytochrome C are effective catalysts.

Haemoglobin acts as a reservoir or source of iron, but the iron bound to haemoglobin does not directly catalyse the production of the hydroxyl radical.

5.3.2 Transferrin

Transferrin is the major iron transport protein containing two iron-binding sites. It binds iron in association with bicarbonate ions, forming a tertiary complex that does not readily dissociate.

5.3.3 Ferritin

Ferritin is a small molecule the structure of which consists of a protein shell (apoferritin) which encloses the iron molecule. The protein shell contains pores, which allow access to the interior. Iron enters ferritin in the ferrous form and is oxidised to the ferric form by the protein. It can be removed from ferritin in its ferrous form by the actions of biological reducing agents. Each ferritin complex can contain up to 4500 ferric ions. The iron is mobilised from ferritin in its ferrous state by reduction.

Ferritin can be degraded in lysosomes, the protein shell being attacked to leave an insoluble product - haemosiderin - a yellowish, intracellular, iron containing pigment that is visible under the electron microscope as dense clusters of ferritin. Grossman (1988) has suggested it is this haemosiderin which contributes to the discolouration of teeth following trauma. This is a potential source of iron for Fenton reactions but is considered a less significant contributor to the *in vivo* generation of hydroxyl radicals. In fact, it has been suggested that the *in vivo*

conversion of ferritin to haemosiderin may represent a protective mechanism against iron toxicity since the iron in haemosiderin is less reactive than the iron in ferritin.

5.3.4 Intracellular iron pool

There exists a small pool of non-protein bound iron moving between transferrin, cell cytoplasm, mitochondria, and ferritin which is capable of providing iron for the Fenton reaction. It is found bound to chelators of low molecular mass (e.g. citrate, ATP, ADP) as di- and tri-phosphate nucleotide-iron complexes.

6.0 Toxicity of the hydroxyl radical

6.1 Reactivity of the hydroxyl radical

The hydroxyl radical is an extremely powerful oxidant capable of readily attacking most organic compounds at diffusion limited rates. As a result the hydroxyl radical will react with almost any type of molecule found in living cells: proteins, lipids, carbohydrates, nucleotides and organic acids to chemically modify and damage their structure. Such a reaction will normally occur within a very small radius of the site of generation of the hydroxyl radical.

Reactions seen are of three main types:

(1) Hydrogen atom abstraction:

e.g. For methanol - $CH_3OH + {}^{\cdot}OH \rightarrow H_2O + {}^{\cdot}CH_2OH$

(2) Addition:

e.g. The hydroxyl radical is capable of adding to an aromatic ring, such as the purine or pyrimidine base of DNA.

(3) Transfer:

e.g. With chloride ions - $Cl^{-} + {}^{\cdot}OH \rightarrow Cl^{\cdot} + OH^{-}$

Therefore if such reactive free radicals are produced *in vivo* in sufficient numbers to

overcome the normally efficient protective mechanisms we could expect to see metabolic and cellular disturbances.

The reactivity of the hydroxyl radical is such that if they are found in biological systems they react with whatever molecule is in their vicinity, producing secondary radicals of variable reactivity. This process increases the potency of the hydroxyl radical, and occurs because the reaction between a radical and a non-radical always begets another radical i.e. amplification of the consequences of the initiating event by a chain reaction.

6.2 Effects of the hydroxyl radical at the molecular level

6.2.1 Effects of the hydroxyl radical on lipid peroxidation

A free radical that has sufficient energy to extract a hydrogen atom from a methane carbon atom of a polyunsaturated fatty acid can initiate a chain reaction in bulk lipid. Biomembranes and subcellular organelles are particularly sensitive to oxidative attack due to the presence of polyunsaturated fatty acids (PUFA) in their membrane phospholipids.

The biological sequence of membrane lipid peroxidation is dependent on the cell population involved, the fatty acid profile of membrane phospholipids and the content of protein bound iron capable of decomposing any generated hydroperoxides. Such an oxidant attack on membrane phospholipid or protein bound PUFA can initiate a complex cascade of events leading to the formation of reactive,

unstable oxidants, long-lived toxic by products and pro-inflammatory mediators capable of propagating damage beyond the confines of the original focus.

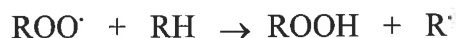
The oxidant's attack is thought to be directed at the hydrogen atoms of methylene (Weiss, 1986). The oxidant removes a hydrogen atom leaving behind an alkyl radical ($R^{\cdot}$). The allylic hydrogen atom requires a strong oxidant for its abstraction. The hydroxyl radical is capable of this abstraction.

Following this abstraction of the hydrogen atom, the carbon radical is stabilised by rearrangement to a conjugate diene which rapidly reacts with oxygen to form the corresponding peroxyl ($ROO^{\cdot}$) radical.

i.e. $RH + OH^{\cdot} \rightarrow R^{\cdot} + H_2O$

$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$

The peroxyl radical has sufficient oxidising potential to attack the allylic hydrogen atom of an adjacent PUFA to form the hydro-peroxide and a new alkyl radical:



$ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$

This reaction thereby initiates an autocatalytic cycle that continues until the collision between two radical species occurs to form non-radical products.

Extensive lipid peroxidation in biological membranes causes loss of fluidity, falls in

membrane potential, increased permeability to ions, and eventual rupture leading to the release of cellular and organelle contents.

These oxidants may also react with albumin-bound fatty acids to mediate pro-inflammatory effects by generating chemotactic substances. For example, in an atherosclerotic lesion, arterial endothelial cells, smooth muscle cells and macrophages have been shown to be capable of oxidising LDL so that macrophages will internalise the LDL faster. (It is these macrophages - containing large amounts of LDL that are referred to as "foam cells" - that form a major component of the atherosclerotic plaque).

6.2.2 Effects of the hydroxyl radical on protein

Amino acids differ in their sensitivity to oxidation. The high level of reactivity of the hydroxyl radical allows it to react with even the least sensitive aliphatic amino acids.

Generation of the hydroxyl radical in the vicinity of a protein does not necessarily result in a biologically significant alteration. The radical must gain access to critical residues controlling biological activity and conformational or cellular processing (Weiss, 1986).

An example of this is the oxidative inactivation of the enzyme glutamine synthetase by hydroxyl radicals generated by a Fenton-like reaction. The hydroxyl radical

attacks one of the sixteen histidine residues and oxidises it to a carbonyl containing derivative. Despite the ability of hydrogen peroxide, hypochlorous acid, and the superoxide radical to oxidise free histidine none of these oxidants are capable of inactivating the enzyme. Its inactivation requires the proper co-ordination of an iron molecule near a critical histidine residue, coupled by the accessibility of the hydrogen peroxide to the bound Fe^{2+} ion. This indicates the site-specific damage and the site specific sensitivity of a biological target to hydroxyl radical induced protein damage.

6.2.3 Effects of the hydroxyl radical on DNA

Exposure of DNA to systems generating superoxide radicals causes extensive strand breakage and degradation of deoxyribose. This is most likely due to the generation of hydroxyl radicals by the superoxide radicals.

Malins *et al.* (1996) found intimate involvement of the hydroxyl modification of DNA in the progression of breast tumours to the metastatic state and the likelihood of the hydroxyl radical being an important aetiological factor in the high degree of heterogeneity and diverse physiological properties characteristic of metastatic cell populations. Weitzman *et al.* (1986) applied 30% hydrogen peroxide twice weekly for periods of up to 22 weeks on the buccal mucosa of rats resulted in increased mitotic activity and hyperplasia in the lining epithelium. These changes were associated with pre-neoplastic lesions. The study also found hydrogen peroxide and other oxygen metabolites applied for short periods of time to cause many genetic

effects including mutations, sister chromatid exchanges and malignant transformations.

6.2.4 Effects of the hydroxyl radical on hyaluronic acid

Hyaluronic acid is the major glycosaminoglycan of synovial fluid and accounts for nearly the entire viscosity of this fluid. It is also a major component of the extracellular matrix of connective tissue. It is normally degraded by hyaluronidase.

The hydroxyl radical can cause the degradation of the hyaluronic acid. (McCord 1974; Greenwald and Moy, 1980). The latter authors proved the role of the hydroxyl radical in the degradation of hyaluronic acid by combining the enzymes superoxide dismutase and catalase which are naturally occurring oxygen derived radical scavengers. This solution provided protection for the hyaluronic acid test solution.

The hydroxyl radicals attack the hyaluronic acid structure at either the glycoside bond or at the C5 hydrogen atom of the pyronese ring (Balaz *et al.* 1967).

6.3 Effects of the hydroxyl radical at the cellular level

6.3.1 Effects of the hydroxyl radical on fibroblasts

Curran *et al.* (1984) reported on the ability of oxygen derived free radicals, particularly the hydroxyl radical, to directly degrade soluble collagen and to have an

indirect effect on collagen degradation at low levels by potentiating the destructive effects of proteinases. This secondary effect is achieved by modifying the structure of collagen which renders it more susceptible to the activities of the proteinases. The authors further investigated this point by studying the effect of the hydroxyl radical on the collagen content of lung tissue. In so doing they demonstrated the direct degradation of soluble collagen and the modification of procollagen which subsequently renders it more susceptible to proteolytic degradation by trypsin.

Physiological damage to the extracellular matrix of tissue is almost always accompanied by inflammation. Inflammatory cells recruited to a site of injury release elastase and other proteinases capable of destroying connective tissue. These inflammatory cells also release highly reactive and destructive oxygen species which are capable of potentiating the effects of the degradative enzymes. The resultant peptides, formed from the breakdown of soluble collagen and extracellular matrix components, are chemotactic for inflammatory cells and fibroblasts. Curran *et al.* (1984) postulated that the effect of oxygen derived radicals may be responsible for the initial recruitment of inflammatory cells to the site of injury.

6.3.2 Effects of the hydroxyl radical on endothelial cells

Varani *et al.* (1988) whose work involved the study of oxygen radical scavengers suggested that endothelial cell injury is mediated not by the myeloperoxidase system but by the formation of hydroxyl radicals. These authors suggested the hydroxyl radical is responsible for the neutrophil dependent tissue injury that is immune

complex induced, and likewise is responsible for the anti-microbial effects of PMN leukocytes. This theory is supported by Miller *et al.* (1996) who found that the addition of hydroxyl radical scavengers (superoxide dismutase, catalase and dimethyl thiourea) to solutions of hydroxyl radicals and endothelial cells reduced the endothelial cell injury by up to 99%.

Ward (1991) stated that the ultimate damage of endothelial cells seems to be related to the production of hydroxyl radical. The most relevant target on endothelial cells for the hydroxyl radical to ensure cell death is unknown. Vercellotti *et al.* (1991), reported that the exposure of endothelial cells to a "bolus" of hydrogen peroxide results in a range of cellular defects and changes which lead to cell death. These changes include alterations in signal transduction and production of endothelial cell prostacyclin (PGI_2), and platelet activating factor (PAF). The sequence of events which occurs following exposure to hydrogen peroxide includes:

- activation of the hexose monophosphate shunt
- oxidation of intracellular glutathione
- decrease in cellular ATP concentration
- depletion of nicotinamide adenine dinucleotide (NAD)

These biochemical alterations are followed by increases in intracellular Ca^{2+} and Na^+ ions, cytoskeletal alterations, cell membrane bleb formation and cell lysis.

7.0 Scavenging of the hydroxyl radical

7.1 Naturally occurring hydroxyl radical scavengers

The production of hydroxyl radicals and other toxic oxygen species by inflammatory cells in the body would result in damage to the soft tissues in the immediate vicinity of their production every time there was such an inflammatory response. The body overcomes this potential threat by producing enzymes capable of inhibiting the production of these radicals. If the production of oxygen radicals is sufficient to overwhelm the body's natural defence mechanisms then their potentially toxic effects may be seen.

Superoxide dismutase is essential for the survival of aerobic cells because of its ability to catalytically scavenge the superoxide radical. This ability indirectly aids elimination of hydroxyl radical production because the superoxide radical spontaneously dismutates to hydrogen peroxide. By eliminating the superoxide radical the production of hydrogen peroxide is reduced thereby permitting less available for the potential Fenton reaction.

Defence against the hydrogen peroxide molecule in eukaryotes comes from several enzymes such as catalase and peroxidase. Catalase dismutates hydrogen peroxide into water and oxygen and is most effective in relatively high concentrations of hydrogen peroxide. Peroxidase is capable of using a diverse array of reductants to reduce hydrogen peroxide to water. The principal peroxidase in mammals is

glutathione peroxidase and this enzyme is thought to have a more profound effect than the catalase enzymes in mammals.

Another form of primary defence offered by the body is the sequestration of free iron and copper ions. There is three times as much transferrin iron-binding capacity in plasma as iron needing to be transported, so that there is essentially no free iron in plasma. Iron ions bound to such proteins as transferrin cannot stimulate lipid peroxidation or hydroxyl radical production. The same is true for copper ions bound to the plasma protein caeruloplasmin or albumin. However, within the confines of the tooth where there is a large concentration of hydrogen peroxide, introduced during the bleaching process, these systems may be overwhelmed.

7.2 Therapeutic scavengers of the hydroxyl radical

There has been an abundance of research aimed at various treatment modalities for oxygen radical-induced injury. Rheumatoid arthritis is closely associated with oxygen radical induced injury has for some time been treated with penicillamine, salicylate, gold sodium thiomalate and chloroquine with varying degrees of success. Iron overload diseases have successfully been treated with desferrioxamine which is effective in the prevention of iron dependent hydroxyl radical production as well as being a powerful inhibitor of iron dependent lipid peroxidation.

A compound which is an effective scavenger of hydrogen peroxide and hydroxyl radicals is thiourea. Detterbeck *et al.* (1990) investigated its use in the scavenging

of these oxygen radicals during reperfusion injury in lung transplant patients and it has been used as an anti-thyroid agent in medicine for some years. Thiourea reacts with hydroxyl radicals to form the sulphydryl form of the thiourea compound. The rate at which this reaction occurs is more rapid than that of the superoxide radical with thiourea and the biological importance of this would becomes dependent on the concentrations of the oxygen species.

8.0 High Performance Liquid Chromatography (HPLC)

8.1 Hydroxylation of salicylate by hydroxyl radicals

Salicylate is the hydroxylation end product of aspirin (o-acetylsalicyclic acid). Grootveld and Halliwell (1986) reported that the attack by hydroxyl radicals upon salicylate produced two products that were not normal products of enzymatic salicylate metabolism. These products were 2,3 – DHB and 2,5 – DHB. Moorhouse *et al.* (1985) reported that smaller amounts of catechol were also detectable following this reaction. Grootveld and Halliwell confirmed the presence of this third reaction product and found the three products 2,3-DHB, 2,5-DHB and pyrocatechol were detected in proportions of 49%, 40% and 11% respectively (Fig. 8.1). These products are capable of being detected by HPLC. However, the reaction between hydroxyl radicals and the aromatic ring of the salicylate may result in further reactions in times of high radical flux, resulting in the production of a greater number of reaction products. These possible reactions require investigation as the reaction products would appear as peaks on the chromatogram if they were produced during the control generation of hydroxyl radicals.

Aromatic hydroxylation as an assay for hydroxyl radical in biological systems was first demonstrated by Richmond *et al.* (1981) who measured hydroxyl radicals in a system generating the superoxide radical and hydrogen peroxide (from a mixture of xanthine and xanthine oxidase) using salicylic acid as the detector molecule.

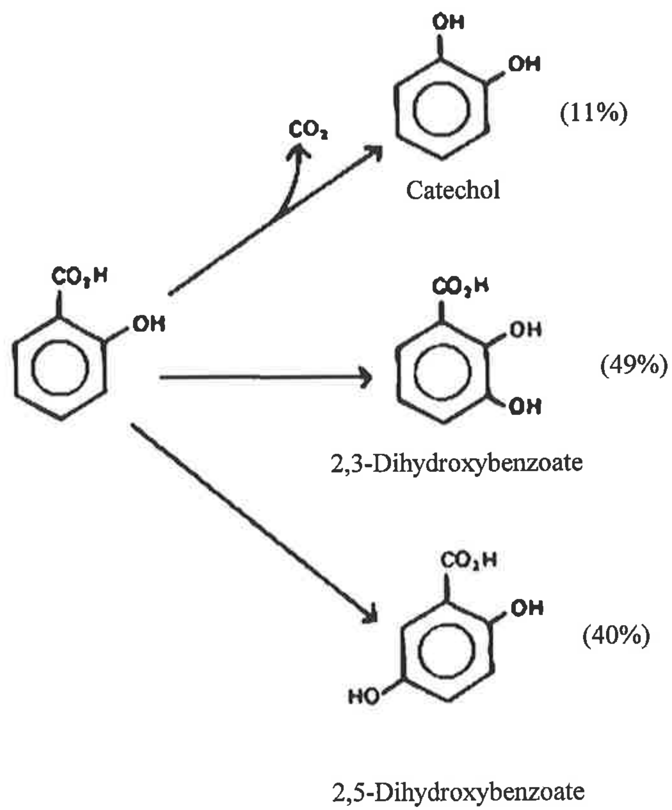


Fig. 8.1 - Chemistry of salicylate breakdown as determined by Grootveld and Halliwell (1986).

The metabolites of salicylate that are administered to humans are reported in the literature to include 2,5 – DHB but not 2,3 – DHB. This would suggest that the hydroxylation of salicylate can be used *in vivo* to assess hydroxyl radical formation by measuring the

amount of 2,3 – DHB only. It has been used as the reaction product to measure the amount of hydroxyl radicals produced during *in vivo* studies (Teismann and Ferger, 2000; Itoh *et al.* 1998; Coudray *et al.* 1995). The authors concluded from each of these studies that 2,3-DHB would be the most accurate measure of hydroxyl radical generation due to it being produced in greater quantities. Davis *et al.* (1983) found that the hydroxylation of salicylate by neutrophils resulted in a greater production of 2,5-DHB than 2,3-DHB and they used this reaction product as the marker of hydroxyl radical production. Lesser amounts of 2,3-DHB were detected. The rate constant of the interaction between the hydroxyl radicals and the salicylate is approximately $10^{-9} - 10^{-10}$ m^{-1} s^{-1} (Hiller *et al.* 1983). As the current study was conducted *in vitro* analysis of 2,5 – DHB was used to quantify the amount of hydroxyl radical produced by the experimental system. This end product was chosen as it was found to be consistently produced in greater quantities than 2,3 – DHB.

Salicylate is referred to as a substituted benzene. The hydroxyl radical reacts with substituted benzenes predominantly by addition to the benzene ring and not by interaction with the substituent (Raghavan and Steenken, 1980). The pattern of the hydroxyl radical attachment to the ring positions is expected to depend on the electron donating or withdrawing properties of the substituent. The attack by hydroxyl radicals on salicylate to produce 2,3 – DHB and 2,5 – DHB occurs by the

addition of hydroxyl groups to either the -3' or -5' position of the aromatic ring. This must occur at a rate which is fast compared to the competing decay reactions. The salicylate is sufficiently reactive to ensure quantitative conversion yet selective enough to prevent rearrangement or decomposition reactions involving the radical intermediates or the final products from occurring (Ravhagan and Steenken, 1980). Maskos *et al.* (1990) investigated the mechanisms involved in the hydroxylation of salicylate using gamma radiolysis. They found that the aromatic ring structure of salicylate was an ortho-para director during the electrophilic attack by the hydroxyl radical. Therefore attack on the aromatic ring of salicylate by hydroxyl radicals will be targeted at the –3' or the –5' positions resulting in the formation of either 2,3 – DHB or 2,5 – DHB. The production of these structure are most likely because their formation involves the addition of a hydroxyl group to either the ortho- or para-position of the salicylate molecule. Maskos *et al.* (1990) reported that the meta-position was essentially deactivated. Anbar *et al.* (1966) described the attack by hydroxyl radicals on aromatic compounds as electrophilic in nature and therefore the attack on the meta position of aromatic compounds carrying ortho-para directing substituents may be neglected. Although addition to the meta-position is possible in times of high radical flux it is unlikely and the authors concluded that very small amounts of the end products would be produced in such conditions. Therefore the production of 2,4 – DHB and 2,6 – DHB are unlikely because they would involve the addition of hydroxyl groups to the deactivated meta- position. Peaks on the chromatogram should therefore not be expected for these reaction products.

Moorhouse *et al.* (1985) suggested that the formation of catechol is the result of

decarboxylation of the salicylate. In the same study Moorhouse *et al.* (1985) reported the formation of catechol and hydroquinone from the hydroxylation of phenol. Hydroquinone is not considered a reaction product of salicylate hydroxylation. However, Dahlstrom (1992) suggested that this reaction is more likely the end result of a simple addition of a hydroxyl group to the aromatic ring structure of the salicylate molecule.

In times of high radical flux it is possible for the first reaction products to undergo further hydroxylation. It is unlikely that hydroxylation occurs at the -meta position and therefore hydroxylation of 2,4 – DHB and 2,6 – DHB to produce the end products 1,3 – DHB (resorcinol) and 1,3,5 – trihydroxybenzoic acid (1,3,5 – THB; phloroglucinol) are unlikely to be formed. Further attack on the reaction product 2,3 – DHB produces 1,2,3 – THB (pyrogallol) by the substitution of a hydroxyl group for the carboxyl group. Both 2,3 – DHB and 2,5 – DHB can be hydroxylated to produce 2,3,5 – THB. The meta-directing (deactivating) properties of the carboxyl group and the ortho/para directing (activating) properties of the hydroxyl groups in dihydroxybenzoic acids would favour the addition of a nucleophile in the 5' position of the 2,3 – DHB or the 3' position of the 2,5 – DHB. Both of these are hydroxylation reactions resulting in the formation of the 2,3,5 – THB. Hydroxylation of the 2,5 – DHB may result in the formation of 1,2,5 – THB. Further hydroxylation of pyrocatechol is unlikely due to the need for the activation of the – meta position by a hydroxyl group. These reaction products and their structures are detailed in figure 8.2.

3,4 – DHB was chosen as the internal standard due to the inability of this compound

to be produced in the hydroxylation of the aromatic ring of salicylate. Grootveld and Halliwell (1986) used 3,4 - DHB as their internal standard for the detection of both 2,3 – DHB and 2,5 – DHB. These authors used different conditions to identify the two reaction products *in vivo*. The internal standard was the only constant between the two methods. Dahlstrom *et al*. (1997) and Wright (1988) used homogentisic acid (HGA) as their internal standard. However Dahlstrom *et al.* expressed concern over the similarity in retention time of HGA and pyrogallol which is capable of being produced in times of high radical flux and suggested 3,4 – DHB or resorcinol as possible alternatives. 3,4 – DHB was chosen resulting from these concerns and for its proven reliability by Grootveld and Halliwell (1986).

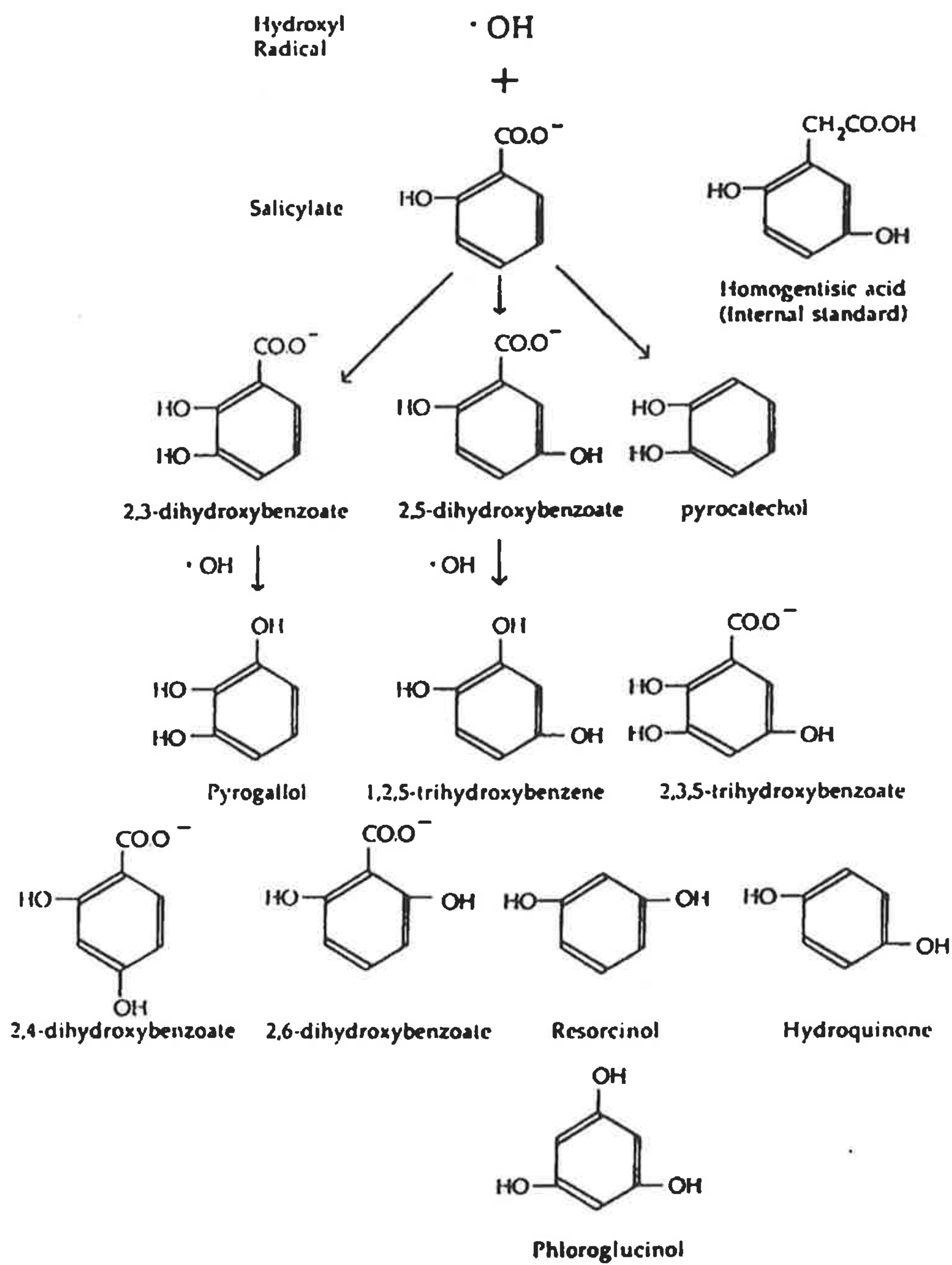


Figure 8.2. Reaction products of the hydroxylation of salicylate by hydroxyl radicals (adapted from Dahlstrom, 1992).

8.2 Detection of hydroxyl radicals using HPLC

The detection of hydroxyl radicals using high performance liquid chromatography and electrochemical detection was first reported by Grootveld and Halliwell in 1986. This process involves the hydroxylation of salicylate, the separation of the end products along the HPLC column and the detection of the end products of this reaction by the electrochemical detector. This method of detection has proved to be relatively simple and extremely sensitive.

The hydroxylation of salicylate by hydroxyl radicals was reported by Halliwell (1977) who hypothesised that the hydroxylation of salicylate could be brought about by hydroxyl radicals which are generated from hydrogen peroxide but the superoxide radical and the peroxyl radical ($HO_2^{\cdot}$) were incapable of this hydrolysis. Salicylate is an aromatic compound that is capable of undergoing an addition reaction in the presence of hydroxyl radicals to produce characteristic products. The overall chemistry is affected by pH and the presence of oxidising agents such as oxygen (O_2), Fe^{3+} ions and Cu^{2+} ions. Aromatic hydroxylation as an assay for hydroxyl radicals in biological systems was reported by Richmond *et al.* (1981) from a mixture of xanthine and xanthine oxidase, using salicyclic acid as the detector molecule.

Richmond *et al.* (1981) reported the production of 2,3-DHB and 2,5-DHB in an *in vivo* study when they tested the production of hydroxyl radicals by the hydroxylation of salicylate. The authors used colorimetric analysis to detect reaction products.

However, the authors reported that several problems resulted from the colorimetric assay technique. Firstly, the chemistry of the colour reaction is unknown. It is thought that the colour is produced from the reaction product 2,3-DHB and therefore the assay measures only the ortho - hydroxylated products, however, as other modes of attack of hydroxyl radicals on salicylate are possible the total amount of hydroxyl radicals produced could be underestimated. Secondly, this system suffers from poor sensitivity. Takemura *et al.* (1992) reported detection limits of 2 – 10 picomoles (pmoles) for the dihydroxybenzoates using UV detection. The authors concluded that the use of electro-chemical detection is a more sensitive method of hydroxyl radical detection and is capable of measuring the entire array of hydroxylated products. Floyd *et al.* (1986) reported that the three reaction products are detectable in fenomolar concentrations. Ste-Marie *et al.* (1996) reported sensitivity using HPLC and ECD in the range of 20-50 femtomoles (fmoles). Coudray *et al.* (1995) found sensitivity as low as 100 fmoles that were linear over five orders of magnitude using this system.

Whilst the use of salicylate has been widely accepted as a relatively simple method of hydroxyl radical trapping it is not without its limitations. Ste-Marie *et al.* (1996) reported that salicylate has several limitations when used for the *in vivo* trapping of hydroxyl radicals. These include (1) Decreased sensitivity of the assay due to the division of salicylate hydroxylation between the formation of the 2,3-DHB and 2,5-DHB. (2) Aspirin, which is the agent used for the generation of salicylate *in vivo,* affects inflammatory responses by the inhibition of cyclo-oxygenase. These inflammatory processes are associated with the generation of reactive oxygen

species. (3) Salicylate is an effective chelator of divalent metal ions including Fe^{2+}. As Fe^{2+} catalyses the decomposition of hydrogen peroxide to hydroxyl radicals this may result in the over-estimation of hydroxyl radical production due to local production. As a result of these limitations the authors, when investigating hydroxyl radical production in *in vivo* models, recommend the use of other hydroxyl radical trapping agents, including phenylalanine. For *in vitro* models these limiting factors of salicylate are not as relevant.

The detection of hydroxyl radicals using hydroxylation of salicylate has been widely used in the medical field to relate the production of these highly reactive radicals to various disease states. Kaur *et al.* (1996) demonstrated that the highly reactive hydroxyl radical is generated in the synovial fluid of patients with rheumatoid arthritis. In their study synovial fluid samples from 18 of 22 patients with rheumatoid arthritis which were aspirated into solutions of salicylate showed evidence of 2,3-DHB and 2,5-DHB. These products of the salicylate hydroxylation were detected using HPLC and ECD. Das *et al.* (1991) used salicyclic acid to aid in the detection of hydroxyl radicals during reperfusion of ischaemic heart muscle. The end products of this hydroxylation reaction; 2,3-DHB and 2,5-DHB were detected using HPLC and ECD. The authors concluded that the hydroxyl radicals are produced during the reperfusion of the heart muscle following ischaemia and that these radicals are responsible for the sustained injury to the heart muscle. These results are supported by the findings of Yang *et al.* (1997) who found increased hydroxyl radical formation following rat myocardial reperfusion. These hydroxyl radicals were detected by the hydroxylation of salicylate to produce 2,3-DHB and

2,5-DHB.

8.3 Other methods of detection of hydroxyl radicals

Several other methods of detection of hydroxyl radicals have been reported which have adequate sensitivity. Electron spin resonance (ESR) trapping has been used for the direct detection of hydroxyl radicals. This technique is highly sensitive with reports of thresholds in the range of 10^{-8}M able to be detected (Kaur and Halliwell, 1997). This technique requires micro-molar concentrations of the radical species being measured. Tosaki *et al.* (1993) reported that there are some difficulties in interpreting which radical species are being produced and that the application of this method is limited by the need for high milli-molar concentrations of the trapping agents. Furthermore in biological systems, the ESR signal is unstable and decreases with time.

Ste-Marie *et al.* (1996) reported on the use of gas chromatography – ion trap mass spectrometry (GC–MS). This method offers excellent sensitivity for the di-hydroxybenzoic acids in the range of 0.2 – 1.0 pmoles. The authors reported that in complex systems this method has the added advantage of positive identification of a peak for the di-hydroxybenzoic acids by its mass spectrum. These results were confirmed by Luo and Lehotay (1997) who found sensitivity in the range of 50fmoles – 1pmole per 1 microlitre injected.

However, the relative simplicity of the HPLC assay and the excellent sensitivity of

electro-chemical detection is the reason for its use in this research. The amount of hydroxyl radicals anticipated to come across the tooth based on the work of Dahlstrom *et al.* (1997) suggested that sensitivity in the range of the limits described by Ste-Marie *et al.* (1996) will be required.

8.4 Theory of High Performance Liquid Chromatography (HPLC)

Heftman (1975) described chromatography as the process by which the flow of solvent gas or liquid promotes the separation of substances by differential migration from a narrow initial zone into a porous sorptive medium. The process of chromatography therefore separates individual components of a liquid or gas mixture.

High performance liquid chromatography is a form of chromatography which offers high resolution of the components of the liquid or gas being separated. The basis of HPLC is the passing of a liquid through a chromatographic column which is packed with sorbent particles, along which solutes within the liquid (referred to as the mobile phase) are separated. These solute bands can be detected at the end of the column by either electro-chemical detection or ultra-violet spectrometry. The fundamental design of HPLC is outlined in Figure 8.3.

Reverse phase chromatography is a form of HPLC which separates molecules based

on their degree of hydrophobicity. Separation of the moieties in reverse phase HPLC therefore occurs by differential hydrophobic interactions of the sample injected onto the column with the hydrophobic functional groups along the column matrix. These functional groups may either bond weakly or strongly to solute molecules and this determines the rate at which the molecules pass along the column. Polar compounds are eluted faster than non-polar compounds i.e. sample molecules that are polar or only mildly hydrophobic will react only slightly with the functional groups along the column. Therefore their retention times will be less than that of hydrophobic molecules which will bind more strongly with column matrix functional groups and will be held longer. The higher the hydrophobicity of the stationary phase surface the longer will be the retention times of the analytes (Hanai, 1999).

Good reproducibility of the retention times will require maintenance of constant temperature. A water bath or an air oven are often employed and Hanai (1999) recommended heating of the mobile phase when temperature control is extremely important. As retention times were stable across a number of mobile phases these recommendations were not deemed necessary for the test conditions.

8.5 Theory of electrochemical detection (ECD)

Electrochemical detectors measure either the conductance of the eluent or the current associated with the oxidation or reduction of solutes. To be able to be detected the solutes must be relatively easy to oxidise or reduce and they must be ionic.

Electrochemical detectors that measure the current associated with the oxidation or reduction of solutes are called amperometric or coulometric detectors. When current is passed through a solution, reactions occur at each electrode in which electron exchange takes place between the electrodes and the substances in solution. At the cathode, substances in solution undergo reduction (gain electrons) and at the anode they undergo oxidation (lose electrons). If a substance can be electrochemically oxidised or reduced it is said to be electrochemically active. The principle of ECD is to measure the current in a flow cell at a fixed point (the column outlet).

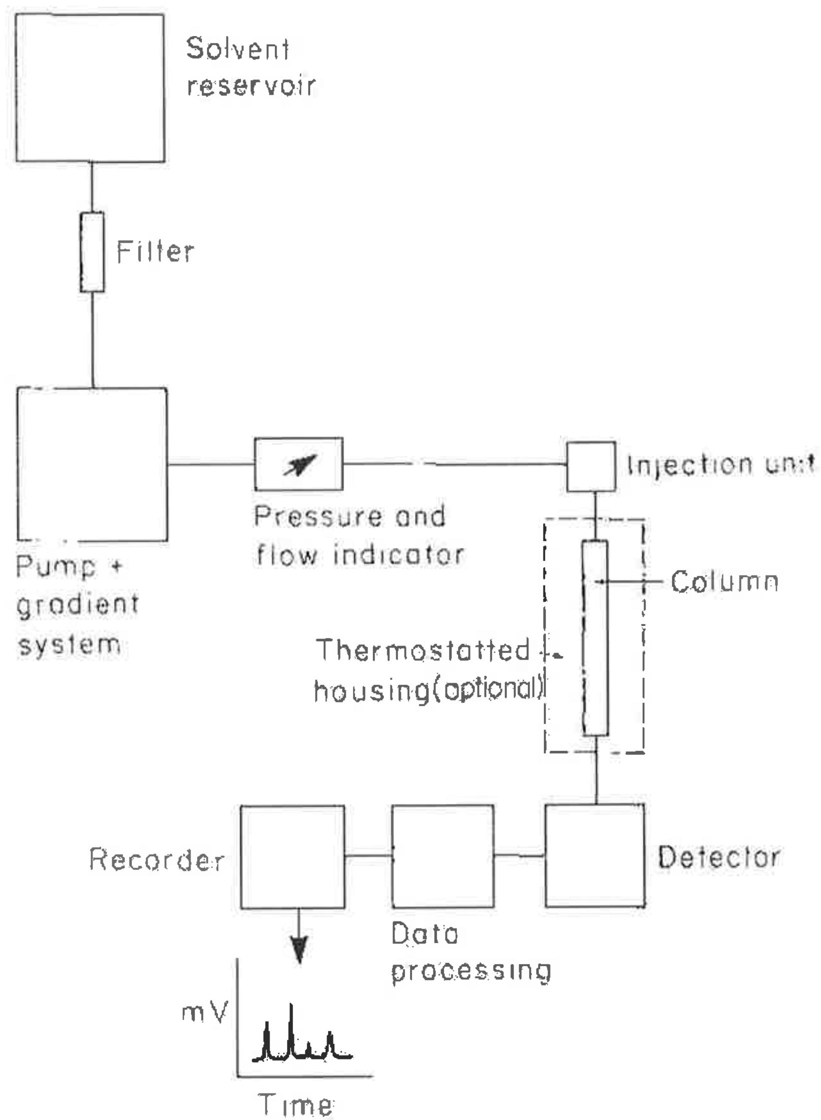


Fig. 8.3 Block diagram of HPLC as used in the experimentation procedure for detection of 2,3 – DHB, 2,5 – DHB and hydrogen peroxide (Lindsay, 1987).

Electric current is measured as the molecules in the solvent undergo either oxidation or reduction at an electrode which is operating at a fixed potential. This potential is usually between 0.5V and 1.0V. If the potential is greater than that required for electrolysis of the solute a measurable charge passes from the electrode to the molecule and vice versa. A positive potential will result in oxidation and a negative potential will result in reduction of the molecules. The selectivity of the detector can be altered by changing the potential of the electrode. The resulting current, as a function of time, is amplified and sent as a 0-10mV signal to the recorder and a chromatogram is produced.

There are two types of electrochemical detectors. Coulometric detection will react with all the electro-active solute passing through the detector. Amperometric detection is more popular as it reacts with a much smaller quantity of the solute passing across the detector (Lindsay, 1987). The currents observed with these latter detectors are very small (nanoamps) and offer high levels of sensitivity.

Three electrodes are used in amperometric detection. The working electrode is the electrode at which the electro-activity is monitored. This electrode is composed of glassy carbon, a pyrolytically prepared form of carbon (Lindsay, 1987) that is inert and electrically conducting. This form of working electrode is particularly useful with mobile phases containing relatively large concentrations of methanol (Kissinger, 1984). Care must be taken to keep the electrode surface smooth so as to maintain a high degree of reproducibility. The reference electrode, a silver – silver

chloride electrode, provides a stable and reproducible voltage to which the potential of the working electrode can be referenced. The third electrode is the auxillary electrode is a current carrying electrode.

9.0 Thiourea (2-thiourea, 2-thiocarbamide, sulphocarbamide)

Thiourea is a colourless, crystalline solid that is soluble in water, alcohol and sparingly soluble in ether (Hampel and Harvey, 1973). It is found naturally in laburnum shrubs. It has been used in the textile industry as a reducing bleaching agent for many years to remove silver tarnish and as a liquefier of animal glues and is used in the medical profession as a scavenger of oxygen species. Thiourea undergoes addition reactions with metals or organic compounds e.g. proteins (Sandmeyer, 1981).

9.1 Commercial uses of thiourea

The commercial use of thiourea usually requires its acidification. The major uses of thiourea commercially are in animal glue liquefiers and silver tarnish removers. In silver polishers it is usually acidified with citric acid or hydrochloric acid. It is widely used in the chemical, rubber and petroleum industries as a vulcaniser (Sandmeyer, 1981). Also as a rust remover, in the production of flame retardant resins, a fixer in photography, for organic synthesis (e.g dyes, drugs and hair preparations) and as a mould inhibitor (Hampel and Harvey, 1973). In the agriculture industry it is used as an insecticide, fungicide and as an accelerator of sprouting in dormant plants.

9.2 Therapeutic uses of thiourea

Thiourea has been used as an anti-thyroid agent by blocking the production of thyroid hormones through the inhibition of thyroid peroxidase. Detterbeck *et al.* (1990) reported on the use of thiourea and DMT as scavengers of oxygen free radicals to decrease the incidence of injury in lung transplant patients. However, Beehler (1994) suggested that the oxygen scavenging effect of the thiourea may be offset by the toxic nature of the thiourea on pulmonary vascular permeability. The use of thiourea as an antidote to anti-cancer agents such as asplatin have been reported by Shibiya *et al.* (1990). Onderwater *et al.* (1998) reported the use of di-substituted thiourea which is under investigation for the treatment of HIV as non-nucleoside inhibitors of HIV-1 reverse transcriptase.

9.3 Toxicity of thiourea

The toxicity of thiourea has yet to be fully researched and this is acknowledged by research groups worldwide. Several states in the USA and many countries throughout Europe have classified thiourea as a carcinogen and recommend caution in the handling of this compound. Current research has been limited to *in vitro* studies and *in vivo* studies on rodents. Target organs following exposure to thiourea include the liver, thyroid gland, lungs and bone marrow. Systemic effects from repeated exposure include bone marrow depression resulting in anaemia, leukopenia, thrombocytopenia and agranulocytosis. It may also cause allergic skin irritations. Long term administration has been associated with the development of

carcinoma (Sandmeyer, 1981). Newcombe and Deane, (1944) described the influence of thiourea fed chronically to rats as inducing hepatic tumours, bone marrow depression and goitres.

Thiourea has an oral LD_{50} toxicity in the rat of 1830mg/kg (Dieke, George and Curt, 1947). Exposure to thiourea is not classified as a significant risk except when found in tarnish remover dips. However, the US FDA prohibit the use of thiourea in food preparations and the Australian Occupational Safety and Health Association (OSHA) list thiourea as a carcinogen.

Mechanisms of thiourea toxicity have not yet been clarified. The first step in bioactivation of thiourea is generally accepted to be oxidation of the thionosulphur. It is believed that flavin-containing mono-oxygenases (FMO) are mostly responsible for this oxidation (Onderwater *et al.* 1998) which results in the formation of sulphenic acids (Guo *et al.* 1992). Intra molecular rearrangement of the sulphenic acids leading to the release of highly reactive singlet sulfur and formation of hydro-disulfides of cysteine residues, was first postulated as the mechanism of thiourea toxicity (Lee *et al.* 1980). However, Decker *et al.* (1992) reported that the release of sulphurmonoxide was the cause of initial toxicity and Miller *et al.* (1988) suggested that the irreversible binding of the corresponding sulphinic or sulphonic acids to cellular proteins via a nucleophilic substitution mechanism was the mechanism of activation. However, Onderwater *et al.* (1998) suggested that the oxidation of cellular non-protein thiols (e.g reduced glutathione - GSH) by sulphenic acids will be the first step in the occurrence *in vivo*. Depletion of the non-protein thiols would

ultimately lead to oxidative stress induced cell death or to irreversible oxidation of essential cellular macromolecular structures. Onderwater *et al.* (1998) showed that α-naphthylthiourea (ANTU) causes a significant depletion of intracellular GSH as well as a significant increase in LDH leakage from the cells, the latter being indicative of cell death.

The effect of thiourea on pulmonary vascular permeability has been well researched. Mackenzie and Mackenzie (1943) reported on the increased pulmonary oedema following a single 1800mg intra-peritoneal injection of thiourea in rats resulting in their death within two hours. The oral route was as effective as the intra-peritoneal route. The authors found that mature rats were more susceptible to the effects of the thiourea but offer no explanation as to the possible cause. Giri *et al.* (1991) offered a possible cause by suggesting that the increased pulmonary vascularity occurs in response to the release of histamine. These authors observed that the administration of thiourea resulted in a close temporal relationship between plasma histamine changes and increased pulmonary vascular permeability. The increased sensitivity of the mature rats may be the result of (1) more binding sites and (2) more histamine available for release. Beehler *et al.* (1994) investigated the role of dimethyl thiourea (DMTU) on lung vascular permeability. These authors found that the administration of DMTU to rats increased lung accumulation of albumin that suggested increased lung vasculature permeability thereby offering further explanation for the increased vascular permeability.

Thiourea is known to increase the potential for thyroid tumours by disrupting one of

the steps in the biosynthesis and secretion of thyroid hormones. Capen (1994) suggested that it is the blockage of organic binding of iodine and coupling of iodothyronines to form thyroxine (T4) and triiodothyronine (T3) by which the thiourea exert its effect. This results in the chronic hypersecretion of TSH which by receptor mediated events places the rodent thyroid gland at greater risk of developing tumours through a secondary mechanism of thyroid oncogenesis. Hurley (1998) reported ethylene thiourea to be an inhibitor of thyroid peroxidase thereby disrupting thyroid – pituitary homeostasis. Shimo *et al.* (1994) reported that the administration of 0.1% thiourea in drinking water to rats results in significantly greater numbers of thyroid proliferative lesions compared to control and other test groups. Graham *et al.* (1975) demonstrated that the introduction of ethylene thiourea (ETU), a commonly used insecticide, increased the incidence of thyroid follicular cell adenomas and adenocarcinomas in both sexes of mice and rats. Hill *et al.* (1989) demonstrated administration of ETU resulted in thyroid hyperplasia, decreased uptake of iodine by the thyroid and decreased serum levels of T3 and T4.

ETU has been reported by Deerfield (1994) to be a developmental toxicant. The author reported that the evidence available at the time of writing cannot discount ETU as being devoid of genotoxic effects. Khera (1987) supported this statement in a review of the teratogenicity and added that ETU is a potent neuroteratogen i.e. an agent that causes, at low teratogenic doses, malformations involving solely or predominantly the CNS or the nervous component of the sense organs.

9.4 Thiourea as a scavenger of oxygen radicals and hydrogen peroxide

Thiourea is a highly cell permeable scavenger of hydroxyl radicals and hydrogen peroxide (Stahl *et al.* 1993). Cederbaum *et al.* (1979) reported that this capacity of thiourea to directly interact with either hydrogen peroxide or hydroxyl radicals renders thiourea unique amongst other hydroxyl radical scavengers e.g. mannitol, benzoate, dimethyl sulphoxide, which are incapable of scavenging hydrogen peroxide. Thiourea is effective at scavenging hydroxyl radicals at lower concentrations than other hydroxyl radical scavengers (Halliwell, 1977). Thiourea reacts with hydroxyl radicals to form the sulphydryl enol form. Kelner *et al.* (1990) was the first to show that thiourea is capable of reacting with the superoxide radical at a rate constant of $2.6 \times 10^{-5} m^{-1} s^{-1}$. The end product of the reduction of the thiourea by the superoxide radical is a sulphydryl compound. The rate of the reaction between thiourea and hydroxyl radicals is several orders above the reaction between the superoxide radical and thiourea. Thiourea reacts with hydrogen peroxide to form thiourea dioxide (sulphinic acid) (Parker *et al.* 1985). The rate of this reaction is slower than the reaction with hydroxyl radicals (Kelner *et al.* 1990). In the presence of excess hydrogen peroxide thiourea is converted to urea. Jackson *et al.* (1988) discussed the potential of thiourea to scavenge hypochlorous acid (HOCl). Although not proven in their study, the authors concluded that thiourea can decrease neutrophil derived HOCl concentrations either by directly scavenging HOCl or by decreasing concentrations of its precursor, hydrogen peroxide. Carrea *et al.* (1991) also reported the ability of thiourea to scavenge HOCl *in vivo*.

Fox (1984) used dimethylthiourea (DMTU), a derivative of thiourea which has been shown to be less toxic to lung vascular tissue than thiourea, to scavenge hydroxyl radicals from lung tissue *in vivo*. The decision to use thiourea derivatives as the hydroxyl radical scavenger of choice was two-fold: (1) thiourea is a highly effective scavenger of hydroxyl radicals at low concentrations (2) thiourea and its derivatives are small enough to be able to penetrate non-perfused areas and inside cells. Other scavengers e.g. superoxide dismutase (SOD) and catalase are large charged proteins which are incapable to provide protection in these areas. Likewise these scavengers may not effectively scavenge hydroxyl radicals when used in concentrations which are not toxic *in vivo*. Carrea *et al.* (1991) added to this list of advantages in using thiourea as the preferred scavenger during *in vivo* studies, the relatively long half-life of 43 hours of thiourea.

The end-product of the reaction between thiourea and hydrogen peroxide is thiourea dioxide (sulfinic acid). Maroulis (1994) showed this product to be an effective bleaching agent in teeth discoloured by blood.

10.0 Materials and methods

10.1 Materials

The materials and techniques used in the preparation of the teeth are those used routinely in the endodontic treatment of teeth. The teeth used in the experimental phase of the research project were collected and stored in phosphate buffered saline with added thymol (an anti-fungal agent). Blood used in the staining of the teeth was obtained from the Red Cross Blood Bank (Adelaide) and consisted of packed red blood cells.

All agents used in the HPLC experimentation protocols were of analytical grade and the methanol used in the mobile phase was of HPLC grade. The crystalline forms of the agents used in the control studies were stored as recommended by their manufacturers i.e. 3,4 – dihydroxy benzoic acid (3,4 – DHB) and 2,3 – dihydroxy benzoic acid (2,3 – DHB) were stored in darkness at below 4^0C; 2,5 – dihydroxy benzoic acid (2,5 – DHB) and sodium salicylate were stored at room temperature in darkness. All aqueous solutions were made up in milliQ water. Hydrogen peroxide is unstable and Hardman *et al.* (1985) showed that hydrogen peroxide is capable of losing more than 50% of its oxidising strength in a six month period. The thirty percent hydrogen peroxide was confirmed to be at the correct concentration by testing the wavelength of the solution with spectrophotometry. Thirty percent (w/v) hydrogen peroxide was made from a 100% stock the day it was required. In this way hydrogen peroxide was maintained at the correct concentration. Hydrogen peroxide

was stored in a light-tight container. Acidified thiourea (1.3M) was made by the formulation outlined in Appendix 2.

Aqueous sodium salicylate solution was made the day of the experimentation and was kept in a light-tight container. Its formulation is outlined in Appendix 2.

All chemicals used throughout the experimentation and their sources are detailed in Appendix 1.

The mobile phase eluting the reverse phase column was composed of 70% aqueous phase and 30% methanol as recommended by Dahlstrom *et al.* (1997). The aqueous phase was comprised of 0.1M potassium di-hydrogen orthophosphate (KH_2PO_4) and 0.1M EDTA made up in 1litre of milliQ water. The pH was kept constant (pH = 2.9) between different batches of the mobile phase.

The HPLC unit was a GBC LC 1150 HPLC pump. This pump was equipped with a ERC-3415 membrane-type degasser designed for continuous feeding of the mobile phase through a gas-permeable membrane to de-oxygenate the solution. The test solutions were injected onto an octadecylsilyl (C18) μBondapak 5μm, 0.4 x 25cm reverse phase column.

The electro-chemical detector was a Bioanalytical Assay Systems (BAS) LC-4A Amperometric Detector. This ECD was equipped with a RE-1 glassy carbon electrode set at an oxidation potential of +0.80V against the Ag/AgCl working

electrode, with a sensitivity range of 5nA per 10 mV of detector output to recorder. The oxidation potential used was 0.8V that was shown by Dahlstrom *et al.* (1997) to provide sufficient sensitivity with minimal background noise. Wright (1988) recommended a potential difference of 0.8V as it produced near maximal response thus minimising the effects of small fluctuations in mobile phase passing between the working and reference electrodes of the ECD. This voltage was also reported by Wright to produce minimal baseline noise and no response to salicylate when a concentration of 100nmol of salicylate is injected onto the column.

10.2 Methods

Thirty-five single canal premolar teeth were used in this study. These teeth had been extracted for orthodontic purposes in such a manner to minimise the extent of damage to the cementum. Sufficient teeth were collected to allow the selection of those teeth that were as anatomically similar as possible and which had intact cementum or as little deficiency of cementum at the level of the CEJ as possible. This was determined by microscopic examination using a Wild Heerbrugg M400 stereomicroscope at 7X magnification. The remaining periodontal ligament was removed from the outer surface of the tooth by gentle rubbing of the outer surface of the tooth with gauze soaked in sterile saline. The teeth were examined under the same stereomicroscope at 7X magnification to ensure complete removal of the periodontal ligament had occurred (Fig 10.1).

The teeth were held in damp gauze saturated with sterile saline throughout their

handling to limit the potential damage to the cemental lining on the root surface. Occlusal access cavities were cut into the teeth using a high speed tungsten carbide bur and the pulp tissue was extirpated from the canal of each of the teeth using endodontic files. The canals were debrided to a size 35 master apical file. The canals were irrigated with sterile saline throughout the debridement process.

Thirty teeth were placed crown first into 10ml polyethylene centrifuge tubes and the tubes were filled with 6ml of the packed red blood cells, completely immersing the teeth. Discolouration of the teeth occurred by the adaptation of a technique devised by Freccia and Peters (1982) and outlined by Marin *et al.* (1997). The teeth, submerged in the packed red blood cells were centrifuged at 10,000 r.p.m. for 10 minutes three times a day for three days. The red blood cells were resuspended between centrifugations by shaking on a Vortex machine. The teeth in their vials were stored at 37^{0}C in an incubator chamber during and after the staining process.

Following visible discolouration (Fig. 10.2) the teeth were removed and irrigated with sterile saline before being obturated with AH26 and gutta percha. A lateral condensation technique was employed in the obturation and radiographic examination of the root canal space confirmed the presence of no radiolucencies in the obturation of these canals (Fig. 10.3). The obturation was finished at a level 1mm below the level of the CEJ and was confirmed by radiographic examination. The pulp chambers of the canals were swabbed with EDTAC (15%) to remove excess sealer and then thoroughly rinsed with sterile saline to remove any traces of the EDTAC solution. The access cavities were lightly cleaned with a No.2 round

bur in a slow speed drill. Each tooth was then set in a 1mm thickness of red boxing wax at the level of the mid-crown of the tooth leaving the CEJ completely below the level of the wax.

The teeth were divided into 7 experimental groups of 5 teeth each:

(1) Unstained teeth, no bleaching protocol (negative control)

(2) Stained teeth, bleached with H_2O_2 - walking bleach technique

(3) Stained teeth, thermocatalytically bleached with H_2O_2

(4) Stained teeth, bleached with acidified thiourea (1.3M, pH = 2.95)

(5) Stained teeth, bleached with acidified thiourea and H_2O_2 (1.3M, pH = 2.95)

(6) Stained teeth, bleached with acidified thiourea and NaOCl (1.3M, pH = 2.95)

(7) Stained teeth, bleached with acidified thiourea, NaOCl and H_2O_2 (1.3M, pH = 2.95)

Teeth subjected to thermocatalytic bleaching had 20 microlitres (μl) of 30% w/w H_2O_2 pipetted into the pulp chamber in which had been placed a sterile cotton pellet. The H_2O_2 was activated with a red-hot ball burnisher which had been heated in a blue flame. The heat was applied for ten seconds intervals. This process was repeated five times over a twenty minute period. The access cavity was sealed with a 2mm layer of Cavit.

Teeth subjected to the walking bleach had 20μl of 30% w/w H_2O_2 pipetted into the pulp chamber of each tooth. A cotton pellet was already seated in the pulp chamber thereby saturating the cotton pellet. The access cavity was sealed with a 2mm layer of Cavit.

Teeth subjected to intra-coronal bleaching with 1.3M thiourea had 20 microlitres of the thiourea solution pipetted into the pulp chamber of the tooth that already had a sterile cotton pellet placed *in situ* thereby saturating the pellet. The access cavity was sealed with a 2mm layer of Cavit.

Test groups (5) – (7) each had 20 μl of the test solutions pipetted into the pulp chamber of the tooth in the order in which they are listed above. The access cavity of each tooth was then sealed with 2mm of Cavit. Following the bleaching procedure, each tooth was immediately suspended in a 2.5ml polycarbonate assay tube filled with sodium salicylate solution. The tooth was sealed in place using sticky wax and the tube was immediately labelled to indicating the test group (Fig 10.4). In this way the cervical margins of the teeth was exposed to the sodium salicylate solution. The teeth were stored at 37^0C in an incubator chamber for 48 hours before samples of each salicylate bath were tested.

Fig.10.1 Photograph of tooth following removal of the periodontal ligament and viewed under the stereo microscope (Scale 1:6)

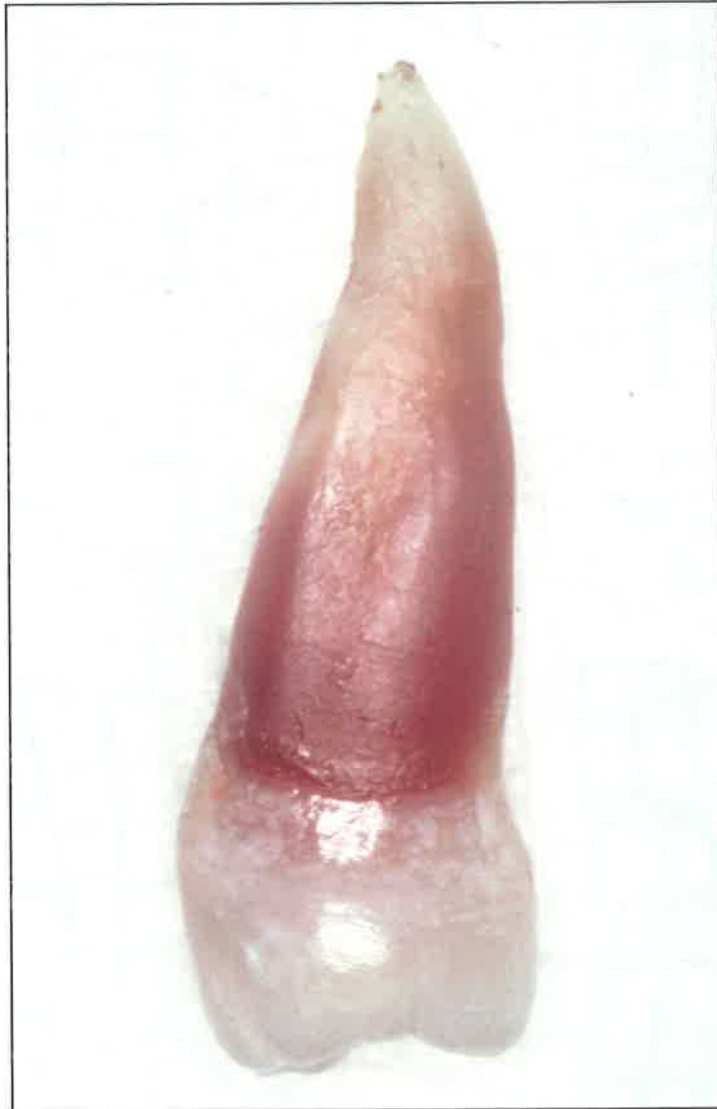


Fig.10.2 Photograph of tooth stained with blood following centrifugation with packed red blood cells. (Scale 1:6)

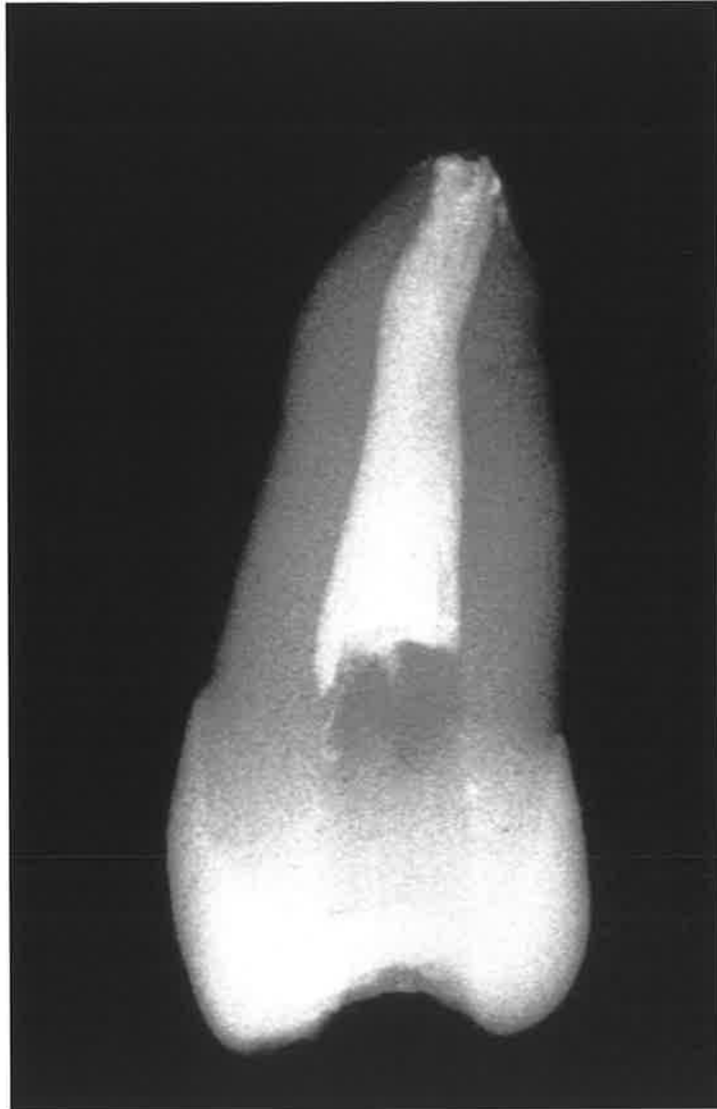


Fig.10.3 Radiograph of tooth, following staining with packed red blood cells and obturated with AH26 and gutta percha (Scale 1:6)

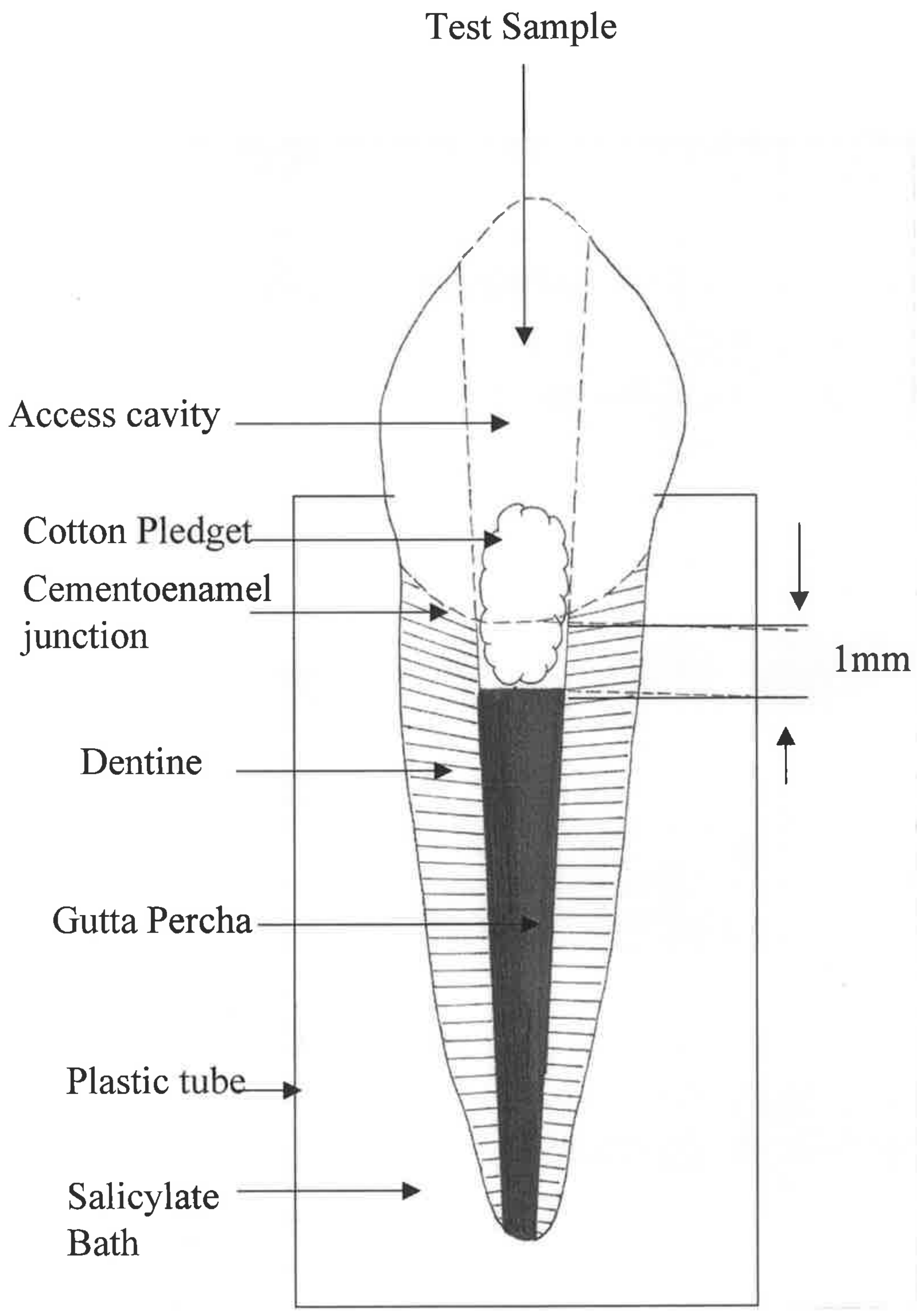


Fig.10.4 Diagrammatic representation of the salicylate model

10.2.1 HPLC detection of reaction products

Detection of the end products of the bleaching protocols tested with the sodium salicylate was based on the technique designed by Wright (1988) and which was adapted by Dahlstrom *et al.* (1997) to suit the intra-coronal bleaching of blood-stained teeth.

A mobile phase was flowed across the column at a rate of 1.0ml min^{-1}. A 20μl sample of each of the test solution in which the tooth was bathing was injected onto the reverse phase column to which was attached the electro-chemical detector. Two samples of each test solution were injected, directly after each other, such that test conditions for both samples were as identical as possible. To the first sample was added a 20μl sample of the internal standard – 2μM solution of 3,4 – DHB. The second sample, injected onto the column immediately after the first sample had passed across the column did not have the internal standard present to ensure that contamination of the internal standard had not occurred.

Diagrammatic outline of the experimental procedure is seen in Fig.10.5.

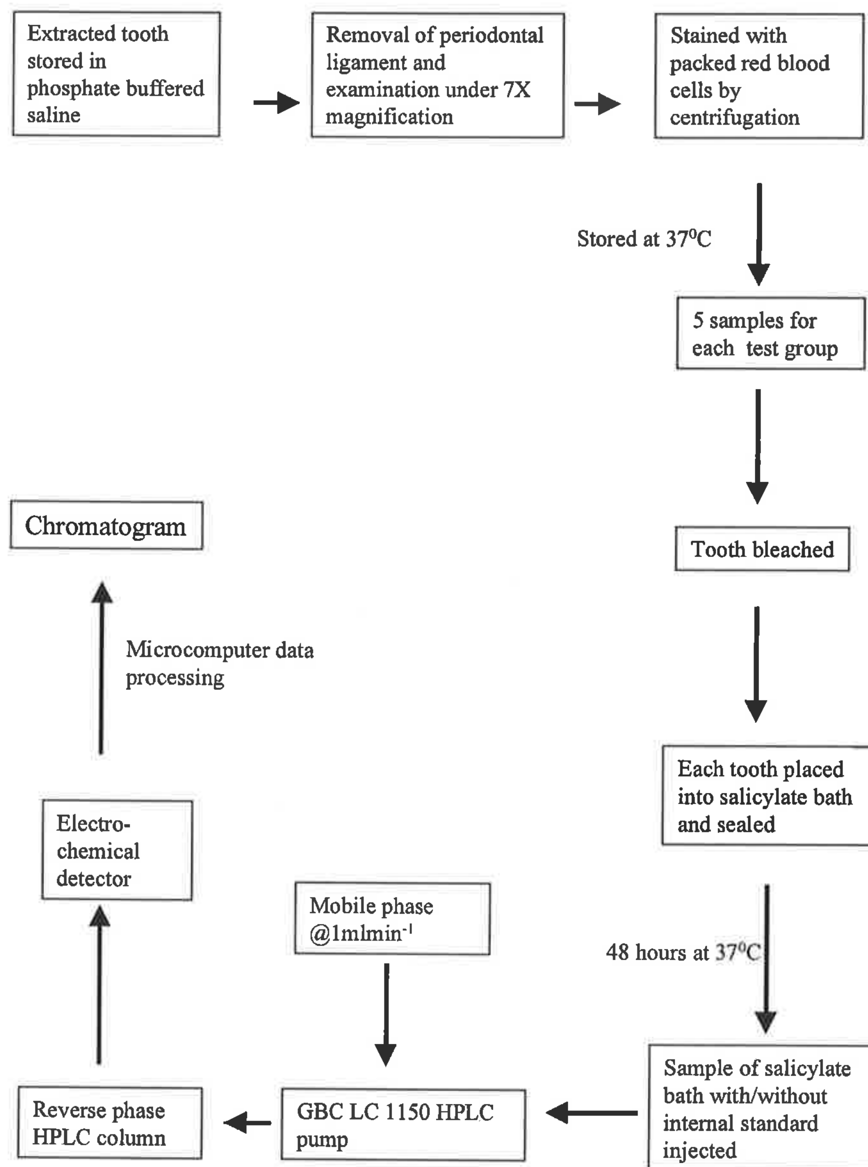


Fig.10.5 Schematic outline of the experimental procedure

10.3 Control experiments

A series of control experiments were run prior to the commencement of the bleaching of the teeth. These experiments can be separated into several categories.

10.3.1 Control generation of hydroxyl radicals

The control generation of hydroxyl radicals was performed by the combination of the following reagents to ensure that these radicals are able to be produced.

(i) Addition of 0.5ml of 30% H_2O_2 to a 500μM $FeSO_4$ solution in 1 litre aqueous 1mM sodium salicylate.

(ii) Addition of 0.5ml of 30% H_2O_2 and 0.5ml of 1.3M thiourea (acidified) to a 500μM $FeSO_4$ solution made up in 1 litre aqueous 1mM sodium salicylate.

(iii) Addition of 0.5ml of 4% NaOCl to a 500μM solution $FeSO_4$ made up in 1 litre aqueous 1mM sodium salicylate.

(iv) Addition of 0.5ml of 30% H_2O_2 and 0.5ml of 4% NaOCl to a 500μM solution of $FeSO_4$ made up in 1 litre aqueous 1mM sodium salicylate.

(v) Addition of 0.5ml of 30% H_2O_2, 0.5ml of 4% NaOCl and 0.5ml of 1.3M thiourea (acidified) to a 500μM solution of $FeSO_4$ made up in 1 litre aqueous 1mM sodium salicylate.

A 20μl sample of each solution was injected onto the column. A fresh mobile phase was employed for each test aspect of the project and collateral runs of the standards were injected before each run to confirm the identification of peaks generated.

10.3.2 Effect of iron concentration on the generation of hydroxyl radical production.

$FeSO_4$ solutions in concentrations of 500μM, 100μM, 50μM and 5μM were made in 1 litre aqueous 1mM sodium salicylate. To each of these solutions was added 0.5ml of 30% H_2O_2. A 20μl sample of these solutions was then immediately injected onto the column. This was repeated at 15 minute intervals for a period of 1 hour for each $FeSO_4$ solution. Concentrations of the resultant 2,5 - DHB and 2,3 – DHB were then plotted to determine the amount of hydroxyl radicals produced over the 1hour time course. The concentration of ferrous iron was varied to determine the effect of different concentration of ferrous ions on the production of hydroxyl radicals.

10.3.3 Generation of other reaction products of salicylate hydroxylation

Theoretically, attack upon salicylate by hydroxyl radicals is capable of producing a greater number of reaction products than only 2,5 – DHB and 2,3 – DHB. In times of high radical flux the 2,5 - DHB and the 2,3 – DHB are capable of undergoing attack by hydroxyl radicals to produce a series of compounds outlined in section 8.1. Therefore it was deemed necessary to obtain retention times for these reaction products 10μl of each of the following were injected on to the column:

10μM hydroquinone (1,4 – DHB)

10μM resorcinol (1,3 – DHB)

10μM pyrocatechol (1,2 – DHB)

10μM phloroglucinol (1,3,5 – THB)

10μM pyrogallol (1,2,3 – THB)

The potential for the end product 2,3,5 – THB to be produced is minimal as it would require activation of the –meta position of the aromatic ring of the salicylate molecule. It was unable to be tested due to it being commercially unavailable.

10.3.4 Detection of thiourea dioxide

In order to do this 20μl of a 5μM solution of thiourea dioxide (equivalent to 1×10^{-10} moles) was injected onto the column to obtain a retention time for thiourea dioxide.

10.3.5 Solutions used in the experimentation procedure

A number of solutions which were to be used in the experimental conditions were tested to determine their potential to generate hydroxyl radicals and thus interfere with the test solutions. 20μl of each of the following solutions was injected onto the column:

(1) 500×10^{-6}M $FeSO_4$ solution (equivalent to 1×10^{-8} moles)

(2) 5×10^{-4}litre 30% H_2O_2 in 1 litre aqueous solution

(3) 1×10^{-3}M sodium salicylate solution (equivalent to 2×10^{-8} moles)

(4) 1×10^{-4}M potassium dihydrogen phosphate solution (equivalent to 2×10^{-6} moles)

(5) 5×10^{-4} litre 4% NaOCl in 1 litre aqueous solution

10.4 Sensitivity assays

In order to determine the degree of sensitivity of the HPLC – ECD and to formulate the quantity of reaction product, sensitivity assays were conducted for hydrogen peroxide, thiourea and the reaction products 2,5 – DHB and 2,3 – DHB as well as the internal standard 3,4 – DHB. A range of 1 – 800 pmoles of the thiourea was injected across the column; 800 femtomoles (fmoles) – 800pmoles of each of 2,5 – DHB, 2,3 – DHB and 3,4 – DHB were injected across the column and a range of 980 – 10 nanomoles (nmoles) of hydrogen peroxide were injected across the column. Standard curves of integration (uVsec) versus the amount of each injected was then plotted for each of these solutions

10.5 Statistical analysis

Statistical analysis of the raw data was carried out to test the significance of differences between the quantities of reaction products of hydroxylation of salicylate by hydroxyl radicals and the influence that thiourea has on the scavenging of these radicals. Two Tailed Student T - Tests were carried out to analyse this data.

Statistical analysis of the raw data was also performed using Two Tailed Student T – Tests to analyse the significance of differences between groups for the quantities

of hydrogen peroxide and thiourea which were able to diffuse through to the outer surface of the teeth.

Examination of the raw data revealed that there were several samples in which amounts of reaction products to be quantified had values that "were extreme". These values consequently distorted the mean values of the affected groups and influenced the statistical analysis of the results. Values of samples which were greater than two standard deviations from the mean value of the raw data of the group were therefore excluded form the statistical analysis. A value excluded from one group was excluded from all statistical analyses involving that group. Possible reasons for these results are discussed in section 12.4.

The amount of the reaction product 2,5 – DHB was used as the measure of hydroxyl radical production in the statistical analysis of the data. This was due to the fact that larger values for this reaction product were obtained throughout the controls and the experimental samples.

11.0 Results

11.1 Sensitivity Assays

Standard curves were generated for the two main products of the hydroxylation of salicylate by hydroxyl radical: 2,3 - DHB and 2,5 – DHB, as well as for the internal standard 3,4 - DHB. Hydrogen peroxide and thiourea also had standard curves generated due to the amount of these compounds that were detected in the salicylate samples taken from around the tooth. Figure 11.1 represents an example of the chromatogram produced following the injection of the three standards onto the column. Figure 11.2 displays the linear relationship between the amount of each of the three standards injected onto the column and the area under the peak generated. The x-axis represents the amount of each of the products injected onto the column and the y-axis represents the area (integration) under the peak generated on the chromatogram. Regression analysis of these standard curves reveals a high degree of linear relationship between the amount injected and the integration value of the peak.

2,3 - DHB: $y = 1.491x + 12.985$ $R^2 = 0.9958$

2,5 - DHB: $y = 2.4237x$ $R^2 = 0.9997$

3,4 - DHB: $y = 1.8539x$ $R^2 = 0.9968$

Standard curves were also generated for pyrocatechol, hydrogen peroxide and thiourea following completion of the experimentation. The area under the peak

generated by each of the three compounds permitted the quantification of each. Figures 11.3– 11.5 display the linear relationship between the amount of each of these compounds injected onto the column and the integration value of the peak generated on the chromatogram. Regression analysis of these standard curves revealed a high degree of linear relationship between the amount of each of the standards and the integration value of the peak.

Thiourea:	$y = 0.876x - 9.139$	$R^2 = 0.992$
H_2O_2:	$y = 0.5134x$	$R^2 = 0.9939$
Pyrocatechol:	$y = 8.3997x$	$R^2 = 0.9973$

The results of these sensitivity assays showed that the practical limit of sensitivity for the 2,3 - DHB, 2,5 - DHB and the 3,4 - DHB to be 800 femtomoles. The limit of the sensitivity for the thiourea was 1 picomole. The practical level of sensitivity for the hydrogen peroxide was 30 nanomoles. Figure 11.6 shows a sample chromatogram of each of the standards at their practical limits of sensitivity. Figure 11.7 shows a sample chromatogram of the practical limit of sensitivity of thiourea and figure 11.8 shows a sample chromatogram of the limit of sensitivity of hydrogen peroxide for the HPLC – ECD.

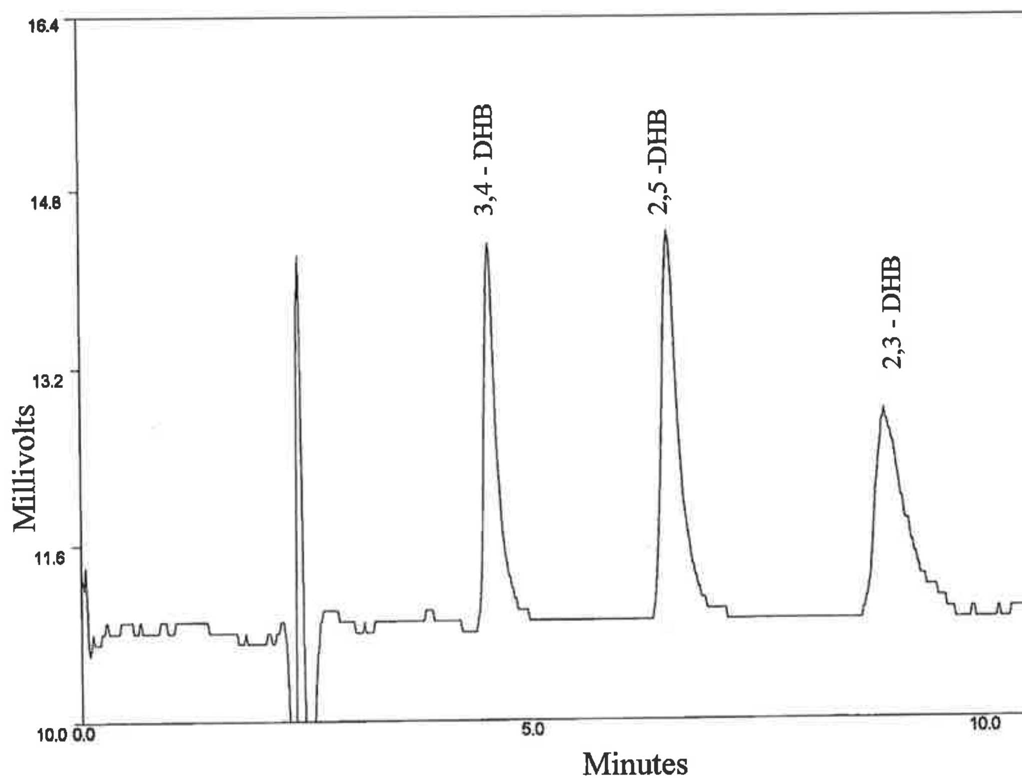


Figure 11.1 Chromatogram of 20 pmoles of the internal standard 3,4 – DHB and the two reaction products 2,5 – DHB and 2,3 – DHB

Standard curves for 2,3 - DHB, 2,5 - DHB and 3,4 - DHB

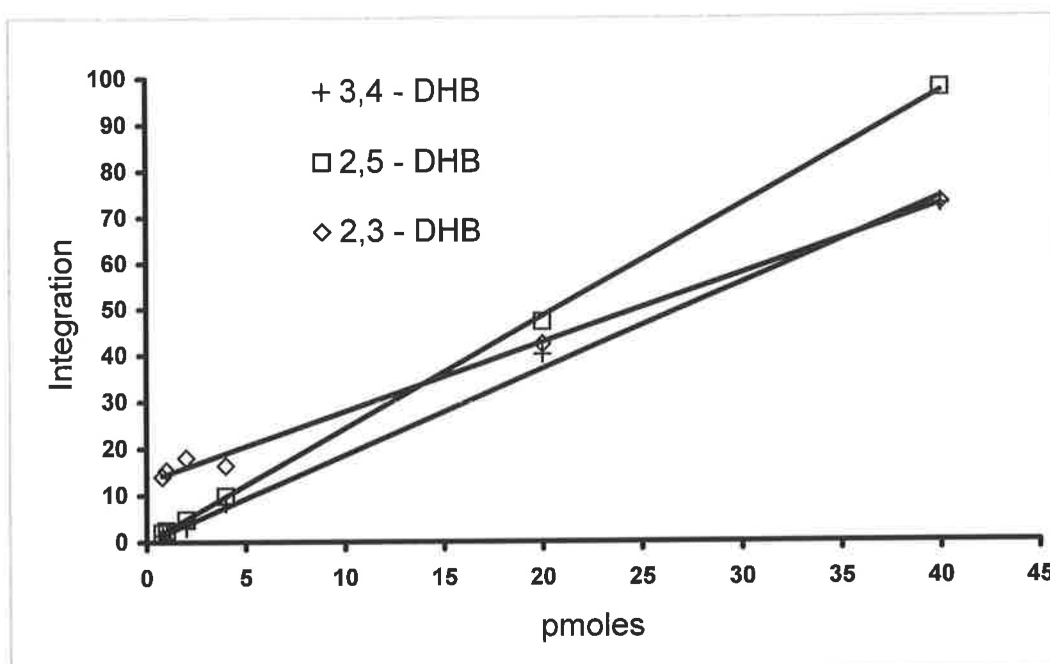


Fig. 11.2 Standard curves for the end products of salicylate hydroxylation by hydroxyl radicals: 2,3 - DHB and 2,5 - DHB and the internal standard 3,4 – DHB over a range of 1 – 40 picomoles.

Standard curve for Thiourea

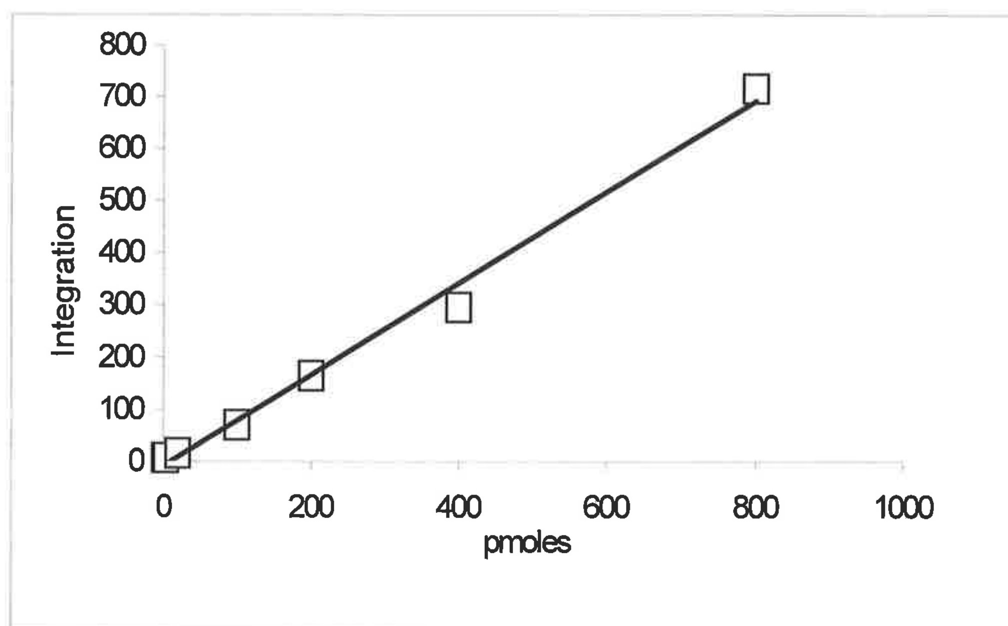


Fig. 11.3 Standard curve for thiourea for a range of 1 – 800 picomoles of the standard injected onto the column

Standard curve for hydrogen peroxide

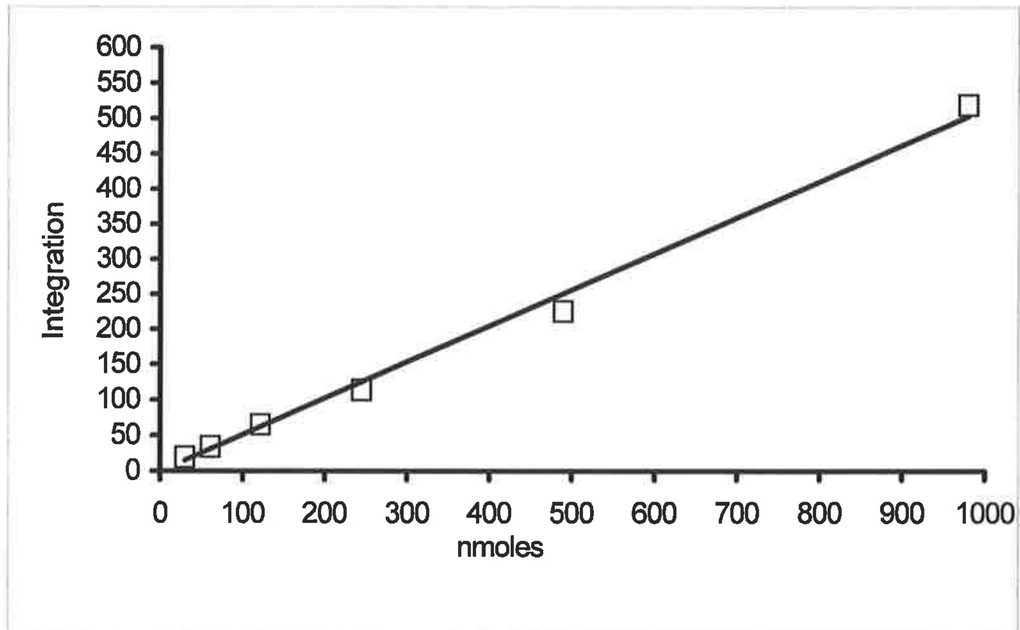


Fig. 11.4 Standard curve for hydrogen peroxide over the range of 30 – 1000 nanomoles of hydrogen peroxide injected onto the column.

Standard curve for pyrocatechol

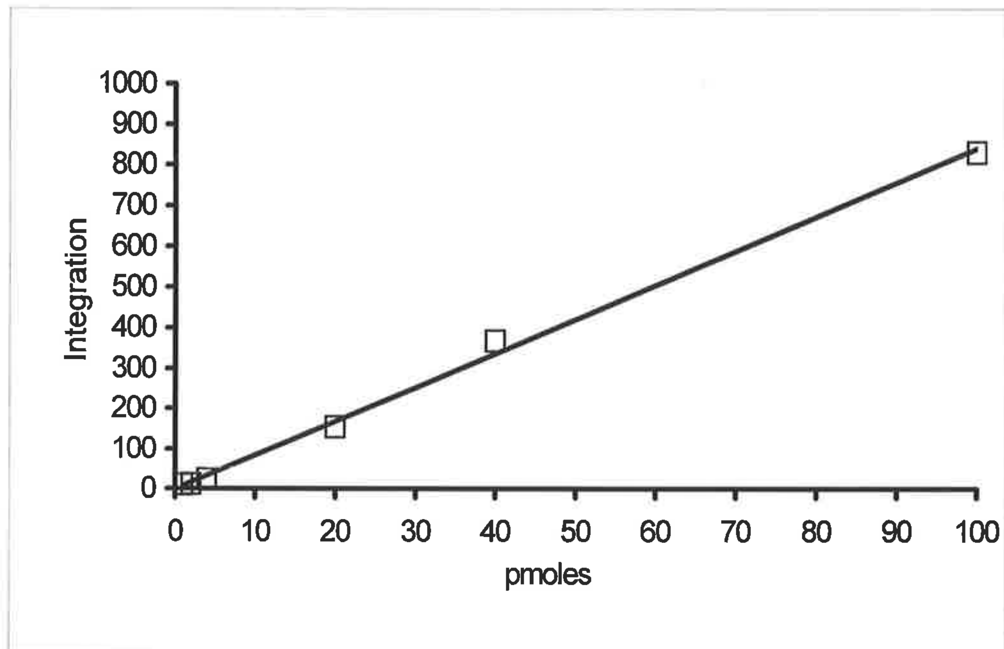


Fig. 11.5 Standard curve for the end product of hydroxylation of salicylate by hydroxyl radicals – pyrocatechol, over a range of 1 – 100 picomoles.

11.2 Control generation of hydroxyl radicals

To ensure that a Fenton reaction occurred following the addition of hydrogen peroxide to ferrous ions the addition of 0.5ml of 30% H_2O_2 to 500μM ferrous sulphate was performed. Three definite peaks, shown in Figure 11.9, were generated. These peaks were identified as pyrocatechol, 2,5 – DHB and 2,3 – DHB. Assuming that all of the reactants were able to react fully, the amount of each of the reaction products which were generated was:

2,5 – DHB: 2747.1 pmoles

2,3 – DHB: 1337.8 pmoles

Pyrocatechol: 300.4 pmoles

The ratio of these reaction products was 1.0 : 0.49 : 0.1.

To determine the effect of thiourea on the scavenging of hydroxyl radicals the addition of 0.5ml of 1.3M acidified thiourea to a solution of 0.5ml of 30% H_2O_2 and 500μM ferrous sulphate in 1 litre of 0.001M sodium salicylate occurred. The reaction between the three compounds yielded amounts of the three reaction products:

2,5 – DHB: 2294.8 pmoles

2,3 – DHB: 1267.0 pmoles

Pyrocatechol: 268.9 pmoles

The ratio of these reaction products was 1.0 : 0.55 : 0.1

To determine the potential of NaOCl to generate hydroxyl radicals the addition of

0.5ml of 4% NaOCl to 500μM ferrous sulphate in 0.001M sodium salicylate occurred. The reaction resulted in a small generation of hydroxyl radicals. The amounts of the three reaction products, as identified by the injection of a collateral run of the three standards on to the column, was determined to be:

2,5 – DHB: 85.8 pmoles

2,3 – DHB: 19.9 pmoles

Pyrocatechol: 1.2 pmoles

The ratio of these reaction products was 1.0 : 0.23 : 0.01.

To determine the influence of NaOCl on the yield of hydroxyl radicals generated during a Fenton reaction, the addition of both 0.5ml of 30% H_2O_2 and 0.5ml of 4% NaOCl to 500μM ferrous sulphate in 0.001M sodium salicylate occurred. The generation of the reaction products 2,5 – DHB, 2,3 – DHB and pyrocatechol, as determined by the injection of a collateral run of these three standards, was found in the amounts:

2,5 – DHB: 2212.7 pmoles

2,3 – DHB: 1146.3 pmoles

Pyrocatechol: 234.3 pmoles

The ratio of these reaction products was 1.0 : 0.52 : 0.1.

The scavenging ability of thiourea on the hydroxyl radicals produced following the addition of 0.5ml of 1.3M thiourea and 0.5ml of 4% NaOCl to 500μM ferrous sulphate in 1 litre of 0.001M sodium salicylate was tested. Production of the reaction products 2,5 – DHB, 2,3 – DHB and pyrocatechol, as determined by the

injection of a collateral run of these three standards occurred in the amounts:

2,5 – DHB: 32.1 pmoles

2,3 – DHB: 5.7 pmoles

Pyrocatechol: 0.9 pmoles

The ratio of these reaction products was 1.0 : 0.17 : 0.02.

The generation of hydroxyl radicals by the addition of 0.5ml of 1.3M thiourea to 0.5ml of 4% NaOCl and 0.5ml of 30% H_2O_2 in a solution of 500μM ferrous sulphate in 1 litre of 0.001M sodium salicylate resulted in production of the reaction products 2,5 – DHB, 2,3 – DHB and pyrocatechol, as determined by the injection of a collateral run of these three standards, in the amounts:

2,5 – DHB: 2379.6 pmoles

2,3 – DHB: 1240.3 pmoles

Pyrocatechol: 277.1 pmoles

The ratio of these reaction products was 1.0 : 0.53 : 0.11.

The addition of 20μl of each of the solutions used in the experimental procedures resulted in peaks being generated for four of the five solutions tested. Hydrogen peroxide was separated on the column early and gave a peak at 2.47 minutes. Sodium hypochlorite was also separated from the column early and had a similar retention time of 2.42 minutes. Thiourea gave off a peak at 2.69 minutes and ferrous sulphate could be detected by generation of a peak at 2.69 minutes. This retention time was similar to that of thiourea. Sodium salicylate was the only test solution that did not generate a peak.

11.3 Effect of ferrous ion concentration on the generation of hydroxyl radicals

The end products of hydroxylation of salicylate by hydroxyl radicals revealed the reaction to be virtually complete within the first 30 minutes. Some end products of the hydroxylation reaction continued to be produced up to 1 hour after the introduction of the hydrogen peroxide. Of concern was the fact that there did not appear to be an accumulative effect of the three reaction products over the time course of the experimentation. This phenomenon will be discussed in section 12.2. Pyrocatechol was detected at all concentrations of ferrous sulphate. Graphs were produced for pyrocatechol, 2,3 – DHB and 2,5 – DHB where the x-axis represents the time elapsed in minutes and the y-axis represents the area under the peak for the given end product. These graphs are shown in figures 11.10, 11.11 and 11.12.

800 femtomoles of 3,4 –DHB, 2,5 – DHB and 2,3 - DHB

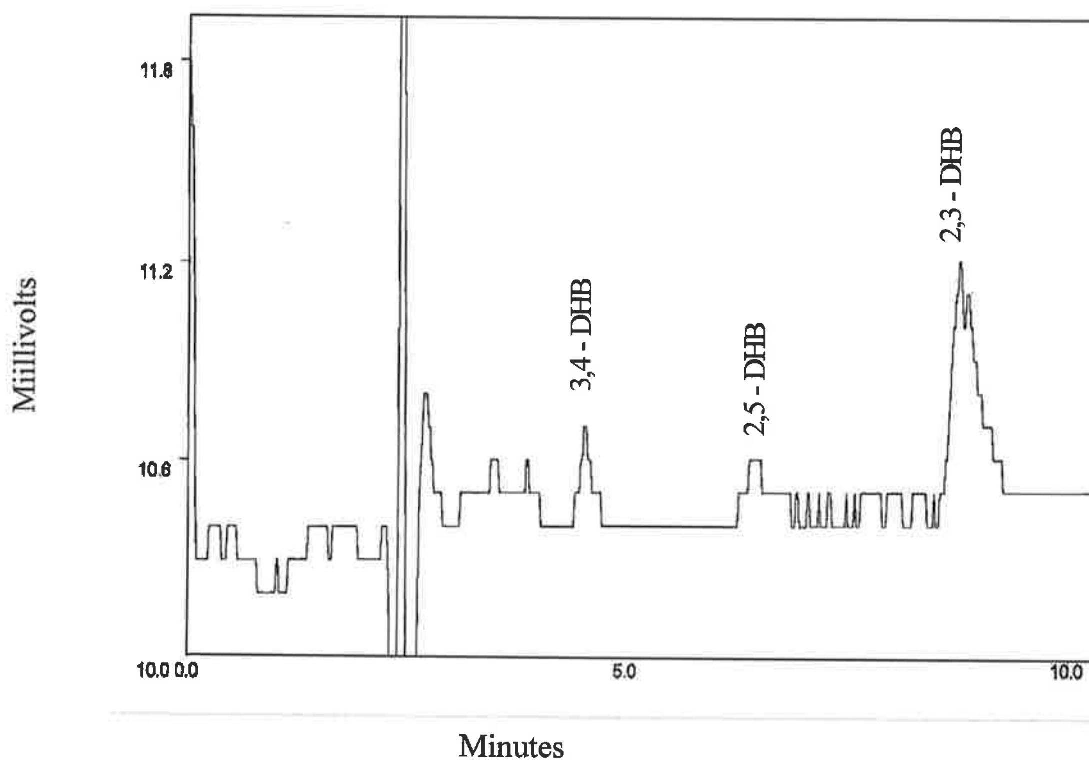


Fig. 11.6 Chromatogram of the internal standard 3,4 – DHB and the reaction products 2,5 – DHB and 2,3 – DHB when 800 fmoles of the stock solution of each was injected onto the column

1 picomole of thiourea

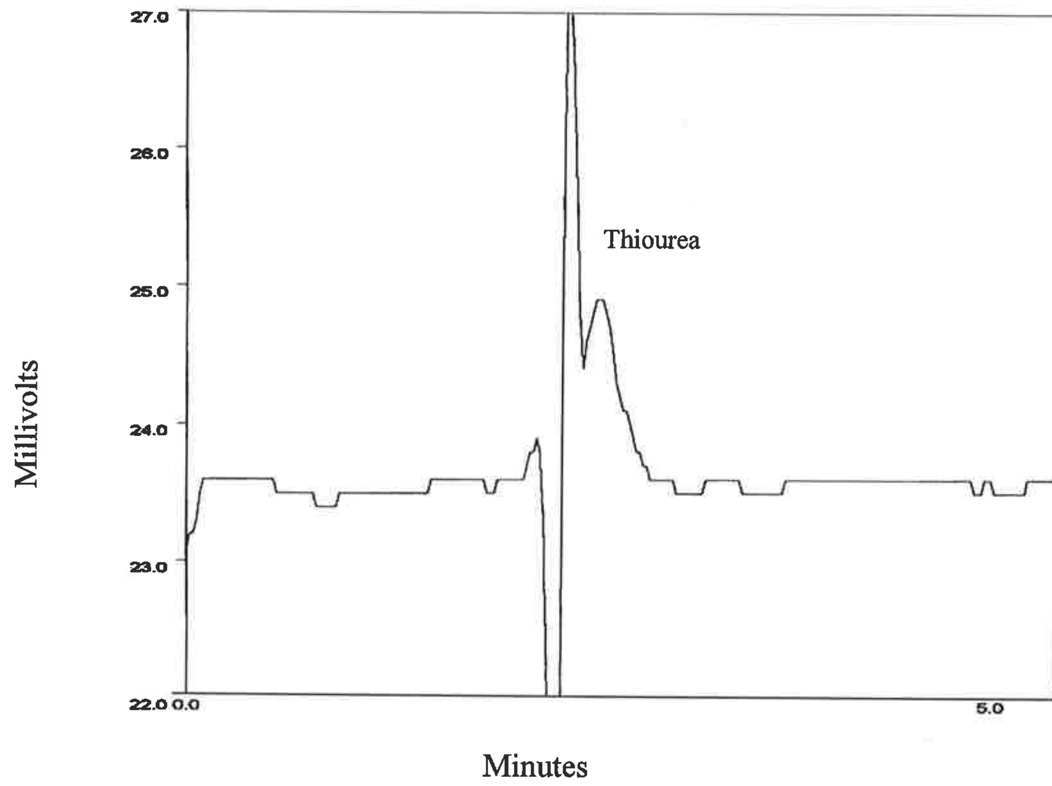


Fig 11.7 Chromatogram of the limit of sensitivity (1 pmole) for thiourea injected onto the HPLC column

30 nanomoles of hydrogen peroxide

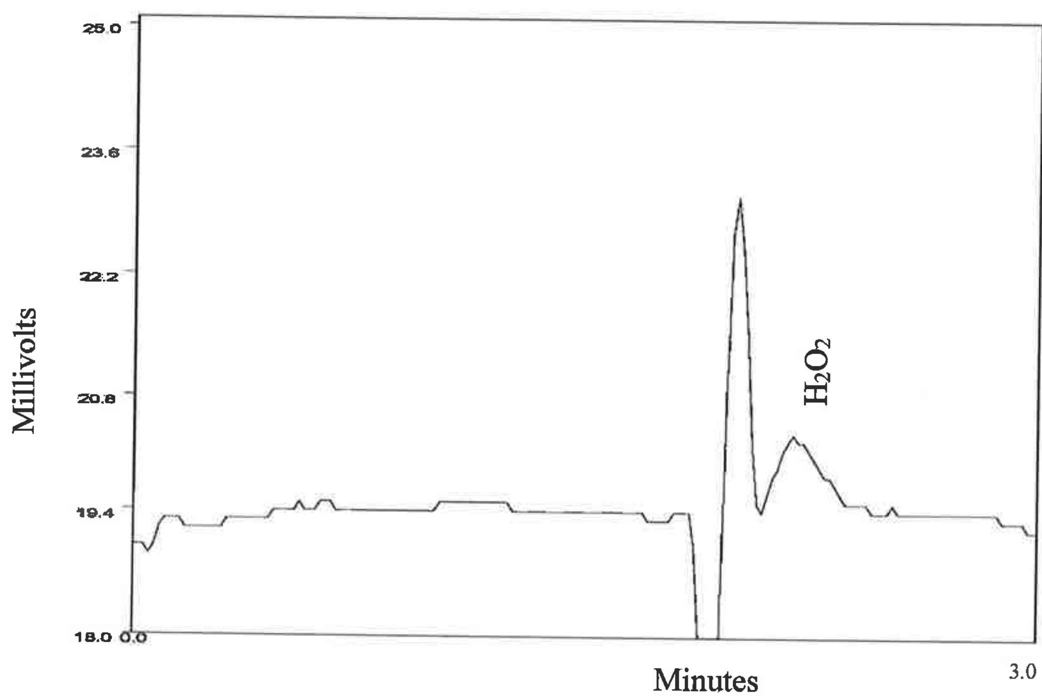


Fig. 11.8 Practical limit of sensitivity (30 nmoles) for hydrogen peroxide injected onto the HPLC – ECD.

Ferrous iron concentration and pyrocatechol production

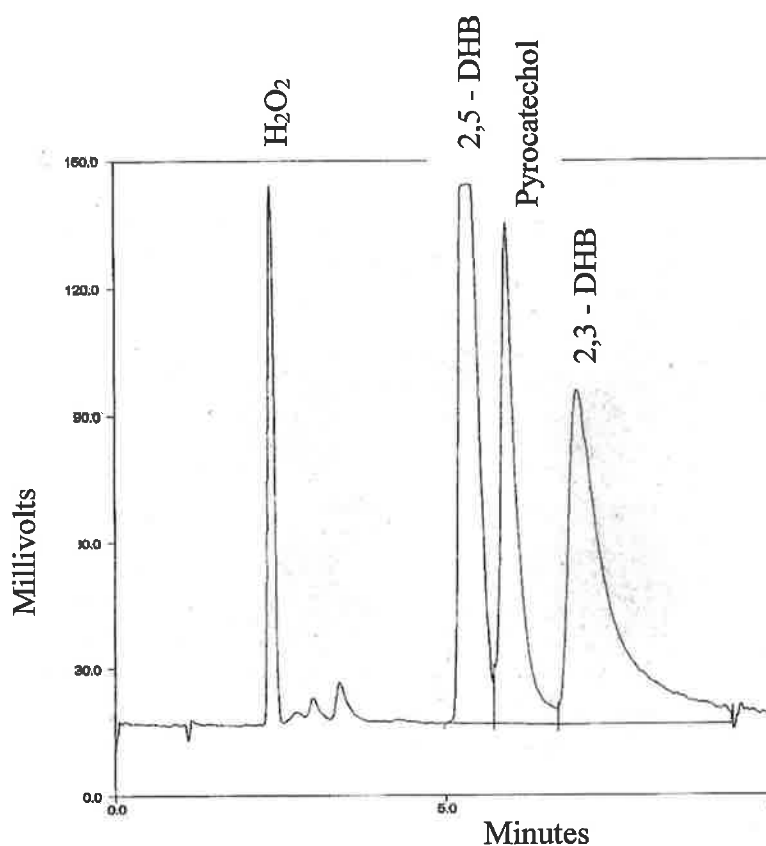


Fig. 11.9 Chromatogram of the control generation of the reaction products of salicylate hydroxylation by hydroxyl radicals generated upon the addition of 0.5ml of 30% H_2O_2 to 500μM $FeSO_4$ in 1 litre of 0.001M sodium salicylate solution.

Ferrous iron concentration and pyrocatechol production

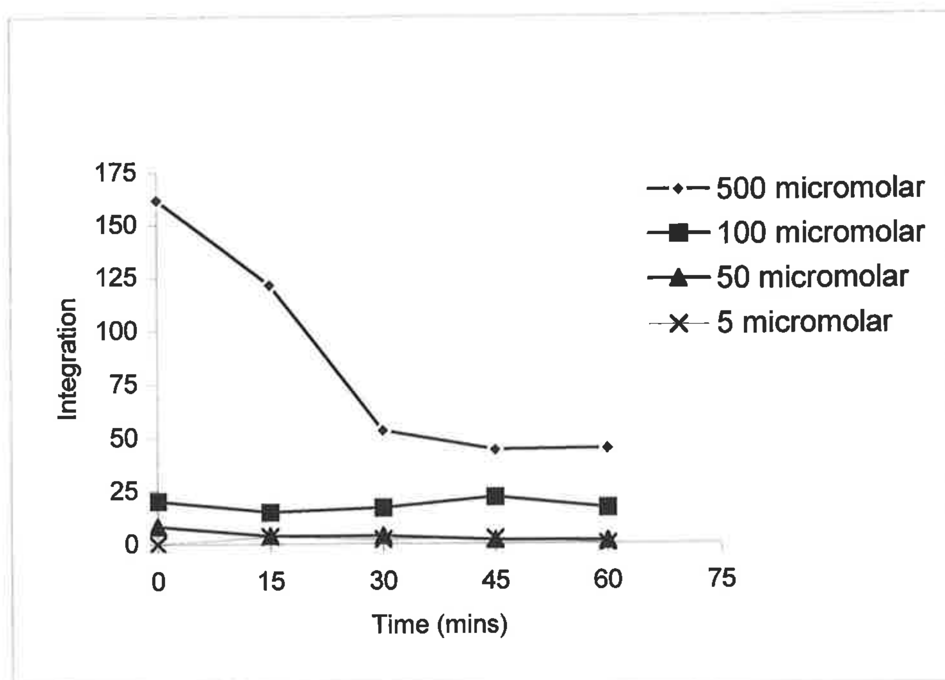


Fig. 11.10 Effect of ferrous iron concentration on the production of pyrocatechol when 20μl of H_2O_2 was injected into a solution of ferrous sulphate and sodium salicylate.

Ferrous iron concentration and 2,3 - DHB production

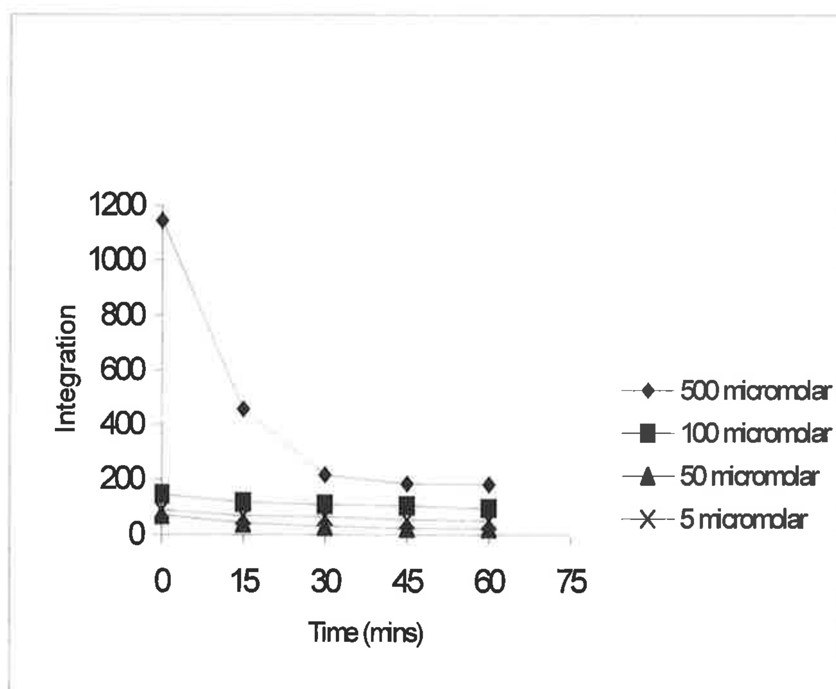


Fig. 11.11 Effect of ferrous iron concentration on the production of 2,3 – DHB when 20μl of H_2O_2 was injected into a solution of ferrous sulphate and sodium salicylate.

Ferrous iron concentration and 2,5 - DHB production

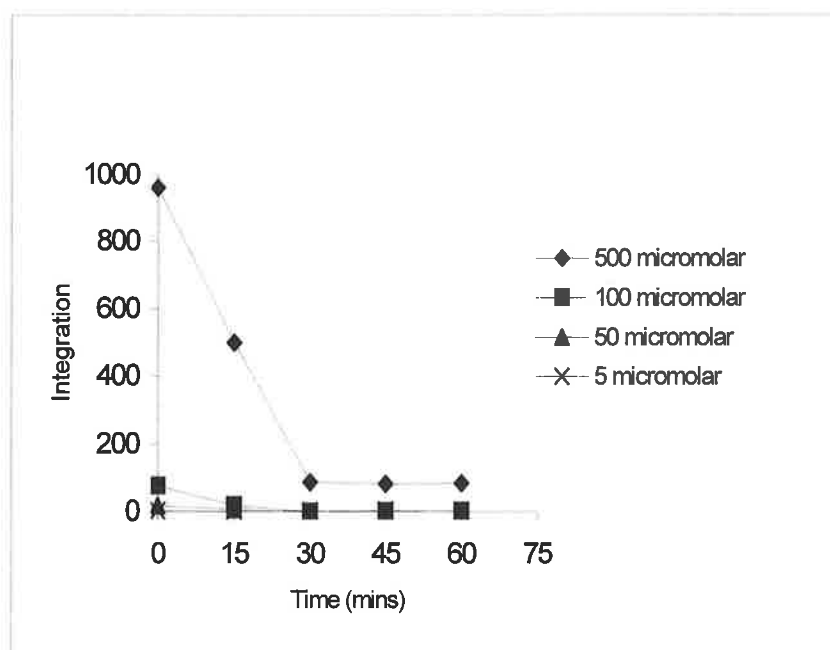


Fig. 11.12 Effect of ferrous iron concentration on the production of 2,5 - DHB when 20μl of H_2O_2 was injected into a solution of ferrous sulphate and sodium salicylate.

11.4 Control generation of thiourea dioxide

A peak was detected (Figure 11.13) with a retention time of 5.85 minutes. This retention time was between that of 2,5 – DHB and 2,3 – DHB but was very close to that of the retention time for pyrocatechol (5.93 mins). As a result the presence of any thiourea dioxide which may be generated on the outer surface of the tooth during the bleaching procedure may not be detected as it will most likely be masked by the generation of pyrocatechol.

11.5 Control generation of other products of hydroxylation of salicylate by hydroxyl radicals

The control generation of potential reaction products other than the three products (2,5 – DHB, 2,3 – DHB and pyrocatechol) were tested to identify if they were separated from the eluent by HPLC. Peaks were detected for each of these reaction products. Table 11.1 outlines the retention times for these peaks and those of the three main reaction products.

Retention time for thiourea dioxide

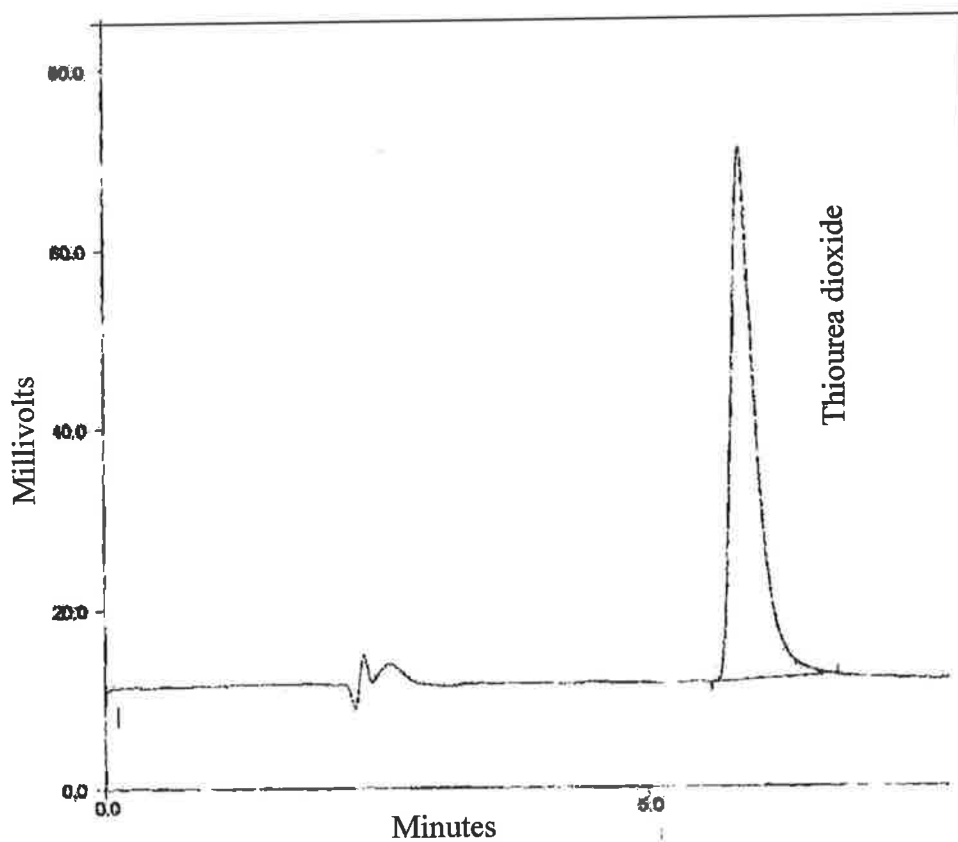


Fig 11.13 Chromatogram of the peak generated when 100 pmoles of thiourea dioxide is injected onto the HPLC column.

11.6 Hydroxyl radical production in bleached root-filled teeth

Integration values for the internal standard 3,4 – DHB were consistent for all samples with only slight fluctuations in the integration values. These fluctuations were deemed insignificant and the sensitivity of the HPLC – ECD and the test conditions were considered standardised for the determination of the quantity of hydroxyl radical production. A second sample of the salicylate bath of each of the teeth was used to calculate the amount of each of the end products of salicylate hydroxylation. This sample was injected immediately after the sample containing the internal standard. This was required due to the possible contamination of the internal standard with 2,3 – DHB and 2,5 – DHB.

The negative control group i.e. non-stained teeth with only a cotton pellet present in the pulp chamber, did not show the presence of any end products of the hydroxylation of salicylate by hydroxyl radicals. This indicates the absence of hydroxyl radicals in the outer radicular dentine and on the outer surface of the tooth. No hydrogen peroxide or thiourea was detected indicating there was no contamination of the control tooth samples with these products.

The amounts of 2,5 – DHB were used to statistically analyse the generation of hydroxyl radicals as this end product was consistently generated in greater quantities than 2,3 – DHB and pyrocatechol. A total of 35 teeth were sampled over a period of 15 hours and the generation of amounts of 2,5 – DHB was measured in picomoles. Each tooth was sampled twice – initially with the internal standard and immediately

after without the internal standard. Figures 11.14 and 11.15 show the relationship of the number of picomoles of each of the two main reaction products within the sample groups.

A total of 22 (62.8%) of the 35 experimental tooth samples generated end products of hydroxyl radical hydroxylation of salicylate. Amounts of 2,5 – DHB ranged from 0.12 pmoles to 21.12 pmoles in each 20μl aliquot. Amounts of 2,3 – DHB ranged from 0.8 pmoles to 18.51 pmoles in each 20μl aliquot. The amount of each of these two reaction products present in the salicylate bath (and therefore the amount of hydroxyl radical generated on the outer surface per tooth) could be calculated by the equation:

$$\frac{\text{No.of pmoles in sample injected onto column} \times (\text{Volume of salicylate bath} \times 10^3)}{\text{Volume of sample}}$$

These results for the reaction product 2,5 - DHB are outlined in Table 11.2. Raw data for each of the test groups is outlined in Appendix 3.

Picomoles/tooth of 2,5 – DHB generated in each sample group

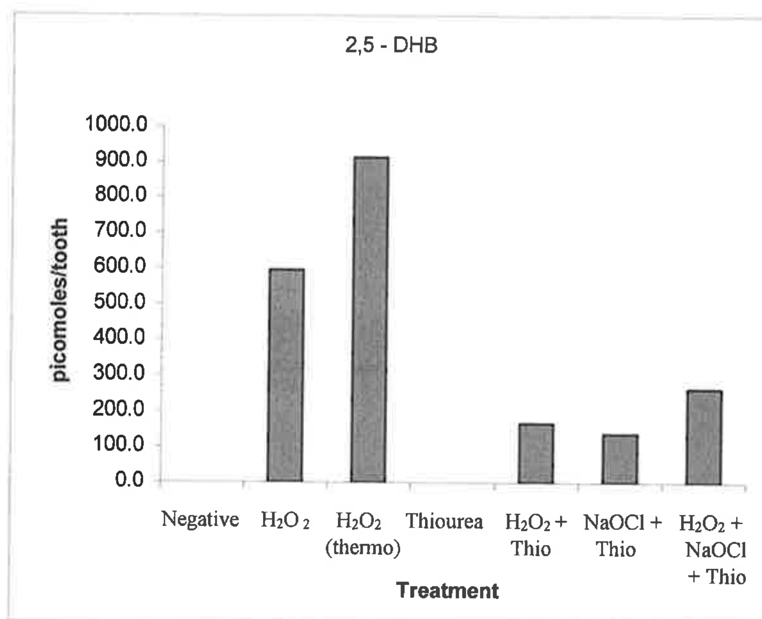


Fig 11.14 Average no. of picomoles/tooth of 2,5 – DHB detected in the salicylate bath for each sample group.

Picomoles/tooth of 2,3 – DHB generated for each sample group

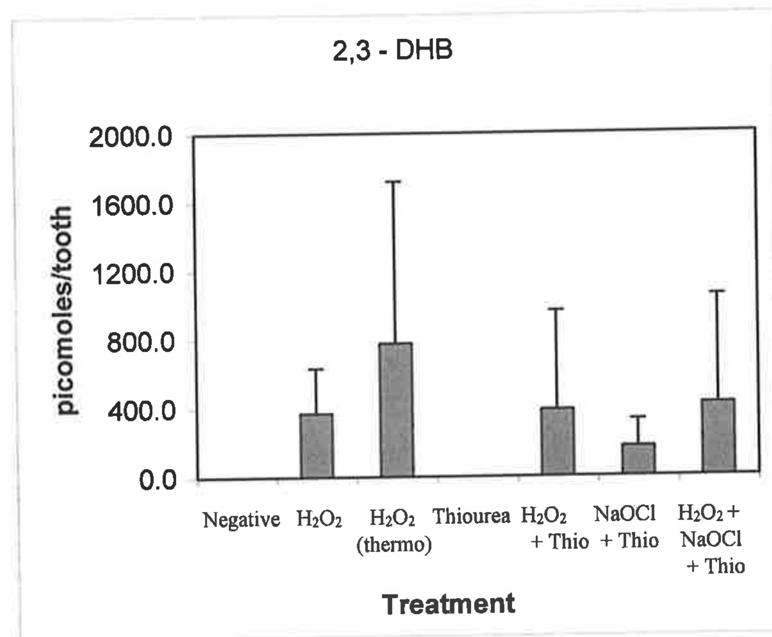


Fig. 11.15 Average no. of picomoles/tooth of 2,3 – DHB detected in the salicylate for each sample group.

Sample No.		Volume (ml)	No. picomoles/sample	average	stdev	No. picomoles/tooth	average	stdev
Negative Control	1	2.80	0.00			0.0		
	2	2.73	0.00			0.0		
	3	2.80	0.00	***0.00***	*0.00*	0.0	***0.0***	*0.0*
	4	3.15	0.00			0.0		
	5	2.70	0.00			0.0		
H2O2	1	2.85	0.83			118.3		
	2	2.65	7.01			928.8		
	3	2.70	0.12	***4.22***	*3.67*	(16.2)	***739.3***	*426.6*
	4	2.70	8.00			1080.0		
	5	2.70	6.15			830.3		
H2O2 (thermo)	1	2.70	0.12			16.2		
	2	2.93	0.00			0.0		
	3	2.85	0.95	***7.11***	*9.66*	135.4	***912.6***	*1220.9*
	4	2.50	21.12			2640.0		
	5	2.65	13.37			1771.5		
Thiourea	1	2.80	0.00			0.0		
	2	2.70	0.00			0.0		
	3	2.85	0.00	***0.00***	*0.00*	0.0	***0.0***	*0.0*
	4	2.65	0.00			0.0		
	5	2.60	0.00			0.0		
H2O2 + Thiourea	1	2.65	0.95			125.9		
	2	2.85	0.00			0.0		
	3	2.60	0.00	***1.22***	*1.88*	0.0	***52.9***	*63.3*
	4	2.60	0.66			85.8		
	5	2.75	4.50			(618.8)		
NaOCl + Thiourea	1	2.58	1.30			167.8		
	2	2.75	1.44			198.0		
	3	2.65	0.00	***1.01***	*0.59*	(0.0)	***172.2***	*27.9*
	4	2.73	0.99			134.9		
	5	2.85	1.32			188.1		
H2O2, NaOCl + Thiourea	1	2.80	2.06			288.4		
	2	2.60	2.52			327.6		
	3	2.58	1.40	***1.95***	*0.68*	180.3	***262.9***	*97.5*
	4	2.80	2.67			373.8		
	5	2.60	1.11			144.3		

Table 11.2 Raw data for the generation of 2,5 – DHB from each sample group expressed in pmoles/sample and pmoles/tooth.

The ratio of 2,5 – DHB : 2,3 – DHB was determined for each of the sample groups and the ratios are displayed in Table 11.3. The ratio of 2,5 – DHB to 2,3 – DHB was approximately 1.6:1.0 for each group without thiourea. The ratio of these two end products when thiourea was included in the bleaching protocol was approximately 0.6:1.0. Therefore the amount of 2,5 –DHB being generated was decreased by almost two-thirds of that which was generated when thiourea was not present.

Pyrocatechol was found to be present in a total of 15 of the 35 samples injected across the column. Pyrocatechol was found to be generated in all teeth in which thiourea had not been introduced (Figure 11.16). In teeth into which thiourea had been introduced as part of the bleaching protocol a total of 5 of a possible 20 samples generated pyrocatechol. In the 5 teeth where thiourea was included in the bleaching protocol there was evidence of a high radical flux as shown by the production of relatively large amounts of 2,5 – DHB and 2,3 – DHB. As a result it can be assumed that the generation of pyrocatechol was only evident in the presence of high radical flux. No evidence of other potential reaction products was detected.

The application of heat to activate the hydrogen peroxide resulted in an increase in the amount of 2,5 – DHB in the salicylate bath of the tooth samples in group 3 when compared to group 2 (Appendix 4). However, due to the large standard

Sample Group	*Ratio of 2,5 – DHB : 2,3 - DHB*
H_2O_2	1.6 : 1.0
H_2O_2 (Thermocatalytic)	1.22 : 1.0
Thiourea	0
H_2O_2 and Thiourea	0.43 : 1.0
NaOCl and Thiourea	0.80 : 1.0
H_2O_2, NaOCl and Thiourea	0.6 : 1.0

Table 11.3 Ratio of 2,5 – DHB to 2,3 – DHB in each of the sample groups

Picomoles /tooth of pyrocatechol in each sample group

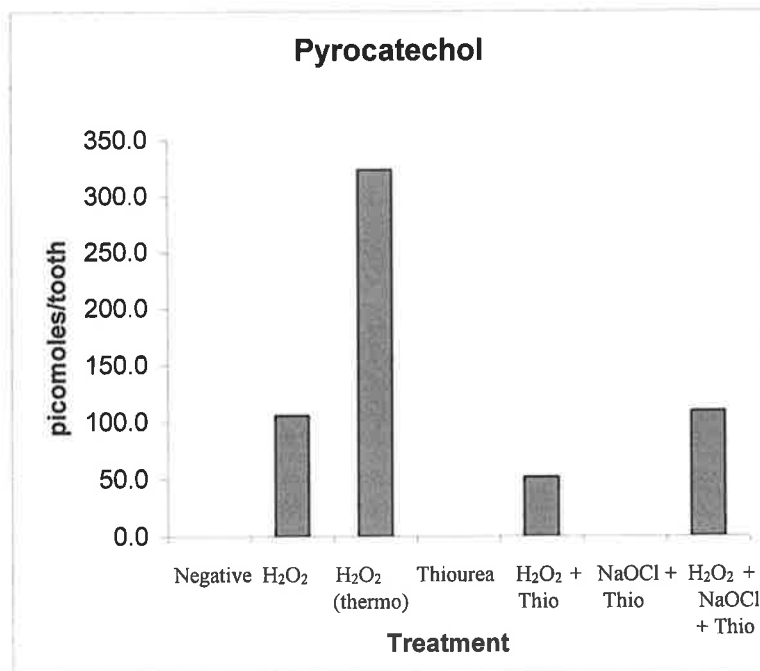


Fig 11.16 Average no. of picomoles/tooth of pyrocatechol detected in the salicylate bath for each sample group.

deviation values of these two groups the difference was not statistically significant at the 5% probability level. The average generation of 2,5 –DHB on the outer surface of the tooth in group 2 was 739.34 pmoles per tooth (4.42 pmoles per 20μl aliquot) compared to the average generation of 912.6 pmoles per tooth (7.11 pmoles per 20μl aliquot) in group 3 (Table 11.2).

The amount of hydrogen peroxide which was detected in the salicylate bath for each sample group was measurable in nanomoles (nmoles)/tooth. The amount of hydrogen peroxide that was detectable in each group can be seen in Fig 11.17. Group 2 samples provided the greatest quantities of hydrogen peroxide (205047 nmoles/tooth) which was significantly greater than the number of nanomoles detectable in any of the groups containing thiourea (Appendix 4). It can be concluded from these results that thiourea has the ability to scavenge hydrogen peroxide.

The difference in the amounts of hydrogen peroxide detected in groups 2 and 3 were statistically significant (Table 11.7). There was a real decrease in the amount of detectable hydrogen peroxide when heat was used to activate the hydrogen peroxide. This decrease would be expected as the heat catalyses the breakdown of hydrogen peroxide. However, the amount of hydrogen peroxide which diffused across the dentine to the outer root surface in the thermocatalytically bleached teeth (Group 3) was not significantly different from those groups in which thiourea was introduced into the bleaching protocol (Appendix 4).

Nanomoles of H_2O_2 /tooth present in each group

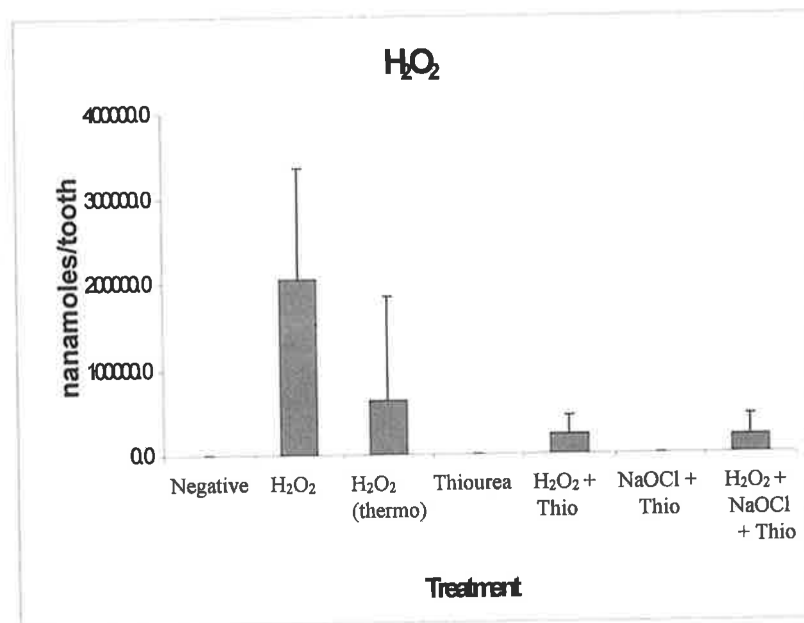


Fig 11.17 Average no. of picomoles/tooth of hydrogen peroxide detected in the salicylate bath for each sample group.

The detectable quantities of thiourea present in the sample groups are seen in Fig. 11.18. There was a statistically significant decrease in the amount of thiourea detectable in the salicylate bath between group 4 and groups 5 – 7 (Appendix 4). This is most likely due to the scavenging effects of the thiourea. As hydrogen peroxide and/or sodium hypochlorite was introduced into the bleaching protocol the amount of thiourea detectable decreased. The least amount of detectable thiourea was in group 7 when both H_2O_2 and NaOCl were present in the pulp chamber and therefore a greater amount of thiourea was consumed during the scavenging of H_2O_2 and hydroxyl radicals within the dentinal tubules.

The ability of thiourea to scavenge hydroxyl radicals present on the outer surface of the tooth or from within the outer layers of radicular dentine should be evidenced by a decrease in the detectable amounts of 2,5 – DHB. Group 5 (H_2O_2 - thiourea) results indicate a statistically significant ($P<0.05$) decrease in the generation of 2,5 – DHB on the outer surface of the tooth compared to group 2 (H_2O_2), as indicated by the decreased detection of this end product in the salicylate samples (Appendix 4). Chromatograms showing the peak heights of the two reaction products of salicylate hydroxylation for group 2 (H_2O_2) and for group 5 (H_2O_2 – thiourea) are present as Figures 11.19 and 11.20 respectively. However, no significant difference was found between group 3 (hydrogen peroxide – thermocatalytic bleaching) and group 5 (Appendix 4).

Nanomoles of thiourea /tooth in each sample group

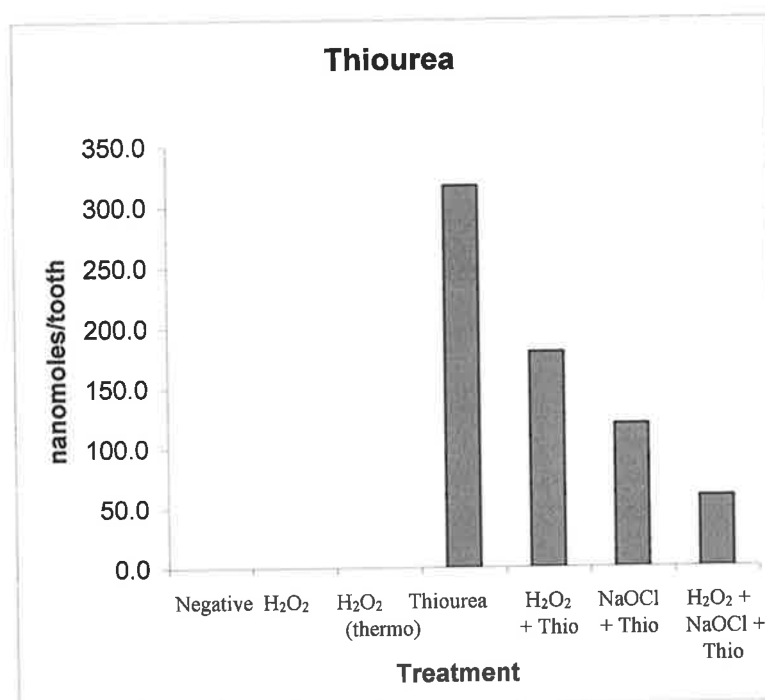


Fig 11.18 Average no. of picomoles/tooth of thiourea detected in the salicylate bath for each sample group.

Chromatogram of Group 2 – H_2O_2

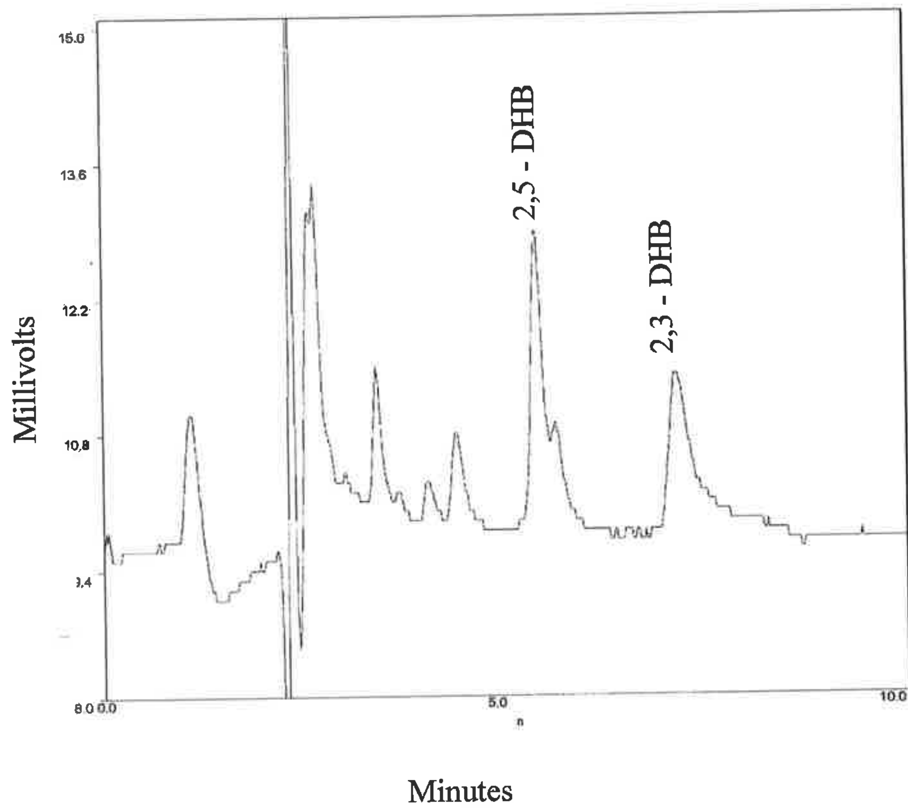


Fig. 11.19 Chromatogram of the introduction into the pulp chamber of hydrogen peroxide and the resultant generation of the reaction products 2,5 – DHB and 2,3 - DHB

Chromatogram of Group 5 – H_2O_2 and Thiourea

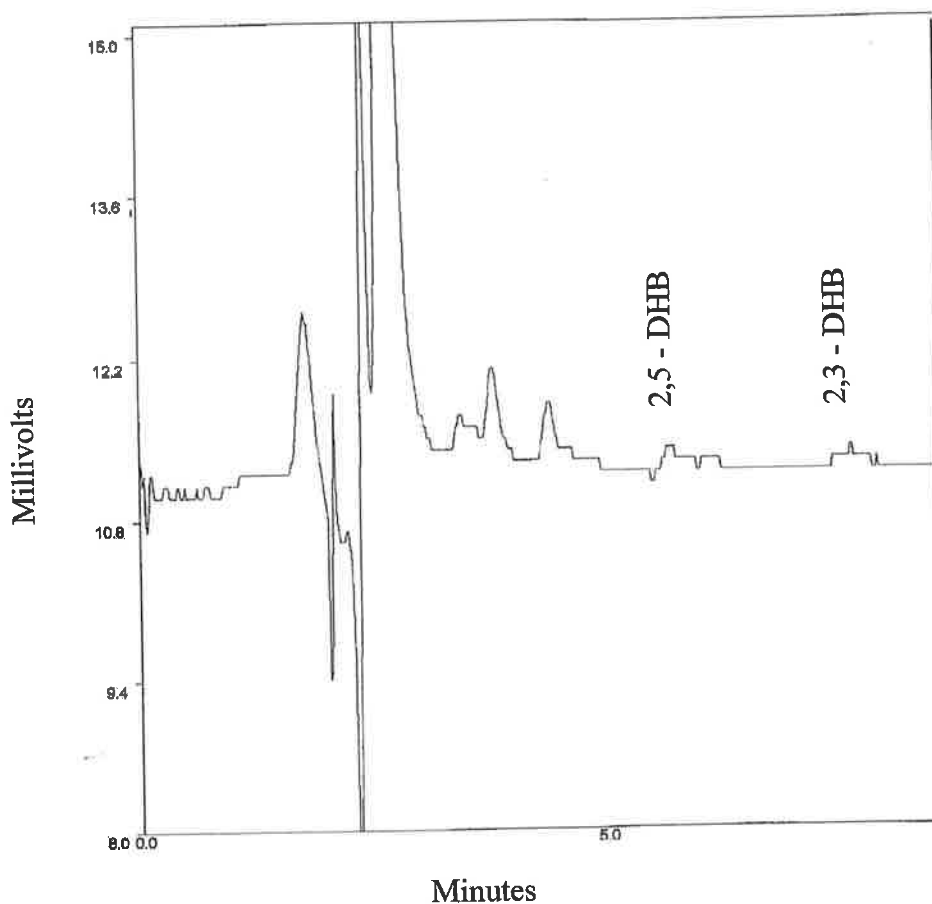


Fig. 11.20 Chromatogram of hydrogen peroxide and thiourea introduction into pulp chamber and the resultant generation of the reaction products 2,5 – DHB and 2,3 – DHB

Group 7 results indicate a reduction in the generation of 2,5 – DHB when compared to those of group 2 (H_2O_2) and group 3 (H_2O_2 – thermocatalytic bleaching). Neither reduction was statistically significant at the 5% level (Appendix 4). The reduction in the generation of these end products is not as great as those of group 5 and the inclusion of NaOCl into the bleaching protocol may have influenced the results. It can be seen from the results displayed in Figs. 11.14 and 11.15 and Table 11.2, that the presence of sodium hypochlorite was capable of generating small amounts of the reaction products, indicative of hydroxyl radical production.

The ability of thiourea to scavenge hydrogen peroxide was statistically significant at the 5% level. There was a significant reduction in available hydrogen peroxide on the outer surface of the tooth following the introduction of thiourea into the bleaching protocol. This was found for group 5 (where H_2O_2 was the active oxidising agent and potential source of hydroxyl radicals) and in group 7 (where both H_2O_2 and NaOCl were the active oxidising agents and potential sources of hydroxyl radicals). This scavenging ability of thiourea has clinical significance that will be discussed in section 12.7.

The presence of sodium hypochlorite in the pulp chamber produced small but significant amounts of hydroxyl radicals on the outer surface of the teeth in group 6. This significance was noted when the amount of hydroxyl radicals detected was compared in groups 5 and 6. The amount of 2,5 – DHB present on the outer root surface in group 5 (H_2O_2 and thiourea) was significantly less than that found in group 6 (NaOCl and thiourea) (Appendix 4).

12.0 Discussion

12.1 Assay Sensitivity

The degree of sensitivity demonstrated by the HPLC – ECD in this research project was found to be in accordance with that found by Dahlstrom *et al.* (1997) and Wright (1988). Sensitivity assays were carried out on the standards 2,5 – DHB, 2,3 – DHB and 3,4 – DHB as well as pyrocatechol, hydrogen peroxide and thiourea. The degree of sensitivity obtainable for the 2,5 – DHB, 2,3 – DHB and the 3,4 – DHB was 800 femtomoles. The degree of sensitivity for the pyrocatechol and thiourea was 1 picomole. These sensitivities are expected using electro-chemical detection and exemplify the extreme sensitivity of the instrumentation used. The degree of sensitivity obtainable for the hydrogen peroxide was not as good as that for the other standards with a limit of 30 nanomoles obtained. Each sensitivity assay completed used a different batch of mobile phase. As a result, the retention times for the reaction products were slightly different for each assay. Standards taken from a stock solution of each of the reaction products was injected onto the column prior to the sensitivity assay commencing so as to ascertain the retention times for these reaction products.

12.2 Control generation of hydroxyl radicals

The control generation of hydroxyl radicals resulted in the production of all three reaction products of salicylate hydroxylation by hydroxyl radicals as demonstrated by Halliwell and Gutteridge (1986). The ratio of the amounts of each of these reaction products generated in the control experiments was consistent. Whenever hydrogen peroxide was introduced into the test solution the ratio of the end products was approximately 2 : 1 for 2,5 – DHB : 2,3 – DHB. This differed to the ratio of these two major end products in the test samples where the ratio was closer to 1.5 : 1 for 2,5 – DHB : 2,3 – DHB. The presence of greater quantities of the 2,5 – DHB compared to the quantity of 2,3 - DHB in the test samples in which thiourea was not present (Groups 2 and 3) was consistent with the results of the control groups.

The ratio of these two reaction products was not influenced by the presence of thiourea in the control groups, however, this ratio was influenced in the test samples where the amount of 2,5 – DHB generated was reduced in the presence of thiourea. The most likely reason for this is that the addition of thiourea to a one litre solution of salicylate prior to the addition of hydrogen peroxide allows the thiourea to dissociate before it has the opportunity to react with and scavenge hydrogen peroxide. This leaves hydrogen peroxide, after it is added to the control solution, to react with the available iron and salicylate virtually unimpeded. The thiourea would therefore have little effect on the generation of hydroxyl radicals in the control solution. However, in the confines of the pulp chamber, the thiourea

would have greater opportunity to react with hydrogen peroxide and generated hydroxyl radicals and therefore the scavenging effect is more pronounced.

The effect of the ferrous ion concentration on the generation of the reaction products of hydroxyl radicals and sodium salicylate revealed that only a concentration of 500μM $FeSO_4$ produced reaction products at sufficient quantities. It is most likely that the $FeSO_4$ at concentrations of 100μM, 50μM and 5μM were insufficient in the presence of 20μl of 30% hydrogen peroxide. Of concern is the fact that each of the products of the reaction between the hydroxyl radicals and sodium salicylate did not accumulate over the hour time course. It is known that the hydroxyl radical is not stable. The reaction products of the salicylation of hydroxyl radicals may likewise be unstable and therefore explain the lack of retention of these reaction products over the time course of the experimentation. If this is the case, the amount of 2,5 – DHB that was measured in the salicylate bath of the test models at 48 hours post-bleaching may be an under-estimation of the true generation of hydroxyl radicals in the bleaching of blood-stained, root-filled teeth.

Pyrocatechol was generated in each of the control group solutions due to the high radical flux generated. Sodium hypochlorite had the ability to react with ferrous iron to generate hydroxyl radicals. The quantity of hydroxyl radicals generated was minor but this small amount must bring into question whether the presence of sodium hypochlorite in the bleaching protocol is warranted. The small effect gained from the inclusion of sodium hypochlorite needs to be questioned when this

solution has the potential to generate hydroxyl radicals in the presence of ferrous iron.

12.3 Presence of other reaction products

The retention times for both phloroglucinol and resorcinol are very similar to those of the peaks generated for hydrogen peroxide and thiourea when these two compounds are included in the bleaching protocol. The large amounts of hydrogen peroxide and thiourea that were able to pass through to the outer surface of the tooth and thereby generate large peaks would almost certainly eliminate the potential for either of these two reaction products to be detected on the resulting chromatogram. This is especially relevant considering the production of either of these reaction products are most likely to occur during times of high radical flux. Such a high radical flux will only occur in these test conditions when hydrogen peroxide is introduced into the bleaching protocol.

The generation of either hydroquinone or pyrogallol, both of which had retention times between that of the hydrogen peroxide and the internal standard 3,4 – DHB, would allow their detection on the resultant chromatogram if they were produced during the hydroxylation of salicylate by hydroxyl radicals. Hydroquinone is not considered a reaction product of the hydroxylation of salicylate by hydroxyl radicals. It can therefore be assumed that any peak detected on the chromatograms which is found between hydrogen peroxide or thiourea and either the internal standard (3,4 – DHB) if present or the reaction product 2,5 – DHB, would represent

the production of pyrogallol (1,2,3 – THB). Pyrogallol is formed by following attack on 2,3 –DHB by hydroxyl radicals where a carboxyl group replaces a hydroxyl group.

12.4 Hydroxyl radical production in bleached root-filled teeth

The internal standard 3,4 – DHB was selected as it was deemed unable to be produced during the hydroxylation of salicylate by hydroxyl radicals. Grootveld and Halliwell (1986) used 3,4 – DHB as the internal standard and found no interference of the peak generated by this internal standard and the retention times of the end products. Likewise, no interference between retention times was found in the results of this study using 3,4 – DHB as the internal standard. Dahlstrom *et al.* (1997) suggested the possibility of using 3,4 –DHB as the internal standard after the authors found that the internal standard used in their research, homogentisic acid (HGA), had a retention time very close to that of 2,5 – DHB. Small fluctuations were detected between the internal standard samples injected onto the column. These fluctuations may have been due to extremely small variations in the volume of the internal standard injected.

The decision to use 2,5 – DHB to measure hydroxyl radical generation was based on the fact that this end product was consistently generated in greater amounts throughout the experimental tooth samples and the control experiments.

condense the root-filling material using both lateral and vertical condensation.

The generation of hydroxyl radicals from teeth in group 2 was not significantly different at the 5% probability from that generated in group three. The application of heat to hydrogen peroxide in the pulp chamber has been used for many years in order to activate the hydrogen peroxide. Tronstad (1988) reported that the application of heat to hydrogen peroxide had two effects: firstly, catalysis of the breakdown of the hydrogen peroxide into its unstable oxidising components; secondly, the imparting of energy to the bleaching solution which may cause it to expand and diffuse more effectively into the dentine tubules of the stained tooth structure. The increased activation of the hydrogen peroxide by heat catalysing its breakdown would suggest a lesser amount of hydroxyl radicals on the outer surface of the tooth due to the extreme reactivity of the hydroxyl radicals which ensures they react with the first ion they encounter. However, Pashley (1983) demonstrated that by increasing the temperature of the radicular dentine by 40^{0}C the hydraulic conductance of the dentine increased 1.8 fold in dentine which had not been etched and 4-fold in dentine which had been etched. The authors concluded that the increased hydraulic conductance evidenced could not be accounted for by a decrease in viscosity in the fluid within the dentinal tubules alone. Thermal expansion induced increases in the tubular diameter were partially responsible for the witnessed increase in hydraulic conductance. Outhwaite *et al.* (1976) found that an increase of 10^{0}C almost doubled dentin permeability. Rotstein *et al.* (1991) reported that higher bleaching temperatures increased the hydrogen peroxide radicular penetration. Rotstein *et al.* found that raising the temperature from 24^{0}C to 37^{0}C

doubled the hydrogen peroxide penetration. In a study on the effects of bleaching agents on the permeability of dentine to bacteria, Heling *et al.* (1995) found hydrogen peroxide increased significantly the ability of bacteria to penetrate through dentine tubules. It was suggested that the ability of hydrogen peroxide to alter the ratio between the organic and inorganic components of the dentine as well as the physical properties of the dentine by reducing its hardness may influence this increase in permeability (Lewinstein *et al.* 1994).

Thus, the application of heat would increase the activation of the hydrogen peroxide catalysing its breakdown and at the same time increase the permeability of the radicular dentine. This would explain the lesser amounts of hydrogen peroxide detected on the outer surface of the tooth in group 3. However, there was an increased amount of hydroxyl radicals detected in the outer layers of radicular dentine and on the outer surface of the tooth in this group. This may not have been expected as the application of heat would suggest increased breakdown of hydrogen peroxide and greater production of hydroxyl radicals closer to the pulp – dentine interface which would then react with components of the dentine and avoid detection on the outer surface of the tooth. However, hydrogen peroxide not immediately broken down into its unstable oxidising components would be capable of more readily diffusing through the expanded dentinal tubules towards the outer surface of the tooth. Likewise, salicylate would be able to more readily diffuse into the tooth to react with the hydroxyl radicals produced in the outer layers of the dentine. These reaction products would then be free to diffuse back out of the tooth into the salicylate bath and be detected as a result of the increased permeability of

the dentinal tubules.

The control generation of hydroxyl radicals using different concentrations of ferrous (Fe^{2+}) ions revealed that most of the reaction occurred in the first 30 minutes. As a result, it could be expected that most of the hydroxyl radicals generated in group 3 following the application of heat would occur within the first thirty minutes. The protocol used for group 3 in this research was to thermocatalytically bleach the blood-stained root-filled teeth and then place the tooth into the salicylate bath. This may have affected the overall yield of hydroxyl radicals able to be detected in the salicylate bath. It may therefore be assumed that the yield of hydroxyl radicals detected on the outer surface of the teeth in group 3 is a minimum detectable amount due to the methodology employed in this research project emphasising the importance of the ability of thiourea to scavenge hydroxyl radicals on the outer surface of the tooth.

The increased production of hydroxyl radicals on the outer surface of the tooth when heat was used to activate the hydrogen peroxide supports the theory that these highly reactive oxygen species may play a role in the initiation of bleaching induced external cervical resorption – referred to as invasive cervical resorption. The highly reactive nature of the hydroxyl radical and its reported toxicity on the major components of the periodontal tissues is well documented and described in Section 4.0. The presence of these radicals within the periodontal tissues will result in injury to these soft tissues and the associated inflammatory response that is capable of activating clastic cell activity.

Thiourea was able to reduce the amount of hydroxyl radicals on the outer surface of the tooth and to reduce the amount of hydrogen peroxide able to diffuse through the dentine from the pulp chamber to the outer surface of the tooth. The average amount of 2,5 – DHB detected within the salicylate bath per tooth in groups 5 and 7 (where thiourea was placed into the tooth prior to the introduction of hydrogen peroxide and/or sodium hypochlorite) was significantly lower than in the absence of the thiourea (groups 2 and 3). The average amount of 2,3 – DHB present in the salicylate bath per tooth in groups 5 and 7 was lower than that of groups 2 and 3, however the reduction was not as great as that found for 2,5 – DHB. Pyrocatechol was found to be present in group 2 and group 3 (in those teeth where high radical flux was found). However in groups 4 – 7, where thiourea was introduced only 5 of 29 teeth exhibited production of pyrocatechol. These teeth also exhibited significantly higher amounts of the reaction products 2,5 – DHB and 2,3 – DHB than other teeth in the respective groups. The influence of thiourea in these teeth was limited and raises questions about the methodology in these individual samples. As a result, the presence of pyrocatechol was not deemed significant in these teeth. The method by which thiourea inhibits the production of 2,5 – DHB, 2,3 – DHB and pyrocatechol has not been reported in the literature. The selective inhibition of the production of 2,5 – DHB by thiourea may explain the greater reduction in this reaction product. Thiourea would have to selectively block or compete for the –5' para position of the aromatic ring of the salicylate thereby preventing the hydroxylation of salicylate at the –5' position. However, if this occurred greater amounts of 2,3 – DHB and pyrocatechol could be expected to be produced. This was not demonstrated. A more likely method of inhibition of the hydroxyl radical

activity by thiourea is the reaction between thiourea and the hydroxyl radical and between thiourea and hydrogen peroxide. The reaction product 2,5 – DHB was consistently produced in greater quantities in groups 2 and 3 and therefore a reduction in available hydroxyl radicals and hydrogen peroxide would result in a more significant reduction in the reaction product 2,5 – DHB. Such a method of inhibition would also account for the reduction in 2,3 – DHB and pyrocatechol seen within the groups. A reduction in hydrogen peroxide resulting from the direct scavenging actions of thiourea ultimately results in a lesser amount of hydrogen peroxide available for Fenton reactions and therefore decreased production of hydroxyl radicals. The more marked decrease in 2,5 – DHB may result from a greater affinity for hydroxyl radical attack on the –3' site of the aromatic ring of the salicylate molecule, and therefore in times of low radical flux greater production of 2,3 – DHB might be expected to occur.

Goldman *et al.* (1981) found that sodium hypochlorite alone does not remove smear layer from within the pulp canal. Fogel and Pashley (1990) likewise found that the application of sodium hypochlorite to dentine slabs had no effect on the hydraulic conductance. This was due to the inability of the hypochlorite to remove smear layer from the surface of each of the dentine slabs. As a result the use of sodium hypochlorite to remove the smear layer prior to the bleaching has no real effect and is not recommended. However, sodium hypochlorite has been proven to be effective in the removal of organic debris from root canals (Byström and Sundqvist, 1983) and has been used as an irrigant in root canal therapy for many years. The aim of including sodium hypochlorite in the bleaching protocol was therefore to aid in the

removal of residual organic debris from within the pulp chamber left behind following the mechanical removal of the pulp tissue. Group 6 of the experimental procedure showed that sodium hypochlorite was capable of generating hydroxyl radicals. The amount of hydroxyl radicals produced on the outer surface of the tooth by sodium hypochlorite, in real terms, was less then that generated by the introduction of hydrogen peroxide. However, the amount of detectable hydroxyl radicals on the outer surface of the tooth was significantly less for group 5 (hydrogen peroxide and thiourea) than group 6 (hypochlorite and thiourea). This may be explained by the ability of thiourea to scavenge hydrogen peroxide and therefore limit the amount of hydroxyl radical production on, or close to the outer surface of the tooth. However, thiourea may have a lesser capacity to scavenge hypochlorite allowing greater volumes of it to diffuse through to the root surface.

The combination of sodium hypochlorite and hydrogen peroxide following the introduction of thiourea into the pulp chamber (Group 7) resulted in a statistically significant greater amount of hydroxyl radicals generated compared to the introduction of hydrogen peroxide and thiourea (Group 5). The significant effect of the introduction of sodium hypochlorite on the production of hydroxyl radicals on the outer surface of the tooth suggests that the efficacy of this irrigant in the removal of residual organic debris from the pulp chamber may be out-weighed by its capacity to generate hydroxyl radicals and therefore its use in the intra-coronal bleaching of root-filled teeth should be reconsidered. Copious irrigation of the pulp chamber with saline to remove all residual sodium hypochlorite is recommended following its application if its use is required.

12.5 Hydrogen peroxide diffusion

The passage of hydrogen peroxide across the tooth was a significant finding. Rotstein *et al.* (1991) found that hydrogen peroxide was able to diffuse through radicular dentine from the pulp chamber to the outer surface of the tooth. Dahlstrom *et al.* (1997) found that out of a total of 40 teeth thermocatalytically bleached with 30% hydrogen peroxide, hydrogen peroxide was found to diffuse through to the outer surface in 5 of these teeth. The results indicated that whenever hydrogen peroxide was introduced into the pulp chamber significant amounts were detectable on the outer surface of the tooth after 48 hours. These results differed to those found by Dahlstrom *et al.* (1997) and Rotstein *et al.* (1991) in two ways: (1) Both previous studies involved the creation of artificial defects on the outer surface of the tooth in the region of the CEJ. Teeth prepared in this study had the CEJ left as intact as possible. Rotstein *et al.* suggested that the presence of cementum impeded the passage of hydrogen peroxide to the outer surface of the tooth, whilst Dahlstrom *et al.* reported that the creation of artificial defects at the CEJ impeded the passage of hydrogen peroxide. The results evidenced in group 2 would indicate that the presence of intact cementum at the level of the CEJ and along the root surface did not impede the passage of hydrogen peroxide. (2) Dahlstrom *et al.* (1997) prepared the teeth in each group with a 2mm base of Cavit. Rotstein *et al.* (1991) did not use a base but relied on the gutta percha to act as a seal. The latter results found hydrogen peroxide to be present in 100% of the teeth and concluded that the gutta percha seal did not act as a sufficient base to inhibit the penetration of hydrogen peroxide. There is a plethora of research that has examined the ability of gutta

percha and sealer acting as a root-filling material to seal the coronal and apical ends of the root canal. There is no doubt that this root filling has the ability to provide an adequate apical seal (Oliver and Abbott, 1998). However, the same authors have shown that gutta percha and AH26 is not capable of creating an adequate coronal seal for the root canal.

The results of the present research may imply that the gutta percha and sealer do not act as a protective barrier to the penetration of hydrogen peroxide down the root canal. However, further research into the ability of the gutta percha – AH26 seal is required. This finding may aid in explaining why large amounts of hydrogen peroxide were found to pass through the radicular tooth structure to the outer surface of the tooth. The use of a 2mm Cavit base by Dahlstrom *et al.* (1997) may explain why a smaller percentage of the sample group in his study exhibited hydrogen peroxide on the outer surface of the tooth. It would appear that cementum did not act to significantly impede the passage of hydrogen peroxide to the outer surface of the tooth.

As expected, the introduction of heat to the hydrogen peroxide catalysed the breakdown of the hydrogen peroxide into its unstable oxidising components. As a result, the amount of hydrogen peroxide present on the outer surface of the tooth was different between groups 2 and 3 when viewing the raw data but due to the degree of variance between samples within each of the two groups the results were not significantly different at the 5% level. Whilst the application of heat will enable available hydrogen peroxide to penetrate further into the tooth, it will become

increasingly unstable and breakdown prior to reaching the outer surface of the tooth. This may explain the greater amount of hydroxyl radical reaction products present in salicylate and the decreased amount of hydrogen peroxide detected in the salicylate.

Fibroblasts are the predominant cell type in gingival connective tissue and within the periodontal ligament. They are responsible for the maintenance and integrity of these tissues. Tipton *et al.* (1995) studied the effects of hydrogen peroxide on human gingival fibroblasts with a specific reference to the viability and the proliferation of the cells, and the production of fibronectin and collagen types I, III and IV. Fibronectin, a glycoprotein, is produced by fibroblasts. It is responsible for mediating the attachment of cells to collagen and other extracellular matrix components. The authors found that hydrogen peroxide at concentrations of 0.0125% or greater, resulted in death of the cells. Concentrations of the hydrogen peroxide as low as 0.006% were sufficient to reduce fibroblast cell proliferation and decrease collagen type I production. Production of fibronectin and collagen types I and III was significantly reduced at concentrations $\geq$ 0.017%. The authors concluded that *in vivo*, even in the presence of protective mechanisms, if hydrogen peroxide is capable of gaining access to connective tissue it may adversely affect fibroblasts and their ability to maintain tissue integrity and participate in healing. The results of the current research project indicate that the amount of hydrogen peroxide in the tooth model which is capable of passing across radicular dentine from the pulp chamber to the outer surface of the tooth is capable of causing cell death. By converting the number of moles of hydrogen peroxide present on the outer surface of the tooth to a percentage concentration (equivalent to grams/100ml), a comparison to Tipton's

results are achievable. Group 2 and group 3 produced 0.256% and 0.079% H_2O_2 on the outer surface of the tooth respectively. The presence of thiourea in the bleaching protocol reduced the concentration of H_2O_2 to 0.027% and 0.026% respectively for groups 5 and 7. However, even the presence of thiourea in this study did not reduce the concentration of H_2O_2 to below the lethal dose for fibroblasts. Woolverton *et al.* (1993) in a similar study to that of Tipton *et al.*(1995) determined the ED_{50} value for hydrogen peroxide to be between 5.0 – 10.0mmol/litre. By converting the number of nanomoles of hydrogen peroxide per volume of salicylate to the number of millimoles per millilitre (mmol/ml) the average amount of hydrogen peroxide which was able to diffuse through to the outer surface of the tooth in group 2 was 0.075mmol/ml (equivalent to 75mmol/L). This value is above the ED_{50} value as determined by Woolverton *et al.* (1993) and therefore consideration needs to be given to the use of hydrogen peroxide if the current test conditions were to be used *in vivo*. The quantity of hydrogen peroxide present in the salicylate bath in group 2 may be explained by the removal of the root filling material to 1mm below the level of the CEJ. This is recommended in order to obtain satisfactory bleaching effect in the region of the cervical margin of the tooth. However, in leaving the root filling at this level the passage of hydrogen peroxide to the outer surface of the tooth may be facilitated. The amount of hydrogen peroxide detected in groups 5 and 7, when thiourea was introduced into the bleaching protocol, was significantly less than that generated in group 2 ($P<0.05$). The average quantity of hydrogen peroxide generated in group 5 samples was 0.008mmol/ml (equivalent to 8mmol/L) and the average quantity of hydrogen peroxide generated in group 7 was 0.0077mmol/ml (equivalent to 7.7mmol/L). These values are found within the ED_{50} value range as determined

by Woolverton *et al.* (1993) and are therefore more acceptable levels of hydrogen peroxide to be passing through the radicular dentine from the pulp chamber to the outer surface of the root. Hanks *et al.* (1993) studied the effects of hydrogen peroxide on the inhibition of succinyl dehydrogenase, an enzyme required for cell metabolism. The authors found the 50% inhibitory dose (ID_{50}) to be dose and exposure time dependent. i.e. the longer the hydrogen peroxide was left *in situ* and the greater the original concentration of the hydrogen peroxide the greater the amount of hydrogen peroxide able to diffuse through the dentine. The ID_{50} was approximately 0.58mmol/L, much less then the amount of hydrogen peroxide which was found to diffuse across the tooth in all test samples. Therefore whilst the thiourea has a significant effect on scavenging the hydrogen peroxide present on the outer surface of the tooth, the concentration of hydrogen peroxide which diffuses across the radicular dentine to the outer surface of the tooth remains above the ID_{50} for hydrogen peroxide on fibroblasts. Scope exists for investigation of the relationship between thiourea concentration and its ability to more effectively scavenge hydrogen peroxide.

12.6 Thiourea diffusion

The detection of thiourea on the outer surface of the tooth suggests that any percentage of thiourea introduced into the tooth as part of the bleaching protocol has the potential to diffuse through radicular tooth structure and into the periodontal ligament. Thiourea was found to pass from the pulp chamber or pulp canal to the outer surface of the tooth in 19 (95%) of the 20 teeth. The amount of

thiourea that was evidenced on the outer surface of the tooth was dependent on whether the thiourea was required to scavenge hydroxyl radicals or other oxygen radicals or reactive oxygen species present within the dentinal tubules. The amounts present are directly proportional to the scavenging effect the thiourea had on either the hydroxyl radicals, hydrogen peroxide or sodium hypochlorite. The average amounts ranged from 395.8 nmoles in group 4 to 47.6 nmoles in the teeth in group 5. The passage of thiourea from the pulp chamber to the outer surface of the tooth is of concern due to the toxic nature of thiourea. In several countries thiourea is classified as carcinogenic. The critical factor to consider when discussing the potential toxicity of thiourea is to compare the reported 50% lethal dose of thiourea (LD_{50}) found in animal studies to the amount of thiourea passing to the outer surface of the tooth. The amount of thiourea passing through the radicular dentine to the outer surface of the tooth was greatest in group 4. An average of 395.8 nmoles of thiourea was found in the salicylate bath from this group. Dieke *et al.* (1947) determined the LD_{50} in rats to be 1830mg/kg. Conversion of the number of nanomoles of thiourea present on the outer surface of the tooth to the number of milligrams of thiourea present per tooth reveals that when thiourea was allowed to diffuse through the tooth unhindered (group 4) the amount present was 8.86mg per tooth. This value decreases to 5.1×10^{-3}mg per tooth when hydrogen peroxide is introduced into the bleaching protocol (group 5) and to 1.66×10^{-3}mg per tooth when both hydrogen peroxide and sodium hypochlorite are introduced into the bleaching protocol. In a 70kg person the toxic dose (LD_{50}) described by Dieke *et al.* (1947) would be 128.1g. Therefore, the amount of thiourea present on the outer surface of the tooth when used in

conjunction with oxidative bleaching agents is safe and not deemed toxic to the periodontal ligament.

12.7 Design of the model

The design of this tooth model for the experimentation was based on the work of Dahlstrom *et al.* (1997). The staining of the teeth was achieved using the method Marin *et al.* (1997) described which is an adaptation of the method devised by Freccia and Peters (1982) and which is outlined in section 10.2. The teeth were satisfactorily stained in this manner.

The design of this model differed to that of the model used by Dahlstrom *et al.* (1997) in several ways. Dahlstrom *et al.* prepared small artificial defects on the outer surface of the teeth at the level of the CEJ. This was done in an attempt to study the effect of the role of cementum on the ability of hydroxyl radicals to present on the outer surface of the tooth. Rotstein *et al.* (1991) and Koulaouzidou *et al.* (1996) both showed that the presence of intact cementum played a role in the inhibition of hydrogen peroxide to the outer surface of the tooth during the intra-coronal bleaching of non-vital teeth. However, Dahlstrom *et al.* (1997) found that the creation of artificial defects at the CEJ had no significant effect on the diffusion of hydroxyl radicals to the outer surface of the teeth, and actually found lesser numbers of these radicals on the outer surface of the teeth in that group with cemental defects. Examination under the light microscope of the teeth in this study was to select teeth with intact cementum or as little cemental damage in the region of the

CEJ. This would provide uniformity amongst the test groups and provide the greatest chance of hydroxyl radical penetration through the radicular tooth structure.

The ideal protocol for the intra-coronal bleaching of non-vital teeth is yet to be determined. Such a protocol should be aimed at maximising the efficacy of the bleaching agent, whilst providing reliability in the bleaching efficacy and optimum protection to the adjacent hard and soft tissues. The use of a base is advocated by several authors (Smith *et al.* 1992, Boksman *et al.* 1983, Hansen-Bayless and Davis, 1992, Rotstein *et al.*1992, Ho and Goerig 1989, Walton and Torebinejad, 1989, Pisano *et al.* 1998). These authors found that the use of a base prevented or decreased the penetration of hydrogen peroxide to the outer surface of the tooth. The use of at least 2mm of a base material by these authors was shown to be an effective barrier to the penetration of hydrogen peroxide along the length of the root canal. They found that the root filling material was insufficient for preventing the passage of hydrogen peroxide along the length of the canal. In the current study, in which no base was used and the root filling was left 1mm below the level of the CEJ, hydrogen peroxide was shown to diffuse through the radicular tooth structure in 95% of the teeth into which it was placed. This differs to the results of Dahlstrom *et al.* (1997) who found - in only 5 (12.5%) of 40 teeth bleached with hydrogen peroxide was the hydrogen peroxide able to be detected on the outer root surface. Dahlstrom *et al.* used a 2mm base of Cavit placed 1mm below the level of the CEJ. These differences may in part, be explained by the presence of this base and add support to the argument for the use of a base in the intra-coronal bleaching of non-vital teeth.

Etching the pulp chamber prior to bleaching is thought to aid the penetration of the hydrogen peroxide through the radicular dentine by opening the orifices of the dentinal tubules. Horn *et al.* (1998) and Casey *et al.* (1989) do not advocate the need to etch the pulp chamber prior to the placement of the bleaching agent. These authors found there is no significant difference in the bleaching efficacy when the pulp chambers of extracted teeth were not etched. For this reason the teeth used in this research were not etched prior to placement of the bleaching agent. The presence of a smear layer was shown to have little or no effect on the penetration of hydrogen peroxide through radicular dentine with hydrogen peroxide passing through radicular dentine in 100% of the bleached teeth.

Sodium hypochlorite was used to aid in the removal of organic debris which may be left following extirpation of the pulp. Smear layer is considered to have an organic component, that NaOCl has the ability to remove. EDTA was not considered in the protocol to remove the smear layer. Dahlstrom *et al.* (1997) found the use of EDTA to clean the walls of the pulp chamber increased the production of hydroxyl radicals most probably due to the auto-oxidation of free iron in the tooth structure and consequently questioned the use of this irrigant in the etching of the pulp chamber walls. Although sodium hypochlorite is a bleaching agent, as seen in concentrations of 4.25% (e.g. household disinfectants) the effectiveness of it in bleaching stained dentine has not been fully explored and little research is available on its ability to bleach teeth. In the 1% concentration used in the current research it is most unlikely to have had any bleaching effect and its role in the bleaching of stained teeth is thought to be minimal in the presence of hydrogen peroxide. Maroulis (1994)

reported that the presence of hydrogen peroxide was effective in the intra-coronal bleaching of non-vital teeth regardless of what it was combined with. Any bleaching effect that 1% sodium hypochlorite might produce could therefore be assumed to be of no clinical consequence.

12.8 Implications in Dentistry

The body produces vast amounts of oxygen species and oxygen radicals every day as part of its natural defence against invading microorganisms. These are normally removed by enzymes that are produced by the body thereby limiting the ability of these radicals and reactive species to harm the host tissues. A fine balance normally exists between the numbers of these oxygen radicals and reactive species that are produced and the ability of the body to remove them. The introduction of a significant increase in oxygen radicals into the body has the potential to overwhelm the body's natural defence mechanisms resulting in potential harm to the soft tissues. It has been well documented that hydrogen peroxide and hydroxyl radicals are capable of causing damage to a range of connective tissue elements – both cellular and non-cellular. Dahlstrom *et al.* (1997) were able to show that the introduction of heat to hydrogen peroxide during the intra-coronal bleaching of non-vital teeth has the ability to generate hydroxyl radicals on the outer surface of the root. Hydrogen peroxide has been shown to have the ability to diffuse through the tooth from the pulp chamber to the outer surface in both thermocatalytic and the walking bleach techniques. Tipton *et al.* (1995) determined that the lethal dose (LD_{50}) for hydrogen peroxide on fibroblasts was 0.00125%. The use of hydrogen peroxide in either

bleaching protocol resulted in the passage of a concentration of hydrogen peroxide which was greater than ten times the lethal dose determine by Tipton *et al.* The introduction of thiourea had a significant scavenging effect to reduce the available concentration of hydrogen peroxide on the outer surface of the tooth. However, the concentration present was still at least two times greater than the LD_{50} determined by Tipton *et al.* Hydroxyl radical generation has been proven to occur on the outer surface of the tooth in both thermocatalytic and walking bleach techniques. The reactivity and toxicity of the hydroxyl radical suggests it may readily initiate an inflammatory response within the periodontal ligament if present in sufficient quantity. Thiourea was able to significantly reduce the amount of hydroxyl radical generated on the outer surface of the tooth. This was most likely due to a direct scavenging effect and an indirect scavenging effect of the thiourea. That is, the thiourea was able to scavenge and therefore reduce the available hydrogen peroxide present on the outer surface of the tooth, which in turn resulted in a lesser generation of hydroxyl radicals. Also, thiourea has a direct scavenging effect on hydroxyl radicals, which would result in a further decrease in the amount of hydroxyl radicals present in the region.

The use of a base over the coronal aspect of the root-filling remains an unresolved issue. The amount of hydrogen peroxide detected and the large number of samples in which it was detected implies that the use of a base would be beneficial in preventing the penetration of hydrogen peroxide along the length of the canal. Further research into the ability of a base, placed over the root-filling material to inhibit the passage of hydrogen peroxide and hydroxyl radicals is required.

The use of heat to activate the hydrogen peroxide has been used for many years in the intra-coronal bleaching of non-vital teeth. The ability of heat to catalyse the breakdown of hydrogen peroxide would suggest that during thermocatalytic bleaching of a blood stained tooth there would be a significant reduction in available hydrogen peroxide for Fenton-like reactions on the outer surface of the tooth. Pashley *et al.* (1983) reported on the effect of heat on the increased permeability of dentine when heat was applied. The tooth model used in the current research resulted in increased dentine permeability that in turn had two effects:

(1) greater diffusion of available hydrogen peroxide through dentine towards the outer surface of the tooth.

(2) increased diffusion of salicylate from the outer surface of the tooth towards the pulp chamber. This may suggest a possible limitation of the experimental model in the current research, as the inward diffusion of tissue fluids would not be expected in the *in vivo* situation. Therefore the *in vivo* generation of hydroxyl radicals close to the outer surface of the tooth but not on the tooth surface, would not be detected due the high reactivity of the hydroxyl radical. There was no significant difference in the generation of hydroxyl radicals on the outer surface of the tooth between the teeth bleached with and without the application of heat. However, the raw datum (Appendix 3) revealed an increase in amounts of hydroxyl radical generation when heat was applied to the hydrogen peroxide. This result suggests that the use of heat to activate hydrogen peroxide during the intra-coronal bleaching of non-vital teeth should be further examined.

The aim of using sodium hypochlorite in the bleaching protocol was to remove

residual organic debris from the pulp chamber that may hinder the bleaching efficacy. However, sodium hypochlorite was shown to generate hydroxyl radicals even in the presence of thiourea. This is supported by Halliwell and Gutteridge (1999) who reported that hypochlorous acid (which breaks down to the hypochlorite ion) readily reacts with ferric (Fe^{3+}) ions to form hydroxyl radicals. The presence of iron in the dentine tubules following their staining with packed red blood cells provides sufficient substrate for Fenton-like reactions to occur involving hypochlorite ions. The ability of thiourea to scavenge sodium hypochlorite in this study was limited which was surprising due to the reported ability of thiourea to scavenge hypochlorite ions (Carrea *et al.* 1991, Jackson *et al.* 1988). This allowed sodium hypochlorite to diffuse though to the outer layers of the tooth where reaction with iron was able to occur leading to the generation of hydroxyl radicals. The advantages of using sodium hypochlorite in a bleaching protocol are outweighed by the increase in hydroxyl radical generation on the outer surface of the tooth.

The ability of thiourea to scavenge both hydroxyl radicals and hydrogen peroxide has been well documented. Its use as a scavenger of these oxygen radicals and reactive oxygen species has been used for many years in medicine. However, its use in dentistry and in particular for the intra-coronal bleaching of non-vital teeth is new. This research has shown that thiourea is an efficient scavenger of hydroxyl radicals and hydrogen peroxide. Maroulis (1994) reported that the end product of the reaction between thiourea and hydrogen peroxide is thiourea dioxide, a powerful bleaching agent. Maroulis further reported that thiourea dioxide is as effective as hydrogen peroxide in the intra-coronal bleaching of blood stained teeth. The ability of thiourea

to reduce the amount of hydrogen peroxide and the amount of hydroxyl radicals on the outer surface of the tooth, combined with its ability to generate thiourea dioxide warrants its consideration as an alternative bleaching protocol. The toxicity of thiourea has been discussed. The amount of thiourea which diffused from the pulp chamber to the outer surface of the tooth in this research is of concern if thiourea was to be introduced by itself. However, when thiourea was introduced as part of a bleaching protocol with hydrogen peroxide, the scavenging effect of the thiourea reduces that thiourea present on the outer surface of the tooth to a level which is below the TD_{50} described by Dieke *et al.* in 1947. Thiourea is a stable compound. There is little research directed at the ability of thiourea to persist within hard and soft tissues. Therefore the ability of thiourea to persist within the dentinal tubules and continue to be released into the adjacent periodontium is unknown. Toxicity studies for thiourea on cellular and non-cellular components of the periodontal ligament and on the rate of clearance of thiourea from the dentinal tubules are required before these concentrations are deemed safe for use in the intra-coronal bleaching of non-vital teeth. Such research should be conducted in the *in vitro* and *in vivo* model and should be conducted using thiourea in concentrations similar to those detected on the outer surface of the tooth.

The question arises as to whether it is necessary to counteract hydroxyl radicals before they can institute the level of damage necessary to trigger such a resorptive response. The association between intra-coronal bleaching and invasive cervical resorption has been well documented and is outlined in section 1.0. The initial insult to the periodontium responsible for the onset of the resorption process is

hypothesised as being the presence of hydroxyl radicals. These radicals are formed by a Fenton reaction between hydrogen peroxide and ferrous (Fe^{2+}) ions which are in turn capable of causing necrosis of the periodontal ligament or impairing the normal function of this tissue. The ability of hydroxyl radicals to cause damage to elements of the periodontal ligament at the cellular and non-cellular level which may result in necrosis of this tissue, have been well established. Lindskog *et al.* (1985) reported the correlation between necrotic periodontal ligament and root resorption thereby offering a possible link between intra-coronal bleaching and invasive cervical resorption. The ability of thiourea to scavenge a significant amount of hydrogen peroxide and to significantly reduce the generation of hydroxyl radicals on the outer surface of the tooth when combined with hydrogen peroxide, along with its known ability to enhance the bleaching efficacy of hydrogen peroxide, (Maroulis, 1994) suggests that thiourea be used as an adjunct in the traditional "walking bleach" technique for more effective bleaching.

Other oxygen radical scavengers have been investigated for their potential use in the intra-coronal bleaching of non-vital teeth. Rotstein (1993) introduced catalase into the pulp chamber following the completion of the bleaching protocol and found it to be an effective scavenger of residual hydrogen peroxide in the dentinal tubules and on the outer surface of the tooth. However, the problem with the use of this agent is that the hydrogen peroxide had been sealed into the tooth for a period of 60 minutes before the introduction of the catalase. The control generation of hydroxyl radicals in this research determined that most of the reaction between hydrogen peroxide and the available ferrous (Fe^{2+}) iron occurs in the first 30 minutes. Rotstein *et al.* (1991)

reported that there was a significant penetration of hydrogen peroxide through the radicular dentine after 60 minutes bleaching time at 37^{0}C. Thus there is the potential for the penetration of hydrogen peroxide and the generation of hydroxyl radicals on the outer surface of the tooth within the first 60 minutes of bleaching. The advantage of thiourea is that it is introduced into the tooth prior to the introduction of hydrogen peroxide and acts as a scavenger of hydrogen peroxide and hydroxyl radicals in the intra-coronal bleaching of non-vital teeth. Thus it is capable of scavenging hydrogen peroxide and hydroxyl radicals which may be generated prior to their penetration to the outer surface of the tooth. Furthermore, Halliwell (1977) reported that thiourea has a much higher rate constant for reaction with hydroxyl radicals and therefore was a more effective inhibitor of hydroxylation by these radicals, compared with other hydroxyl radical scavengers, when present in much lower concentrations.

13.0 Conclusions

Several conclusions can be drawn from this research project

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(1) Both the "walking" and "thermocatalytic" bleaching techniques are capable of generating the production of hydroxyl radicals on the outer surface of the tooth. There was no significant difference in the quantity of these radicals generated by the two bleaching protocols, however, application of heat to the hydrogen peroxide appeared to generate greater quantities in real terms when the raw data was observed.

(2) Hydrogen peroxide was capable of diffusing through the radicular dentine to the outer surface of the teeth following its introduction into the pulp chamber. Significantly greater quantities of hydrogen peroxide were found on the outer root surface in teeth in which the hydrogen peroxide was sealed into the tooth. The application of heat appeared to catalyse the breakdown of hydrogen peroxide into its unstable oxidising components. The amount of hydrogen peroxide capable of diffusing through the tooth was above the TD_{50} for hydrogen peroxide and was therefore deemed capable of inducing toxic effects on the periodontal tissues.

(3) Thiourea was capable of significantly reducing the quantities of hydrogen peroxide and reaction products of hydroxyl radicals detectable on the outer surface of the tooth. The scavenging of hydrogen peroxide was sufficient to

lower the detectable quantity down below the level ascertained by Woolverton *et al.* (1997) to cause toxic effects on fibroblast cells. Thiourea was likewise capable of significantly reducing the quantity of reaction products of hydroxyl radicals on the outer surface of the tooth. It was not capable of eliminating all hydrogen peroxide nor all hydroxyl radicals.

(4) The quantity of thiourea which was able to diffuse through radicular dentine to the outer surface of the tooth was well below the TD_{50} level ascertained by Dieke *et al.* (1947). The amount of thiourea available to diffuse through to the outer surface of the tooth following the introduction of hydrogen peroxide into the tooth resulted in a detectable quantity that was even lower than the LD_{50} described by Dieke *et al.* (1947). Therefore, thiourea introduced into the pulp chamber to scavenge excess hydrogen peroxide is concluded as being non-toxic to the periodontal ligament tissues.

(5) The technique employed in this research project is sensitive for the detection of hydrogen peroxide and thiourea and all of the generated reaction products. This technique is relatively simple and the results are reproducible. However, there are several concerns regarding the methods employed in this protocol which surfaced during the experimental procedures and which have been addressed in the discussion section.

The generation of hydroxyl radicals and the passage of hydrogen peroxide during the intra-coronal bleaching of root-filled teeth has been demonstrated in this study.

The ability of thiourea to scavenge both hydrogen peroxide and hydroxyl radicals has also been demonstrated. Hydrogen peroxide and hydroxyl radicals are known to be toxic to elements of the periodontal ligament and there is an association between intra-coronal bleaching of root-filled teeth and the condition of invasive cervical resorption. The ability of thiourea to scavenge these oxygen radicals and reactive oxygen species, when introduced into the bleaching protocol, may help reduce the incidence of this type of resorption.

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Appendix 1

Sources and components of chemicals and equipment

Chemical and Source	Components
AH-26:	Epoxy resin
De Trey – Dentsply, Switzerland	Bismuth oxide
	Hexamethylene tetramine
	Titanium dioxide
	Bis-phenol di-glycidyl ether
Gutta percha:	gutta percha
Progress, Rudolph Gunz, Australia	zinc oxide
	Heavy metal sulphates
	Plasticizers
Cavit:	calcium sulphate
Espe, West Germany	zinc oxide
	glycol acetate
	Polyvinyl acetate
	Polyvinyl chloride acetate
	Triethanolamine

Modelling wax:	ceresin
Ainsworth Dental Co. Australia	beeswax
	carnauba wax
	synthetic resins
	synthetic waxes

Sodium chloride: BDH, Australia

Packed Red Blood Cells : Blood bank - Red Cross, Australia

Potassium dihydrogen orthophosphate (K_2HPO_4) (Analar) : BDH, Aust.

Ethylene diamine tetra-acetic acid (EDTA) : J.T. Baker Chemical Co., USA

Methanol (HPLC grade) : Mallinckrodt, USA

Hydrochloric acid (Analar) : BDH, Australia

Hydrogen peroxide (H_2O_2) 100% w/v : BDH, Australia

Thiourea (Analar) : BDH Lab Supplies, England

Sodium hypochlorite 4% (NaOCl): BDH, Australia

Formamidinesulfinic acid (thiourea dioxide) (Analar) : Sigma Chemical Co. USA

2,3 –dihydroxybenzoic acid (Analar) (2,3 – DHB) : Sigma Chemical Co. USA

2,5 – dihydroxybenzoic acid (Analar) (2,5 – DHB) : Sigma Chemical Co. USA

3,4 – dihydroxybenzoic acid (Analar) (3,4 – DHB) : Sigma Chemical Co. USA

Resorcinol (Analar) (1,3 – DHB) : BDH, Australia

Pyrocatechol (Analar) (1,2 – DHB) : BDH, Australia

Pyrogallol (Analar) (1,2,3 – THB) : Aldrich Chemical Co., USA

Phloroglucinol (Analar) (1,3,5 – THB) : Aldrich Chemical Co., USA

Hydroquinone (Analar) (1,4 – DHB) : BDH, Australia

Sodium salicylate (Analar) : Aldrich Chemical Co., USA

Ferrous sulphate (Analar) : BDH, Australia.

Appendix 2

Preparation of test solutions for HPLC injection

Mobile phase:

A 70% aqueous phase of 0.1M KH_2PO_4 (Mwt = 136.0) and 0.001M EDTA (Mwt = 372.2) was added to 30% methanol at a constant pH of 2.9.

Aqueous phase was made by the addition of 13.6g of KH_2PO_4 and 0.0372g of EDTA to 1 litre of milliQ water. 0.7 litre of this stock solution was than added to 0.3 litre of methanol and the pH adjusted to 2.9 with concentrated HCl, to form the mobile phase for the HPLC.

0.001M Sodium Salicylate stock solution:

(Mwt = 160.11)

0.160g was added to 1 litre of milliQ water to form a 0.001M solution of sodium salicylate

0.003M 2,3 – DHB ; 2,5 – DHB and 3,4 – DHB stock solutions:

(Mwt = 154.1)

4.6mg was added to 1 litre of milliQ water (equivalent to 4.6 x 10^{-8}g / 10ml) to obtain a 0.003M stock solution of each of these standards. A dilution cascade was then made from each stock solution to obtain the required molar concentration of each standard.

1.3M Thiourea (acidified) stock solution:

100ml of 33% hydrochloric acid (Mwt = 34.1) and 100g of thiourea (Mwt = 76.12) was added to 1 litre of milliQ water to form a stock solution of 1.3M acidified thiourea (pH =2.95).

0.001M stock solutions of hydroquinone, pyrocatechol and resorcinol:

(Mwt = 110.11)

1.1g of each of these standards was each added to 1 litre of milliQ water to make a 0.001M stock solution of each of hydroquinone, pyrocatechol and resorcinol. A dilution cascade was then made from each stock solution to obtain the required molar concentration of each standard.

0.001M stock solutions of phloroglucinol and pyrogallol:

(Mwt = 126.11)

1.26g of each of these standards was added to 1 litre of milliQ water to make a 0.001M stock solution of each of phloroglucinol and pyrogallol. A dilution cascade was then made from each stock solution to obtain the required molar concentration of each standard.

50×10^{-6}M stock solution of Ferrous Sulphate:

0.139g of ferrous sulphate was added to 1 litre of milliQ water to make a 50 x 10-6M stock solution of ferrous sulphate.

Appendix 3
Raw data from the HPLC - ECD test solutions

Sample No.		Volume(ml) of each sample	No. picomoles/tooth			No. nanomoles/tooth	
			2,3 - DHB	2,5 - DHB	Pyrocatechol	H2O2	Thiourea
Negative	1	2.80	0.0	0.0	0.0	0.0	0.0
	2	2.73	0.0	0.0	0.0	0.0	0.0
	3	2.80	0.0	0.0	0.0	0.0	0.0
	4	3.15	0.0	0.0	0.0	0.0	0.0
	5	2.70	0.0	0.0	0.0	0.0	0.0
H2O2	1	2.85	210.9	118.3	230.9	123830.0	0.0
	2	2.65	791.0	928.8	107.3	355045.6	0.0
	3	2.70	144.5	16.2	70.2	119643.7	0.0
	4	2.70	452.3	1080.0	86.4	339445.4	0.0
	5	2.70	253.8	830.3	32.4	87273.5	0.0
H2O2 (thermo)	1	2.70	507.6	16.2	487.4	277520.8	0.0
	2	2.93	196.0	0.0	564.5	8716.5	0.0
	3	2.85	391.9	135.4	122.6	6855.7	0.0
	4	2.50	335.0	2640.0	256.3	9130.0	0.0
	5	2.65	2452.6	1771.5	184.2	13239.4	0.0
Thiourea	1	2.80	0.0	0.0	0.0	0.0	221.0
	2	2.70	0.0	0.0	0.0	0.0	326.7
	3	2.85	0.0	0.0	0.0	0.0	547.1
	4	2.65	0.0	0.0	0.0	0.0	0.0
	5	2.60	0.0	0.0	0.0	0.0	488.4
H2O2 + Thiourea	1	2.65	390.9	125.9	31.8	29395.1	122.7
	2	2.85	0.0	0.0	0.0	5883.8	10.8
	3	2.60	0.0	0.0	0.0	0.0	705.0
	4	2.60	174.2	85.8	26.0	19629.3	25.7
	5	2.75	1375.0	618.8	199.4	57099.6	31.3
NaOCl + Thiourea	1	2.58	267.8	167.8	0.0	0.0	40.1
	2	2.75	276.4	198.0	0.0	0.0	74.9
	3	2.65	0.0	0.0	0.0	0.0	341.3
	4	2.73	0.0	134.9	0.0	0.0	36.6
	5	2.85	314.9	188.1	0.0	0.0	102.2
H2O2, NaOCl + Thiourea	1	2.80	112.0	288.4	315.0	8535.8	21.5
	2	2.60	1534.0	327.6	0.0	60568.3	155.7
	3	2.58	242.1	180.3	0.0	18708.7	44.2
	4	2.80	0.0	373.8	229.6	10444.0	38.6
	5	2.60	218.4	144.3	0.0	4988.1	33.6

Appendix 4
Statistical analysis of the raw data

2,5 - DHB

H2O2	H2O2 + Thio
118.3	125.9
928.8	0.0
	0.0
1080.0	85.8
830.3	
739.3	52.9
426.6	63.3
0.05	

H2O2	H2O2 -thermo
118.3	16.2
928.8	0.0
	135.4
1080.0	2640.0
830.3	1771.5
739.3	912.6
426.6	1220.9
0.78	

H2O2	H2O2,NaOCl, Thio
118.3	288.4
928.8	327.6
	180.3
1080.0	373.8
830.3	144.3
739.3	262.9
426.6	97.5
0.11	

H2O2+ Th	NaOCl+Th
125.9	167.8
0.0	198.0
0.0	
85.8	134.9
	188.1
52.9	172.2
63.3	27.9
0.02	

H2O2 -thermo	H2O2 + Thio
16.2	125.9
0.0	0.0
135.4	0.0
2640.0	85.8
1771.5	
912.6	52.9
1220.9	63.3
0.19	

2,3 - DHB

H2O2	H2O2 + Thio
210.9	390.9
791.0	0.0
144.5	0.0
452.3	174.2
253.8	1375.0
370.5	388.0
261.6	574.7
0.95	

H2O2	H2O2 -thermo
210.9	507.6
791.0	196.0
144.5	391.9
452.3	335.0
253.8	2452.6
370.5	776.6
261.6	943.6
0.40	

H2O2	H2O2,NaOCl, Thio
210.9	112.0
791.0	1534.0
144.5	242.1
452.3	0.0
253.8	218.4
370.5	421.3
261.6	629.4
0.87	

H2O2+ Th	NaOCl+Th
390.9	267.8
0.0	276.4
0.0	0.0
174.2	0.0
1375.0	314.9
388.0	171.8
574.7	157.8
0.46	

H2O2

	H2O2	H2O2 + Thio
	123830.0	29395.1
	355045.6	5883.8
	119643.7	
	339445.4	19629.3
	87273.5	57099.6
mean	205047.6	28002.0
st. dev	130693.4	21663.6
t test	0.04	

	H2O2	H2O2 -thermo
	123830.0	
	355045.6	8716.5
	119643.7	6855.7
	339445.4	9130.0
	87273.5	13239.4
mean	205047.6	9485.4
st. dev	130693.4	2691.1
t test	0.03	

	H2O2	H2O2,NaOCl, Thio
	123830.0	8535.8
	355045.6	60568.3
	119643.7	18708.7
	339445.4	10444.0
	87273.5	4988.1
mean	205047.6	20649.0
st. dev	130693.4	22877.1
t test	0.03	

	H2O2	H2O2,NaOCl, Thio
	123830.0	8535.8
	355045.6	60568.3
	119643.7	18708.7
	339445.4	10444.0
	87273.5	4988.1
mean	205047.6	20649.0
st. dev	130693.4	22877.1
t test	0.03	

	H2O2+Th	H2O2,NaOCl+Th
	29395.1	8535.8
	5883.8	60568.3
		18708.7
	19629.3	10444.0
	57099.6	4988.1
mean	28002.0	20649.0
st. dev	21663.6	22877.1
t test	0.75	

	H2O2 -thermo	H2O2 + Thio
		29395.1
	8716.5	5883.8
	6855.7	
	9130.0	19629.3
	13239.4	57099.6
mean	9485.4	28002.0
st. dev	2691.1	21663.6
t test	0.19	

	H2O2 -thermo	H2O2,NaOCl+Th
		8535.8
	8716.5	60568.3
	6855.7	18708.7
	9130.0	10444.0
	13239.4	4988.1
mean	9485.4	20649.0
st. dev	2691.1	22877.1
t test	0.34	

Thiourea

	Thio	H2O2+Thi
	221.0	122.7
	326.7	10.8
	547.1	
		25.7
	488.4	31.3
mean	395.8	47.6
st.dev	149.2	50.8
t test	0.01	

	Thio	NaOCl+Thi
	221.0	40.1
	326.7	74.9
	547.1	341.3
		36.6
	488.4	102.2
mean	395.8	119.0
st.dev	149.2	127.1
t test	0.03	

	Thio	H2O2,NaOCl+Thi
	221.0	21.5
	326.7	155.7
	547.1	44.2
		38.6
	488.4	33.6
mean	395.8	58.7
st.dev	149.2	54.9
t test	0.02	