HYDROXYL RADICAL ACTIVITY IN BLEACHED ROOT-FILLED TEETH

by

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DECLARATION

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SUMMARY

Bleaching procedures have been carried out on both vital and root-filled teeth for many years. These bleaching procedures have generally been considered to be both effective and safe. The most commonly used bleaching agent in the treatment of discoloured root-filled teeth has been 30% hydrogen peroxide. This is sometimes used in combination with sodium perborate. Harrington and Natkin (1979) were the first to report an association between bleaching procedures in root-filled teeth and the condition of invasive cervical root resorption. Reports then followed from other authors supporting this observation. Harrington and Natkin suggested that the cause of bleaching-related root resorption could be the leakage of bleaching agents from the pulp chamber through patent dentinal tubules into cervical periodontal tissue during the bleaching procedure. This could damage periodontal tissue and initiate root resorption. This process may well be facilitated by traumatically or developmentally produced defects in the cementum layer in the cervical region of the tooth. A recent study (Rotstein et al., 1991) has indeed demonstrated the passage of hydrogen peroxide from the pulp chambers of root-filled teeth to the external root surface. In this study, greater concentrations of hydrogen peroxide were detected when there were defects in the cementum layer.

Experimental and clinical reports have served to demonstrate the toxic nature of hydrogen peroxide (Seale et al., 1981 ; Weitzman, 1986). Hydrogen peroxide is considered to be a reactive oxygen species (Halliwell, 1989b) and has been demonstrated *in vitro* and *in vivo* to cause the generation of hydroxyl radicals in the presence of transition metals

such as iron. Ferrous salts combined with hydrogen peroxide will generate hydroxyl radicals (Halliwell, 1978). Although most iron in the body is bound to either haemoglobin, myoglobin, transferrin or ferritin, in some disease states, iron is free to participate in such a reaction. Furthermore, iron may be made available for this reaction when haemoglobin is exposed to hydrogen peroxide.

The hydroxyl radical is one of a number of oxygen-derived free radicals and is considered extremely reactive. Its role in the destruction of connective tissue components, collagen and hyaluronic acid, is well documented (Greenwald, 1981). Hydroxyl radicals are also capable of altering deoxyribosenucleic acid (DNA) through strand breakage as well as destroying cell membranes through lipid peroxidation.

In traumatised teeth, the development of deep discolouration has been attributed to the extravasation of blood components from the pulp chamber into surrounding dentinal tubules with possible subsequent decomposition of these components (Grossman, 1988). Freccia and Peters (1982a) further suggested that, with the breakdown of extravasated erythrocytes, iron is released. Combination of this iron with hydrogen sulphide produced by bacteria may also result in the formation of brown - black iron sulphide, thus adding to the degree of discolouration of the tooth.

The aim of the present study was to determine whether hydroxyl radicals are generated when hydrogen peroxide is used to bleach root-filled teeth which have been discoloured by blood components.

Forty extracted human premolar teeth were root-filled with gutta percha and AH26^{R} sealer cement. Twenty of the teeth were experimentally discoloured by blood components. All teeth were then

thermocatalytically bleached using 30% hydrogen peroxide while the tooth roots were suspended in a test solution of sodium salicylate. Hydroxyl radical generation was determined by the detection of products of the reaction of this radical with salicylate using high performance liquid chromatography with electrochemical detection.

The presence of reaction products was detected in twenty five of the teeth. A significant association was found between the presence of tooth discolouration caused by blood components and the production of hydroxyl radicals. There was no association between the presence of cementum defects and the production of hydroxyl radicals. If anything, there was an apparent inhibition of hydroxyl radical production by cementum defects. Greater yields of hydroxyl radicals were detected in teeth which were discoloured by blood but which did not have detectable defects in the cementum at the cemento-enamel junction (CEJ). Overall there was no significant statistical association between the use of ethylenediamine tetra-acetic acid (EDTA) to flush the pulp chamber before the bleaching procedure and the production of hydroxyl radicals. However, the greatest individual yields of hydroxyl radicals found in this study occurred in teeth in which EDTA had been used to flush the pulp chamber prior to the bleaching procedure.

Supplementary experiments in the present study demonstrated that intense U.V light increased the production of hydroxyl radicals from sodium salicylate solution alone and in sodium salicylate solution which contained hydrogen peroxide.

There appeared to be a minor reaction between hydrogen peroxide and sodium salicylate solution itself. This phenomenon is discussed as it may have had some influence on the results of the present study and it

may hold implications for other studies which have employed this technique for the detection of hydroxyl radicals.

It was concluded that hydroxyl radicals are generated during bleaching of root-filled teeth. There is a significant association between the presence of tooth discolouration due to blood components and the production of hydroxyl radicals. The presence of EDTA may result in a greater yield of hydroxyl radicals in discoloured, root-filled teeth undergoing bleaching. The generation of hydroxyl radicals could be one mechanism underlying periodontal tissue destruction and invasive cervical root resorption that may occur after intracoronal bleaching of discoloured root-filled teeth.

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1.1 General Aetiology

Invasive cervical resorption is a form of external root resorption which occurs in the cervical region of the tooth. It can occur in vital teeth as well as teeth which have been root-filled. In most cases, it is seen as a late complication of traumatic injury to the tooth (Chivian, 1987) but resorption may also be due to intracoronal bleaching, orthodontic tooth movement, dento-alveolar surgery or periodontal treatment (Tronstad, 1988).

Numerous aetiologies have been proposed for this condition. Earlier discussion of the condition mainly concerned the condition as it affected vital teeth. Southam (1967) believed that the cervical area of the tooth could become susceptible to resorption due to a developmental defect which exposed the cervical dentine to the tissue of the periodontal ligament. He proposed this may be due to either the failure of cementum and enamel to meet at the cemento-enamel junction; to premature degeneration of reduced enamel epithelium; or to a differential rate of cementum deposition in the cervical region leaving an area devoid of cementoid, thus exposing root dentine. He also believed that adjacent gingival inflammation was a result of the resorption process, but not the converse.

Sullivan and Jolly (1957) postulated that ideopathic resorption (sic) is brought about by any factor that causes local destruction or change of surface protective tissues such as cementoid or odontoblasts, followed by direct apposition of granulation tissue to the exposed calcified surface.

Makkes and Thoden van Velzen (1975) proposed that the probable cause of "cervical external resorption" was a primary local chronic inflammation. They believed this may result from such circumstances as periodontal disease or the pressure of an adjacent impacted tooth.

1.2 Clinical Features

Pindborg (1970) considered invasive cervical resorption as essentially an external inflammatory resorption, involving mainly cementum and dentine, which is found in connection with the periodontal ligament (PDL). Clinically, the condition is usually asymptomatic until the late stages. In advanced cases, the crown of the tooth can become completely undermined leading to eventual fracture. There may also be an intervening bacterial infection manifesting in abscess and sinus tract formation. Pindborg claimed that even large resorption cavities would be difficult to detect clinically if the entrance to the defect lay beneath the gingival attachment. Sullivan and Jolly (1957), however, believed that the loss of attachment of Sharpey's fibres followed by pocket formation would allow the defect to be readily probed.

In advanced cases of invasive cervical resorption, enamel may also be resorbed and this may produce a so-called "pink spot" as the highly vascular resorptive tissue shines through the enamel of the crown. Invasive cervical resorption characteristically displays a mottled radiographic appearance with an irregular outline (Makkes and Thoden van Velzen, 1975). In such cases, invasive cervical resorption may be confused with the metaplastic type of internal resorption (Heithersay, 1985). This latter condition, which Andreasen (1981) termed internal replacement resorption, is also characterised radiographically by an

irregular enlargement of the pulp chamber and a mottled radiographic appearance, but histologically is characterised by a metaplastic change of normal pulp tissue into cancellous bone. In contrast, another resorptive condition, internal inflammatory resorption, usually has a uniform radiolucency and appears radiographically as a symmetrical, spherically shaped enlargement of the pulp chamber or root canal which gradually blends in with the contours of the canal.

It is important to differentiate between the above conditions because treatment modalities can differ markedly. A differential diagnosis can often be made on the basis of radiographic appearance. Andreasen (1981) considered it essential to take mesial and distal eccentric radiographs of the tooth. Where the resorption lesion did not move in relation to other structures on radiographs taken at different angulations, it could be considered an internal resorption. Furthermore, in most cases of invasive cervical resorption, the outline of the pulp chamber and root canal can be traced through the resorption lesion on a radiograph, particularly where the lesion is on the buccal or lingual surface of the tooth (Makkes and Thoden van Velzen, 1975). Resorption of adjacent marginal bone is generally not evident with invasive cervical resorption although, in cases where a supervening infection occurs, bone destruction can occur, giving the appearance of a periodontal pocket (Cvek and Lindvall, 1985; Tronstad, 1988).

1.3 Histology

Histologically, invasive cervical resorption presents a complicated picture. Destruction appears to be caused by highly vascular granulation tissue which originates in either gingiva or periodontal ligament

(Pindborg, 1970). Resorption generally involves cementum and dentine but, in advanced cases, enamel may be resorbed. Pindborg (1970) believed that the entry point of resorptive tissue was located below the free margin of the gingiva. Tronstad (1988) supported this, believing the lesion occurred in response to injury of the cervical periodontium below the epithelial attachment. Friedman et al. (1988), in a study of invasive cervical resorption in pulpless teeth, found that resorption commenced just apical to the cemento-enamel junction.

Resorptive tissue in this condition spreads deep into dentine with extensive tunnel-like ramifications. Usually, however, predentine and circumpulpal dentine are not affected until the late stages of the condition (Pindborg, 1970; Rushton, 1970). The pulp in vital teeth therefore remains covered by a thin shell of dentine and predentine. Yacoub (1980) proposed that this resistant shell was due to the lower degree of mineralisation in predentine and circumpulpal dentine. Wedenberg (1987), on the basis of experimental evidence, believed that there was an extractable inhibitor which may be responsible for the resistance of predentine and circumpulpal dentine to resorption.

Makkes and Thoden van Velzen (1975) described two cases of invasive cervical resorption in vital teeth and provided a detailed histological description. They reported that the resorption exhibited ramifying tunnels containing loosely organised but highly vascular tissue, with few inflammatory cells present. The pulp chamber was generally separated from resorptive tissue by a thin layer of dentine, except in one case where a small pulpal exposure was detected. They found that the entrance cavity into the root surface was a small perforation approximately 500 micrometres in diameter located "a few millimetres"

below the cemento-enamel junction. The root surface around the entrance tunnel displayed an irregular cementum layer with corrugations and invaginations where dentine was occasionally involved. The tissue in these extensive tunnels contained macrophages, lymphocytes and the occasional fibroblast. The presence of polymorphonuclear neutrophils was not noted. Along the walls of the tunnels were extensive resorption lacunae containing numerous multinucleated giant cells, presumably the cells responsible for the resorption. Extensive deposits of bone tissue (osteoid) were also noted in the resorption lacunae. A histological examination of the pulp revealed essentially normal tissue except at the site of perforation by the resorptive tissue. Macrophages, lymphocytes and the occasional neutrophil were visible at this site.

Lado et al. (1983) reported a case of invasive cervical resorption in a bleached root-filled tooth, which had failed to respond to treatment and was subsequently extracted. A histopathological report of the resorption area described it as being filled with chronically inflamed, vascular connective tissue with numerous dentinoclasts seen in lacunae, indicative of an active resorption process.

1.4 Invasive Cervical Resorption in Bleached Root-Filled Teeth

Harrington and Natkin (1979) were the first to report cases demonstrating an association between invasive cervical resorption and bleaching in root-filled teeth. Following this initial report, further cases from other clinicians were reported in the literature.

Friedman et al. (1988) in a study of 58 bleached root-filled teeth reported an incidence of invasive cervical resorption of 6.9% over a period of one to eight years. Chivian (1987) believed that the incidence

could be higher than generally believed, depending on a clinician's ability to detect the condition.

In general, bleaching-related invasive cervical resorption has been found to be progressive. In several reports, however, cases of spontaneous arrest have been mentioned (Friedman, 1988; Cvek and Lindvall, 1985), although it is open to conjecture as to whether these were true cases of invasive cervical resorption.

Harrington and Natkin pointed out a number of factors which were common to the cases they presented . These were :

1. All patients were in their early teens (11 - 15 years)
2. All resorptions occurred in the cervical area of the tooth
3. All teeth had a history of trauma leading to a loss of vitality with no subsequent history of trauma
4. All teeth had been bleached with either a thermocatalytic or 'walking bleach' technique (see Section 1.6), both of which employed 30% hydrogen peroxide.
5. Detection of the resorption occurred between two and seven years after the bleaching procedure was carried out.
6. Bleaching was usually carried out several years after the original trauma.

From these factors, Harrington and Natkin suggested that the cause of bleaching-related invasive cervical resorption may be one of the following :

1. Bleaching agents, such as hydrogen peroxide, may inadvertently leak directly onto gingival tissues causing an inflammation of the gingival attachment and initiating a resorptive response.

2. Leakage of hydrogen peroxide through patent dentinal tubules from the pulp chamber into the cervical periodontium may occur during the bleaching procedure and could initiate an inflammatory response resulting in resorption.
3. The original trauma to the tooth, resulting in loss of vitality. This was considered an unlikely possibility.
4. Heat produced during the bleaching procedure, which may cause periodontal tissue injury resulting in resorption. This also was considered less likely.

Harrington and Natkin believed that the age of the patient was significant. Pulp spaces in those teeth affected would have been larger at these ages than in more mature teeth and dentinal tubules more open, potentially allowing the passage of bleaching agent from the pulp chamber to the cervical periodontium.

Trauma may also have played a part in the initiation of bleaching-related invasive cervical resorption. Webber (1983) believed that teeth with a history of trauma have a potential for resorption which may prevail throughout the patient's life. He believed that since cementum is resorbed after trauma, patent dentinal tubules would be exposed to periodontal tissue. Application of toxic bleaching agents could re-initiate an inflammatory response leading to resorption through this defective barrier. This hypothesis is supported by the findings of Lindskog and Hammarström (1983) who showed that in experimentally produced resorption cavities on the root surface of replanted teeth, reparative cementum was frequently separated from dentine, yet was firmly bound

to intermediate cementum. This may result in an incomplete barrier between root dentine and the periodontal ligament. In the cases reported by Harrington and Natkin there was no subsequent trauma after the traumatic episode which resulted in loss of vitality. Furthermore, in these cases, there was usually a substantial time period (6-15 years) between the original trauma and the detection of resorption.

Harrington and Natkin cited studies by Avny et al. (1973) and Taylor (1976) which demonstrated the diffusion of parachlorophenol from the pulp chamber through dentine. Harrington and Natkin believed that in teeth of young individuals with large, open dentinal tubules there was a real possibility that hydrogen peroxide could diffuse from the pulp chamber to the periodontal ligament and initiate resorption. The observation that in 10% of teeth cementum and enamel did not meet at the cervical area (Armitage, 1976) supposedly increased this possibility. Subsequent studies (Schroeder and Scherle, 1988) have, however, suggested that the incidence of developmental cementum defects at the cemento-enamel junction may be as high as 25%. A recent study (Rotstein et al., 1991) has indeed demonstrated the diffusion of hydrogen peroxide from the pulp chambers of root-filled teeth through to the external root surface, even with the presence of an apparently intact cementum layer. Diffusion was significantly greater, however, when artificial 'defects' were placed in the cementum layer.

Cases reported after the initial report by Harrington and Natkin served to demonstrate the variable nature of factors associated with bleaching-related invasive cervical resorption. Lado et al. (1983) reported the case of a 50 year-old woman who had undergone a combination of thermocatalytic and 'walking bleach' procedures (a detailed description of

these procedures is given in Section 1.6) in a root-filled tooth. This case differed from those of Harrington and Natkin in that there had been no history of trauma and that the patient was significantly older when the bleaching procedures had been carried out. They proposed that the bleaching agent may denature dentine in the cervical region where a developmental defect of the cemento-enamel junction existed (ie cementum and enamel did not meet). Once denatured, the dentine would be attacked as a foreign body by elements in the gingival tissue.

Shearer (1984) also reported a case of bleaching-related invasive cervical resorption in which there had been no associated history of trauma. Resorption was detected 16 months after a combination of thermocatalytic and 'walking bleach' techniques.

Cvek and Lindvall (1985), in a retrospective study of root-filled teeth which had been bleached with 30% hydrogen peroxide, found that a number of teeth proceeded to a progressive cervical resorption. Some of these teeth displayed ankylosis, providing evidence of periodontal ligament necrosis. Radiolucency was also noted in the adjacent marginal bone or on the opposite side of the tooth. They postulated that hydrogen peroxide diffusion into the periodontal ligament was aided by the loose adhesion of reparative cementum to dentine (Lindskog et al., 1983). They also believed that those teeth that proceeded from ankylosis to progressive inflammatory resorption did so because of bacterial infection and that these bacteria could have originated from either the pulp chamber or the gingival crevice. This study involved teeth in which silicate cement was used to restore the endodontic access cavity, so the likelihood of a cervical inflammatory resorption is high. The poor marginal seal of the silicate cement could well have allowed bacterial

ingress into the pulp chamber, resulting in cervical inflammatory resorption. Cvek and Lindvall further reasoned that the establishment of ankylosis made it unlikely that denaturation of dentine with subsequent foreign body reaction could account for this type of resorption.

Goon et al. (1986) presented a case of a 15 year-old girl who was treated with a 'walking bleach' procedure only. This report thus served to demonstrate resorption in a case where no heat had been used in the bleaching procedure.

Further experimental evidence has added to the discussion of the causes of bleaching related invasive cervical resorption. Kehoe (1987) demonstrated that the pH of the external cervical root surface decreased after the insertion of 30% hydrogen peroxide into the pulp chamber and coronal root canal of a root-filled tooth. This pH change was then counteracted by the insertion of calcium hydroxide. He suggested that a decrease in the pH caused by the bleaching agent may favour osteoclast activity and may be sufficient to initiate a resorption process. In addition, he proposed that calcium hydroxide should be inserted into the pulp chamber immediately after the bleaching procedure in order to counteract the acidifying effect of the bleaching agent. Madison and Walton (1990) carried out bleaching procedures on the root-filled teeth of dogs *in vivo* and concluded that it was the combination of 30% hydrogen peroxide and applied heat that was associated with the onset of invasive cervical resorption. They suggested that heat drives hydrogen peroxide through dentinal tubules to chemically alter cementum and elicit a foreign body reaction. Like Cvek and Lindvall (1985), they also found that some teeth displayed ankylosis in the cervical region.

1.5 Causes of Tooth Discolouration

Bleaching procedures are carried out on both vital and root-filled teeth to remove discolorations or stains and to restore the tooth to a natural colour. Tooth discoloration may be due to either extrinsic or intrinsic stains, or to intra-canal calcification after traumatic injury to the tooth.

The most common causes of intrinsic tooth staining are :

1. Systemic drugs or chemicals, such as tetracycline or fluoride
2. Trauma, with subsequent haemorrhage into the pulp space
3. Systemic illnesses such as porphyria, postnatal jaundice, erythroblastosis foetalis and vitamin A and D deficiencies.
4. Congenital abnormalities, such as amelogenesis imperfecta
5. Dental medicaments and materials, such as silver-containing root canal sealer or amalgam.

The most common cause of tooth discoloration is pulpal decomposition (Grossman, 1988). The discoloration associated with pulpal decomposition occurs gradually in the ensuing months after pulpal death. Grossman, however, believed that, in traumatically injured teeth, the pronounced dark discoloration was due to pulpal haemorrhage. As a result of the traumatic injury, extravasated blood would be hydraulically forced up into dentinal tubules with subsequent breakdown of its components, particularly haemoglobin. He also considered that hydrogen sulphide from bacteria may combine with haemoglobin to further darken the tooth. In contrast, a study by van Wyk (1987), who examined pink tooth discoloration in post-mortem specimens by

spectrophotometric analysis, found that discoloration was due to intact haemoglobin, not its breakdown products.

1.6 Bleaching of Root-Filled Teeth

Two bleaching techniques have been associated with the occurrence of invasive cervical resorption. These are the thermocatalytic and 'walking bleach' procedures, both of which are procedures that are carried out on root-filled teeth. Many of the reported cases to date have employed a combination of the two.

The thermocatalytic technique requires the use of 30% hydrogen peroxide solution which is delivered by a needle and syringe to a cotton pellet in the prepared pulp chamber of a root-filled tooth. This cotton pellet is saturated with the hydrogen peroxide solution. It is then heated using either a red-hot metal instrument or a photolamp to "activate" the hydrogen peroxide. An ultra-violet lamp can also be used for this purpose. Heating is generally carried out for two to five minute periods for a total of 20-30 minutes per appointment. Several authors have added variations to this technique. Freccia and Peters (1982a) advocated the additional application of hydrogen peroxide on cotton wisps to the external buccal surface of the tooth, while Boksman et al. (1983) suggested the etching of the pulp chamber and root canal walls with 37% phosphoric acid solution prior to bleaching in order to enhance the patency of the dentinal tubules. The flushing of the pulp chamber with EDTA to remove the smear layer prior to bleaching has also been advocated in recent times. Smear layer is a layer of debris covering the root canal walls composed of the organic and mineral components of dentine. It is produced by the contact of instruments with the dentine

surface and is considered to reduce the permeability of the dentine (Pashley, 1984). Removal of the smear layer is achieved by irrigating with EDTA solution which allows better diffusion of hydrogen peroxide through the tooth.

The 'walking bleach' technique was first described by Nutting and Poe in 1960 (quoted in Nutting and Poe, 1967). The original technique employed a cotton pellet saturated in 30% hydrogen peroxide sealed into the pulp chamber between appointments. The bleaching agent would then "walk" through the tooth during this time. Spasser (1961), concerned about the toxicity of hydrogen peroxide, substituted sodium perborate solution using the same technique. It is interesting to note here that, when combined with water, sodium perborate forms sodium metaborate and hydrogen peroxide. Nutting and Poe (1963), in an attempt to promote a synergistic effect, mixed 30% hydrogen peroxide with sodium perborate into a thick paste, which was then sealed into the pulp chamber between appointments. The latter procedure has been generally accepted as the 'walking bleach' procedure widely used in dental clinical practice today.

A method of bleaching of tetracycline-stained teeth was introduced by Abou-Rass (1982) in which teeth that had failed to respond to vital bleaching procedures (bleaching of the external surfaces of the tooth only) were intentionally devitalised and root-filled. The gutta percha was then removed to a level two to three millimetres below the cemento-enamel junction, but no base was placed. Teeth were then treated using a 'walking bleach' technique employing 30% hydrogen peroxide with sodium perborate. Bleaching procedures were repeated depending on the severity of the tetracycline staining present. The results were generally aesthetically favourable and other authors reported similar successes

(Fields, 1982). The procedure is claimed to be safe and predictable. A recent four year longitudinal study (Anitua et al., 1991) showed excellent aesthetic results with no apparent complications. However, Johnson (1985) reported a case of invasive cervical resorption in a tetracycline stained tooth which had been intentionally devitalised and bleached using a 'walking bleach' technique. Montgomery (1983) voiced concern over this technique and pointed out that patients seeking treatment for tetracycline staining are generally young with teeth that probably have wide dentinal tubules that may allow easy passage of bleaching agent to the cervical periodontium, with the attendant risk of initiating invasive cervical resorption.

2. HYDROXYL RADICAL CHEMISTRY

2.1 General

According to Halliwell (1989b), a free radical may be defined as any chemical species which is capable of independent existence with one or more unpaired electrons, where each unpaired electron is alone in its own orbital. Such electrons are able to determine their own direction of spin. Since unpaired electrons tend to create instability within a chemical structure, the presence of the unpaired electron in the radical makes it quite reactive. The fate of such a radical may therefore be to combine with another radical and form a covalent bond, to donate the unpaired electron to another molecule or to steal an electron from another molecule. By donating to, or removing an electron from, another molecule, the original radical is able to produce yet another radical. By virtue of this mechanism, a chain reaction of radical production can occur.

2.2 Oxygen Radicals

The study of radical reactions in chemistry is not new but after the discovery of the enzyme superoxide dismutase (SOD) (Fridovich, 1974), researchers involved in the biological sciences became increasingly interested as it became clear that oxygen radicals were intrinsic to many biological processes and were strongly implicated in a number of human disease processes.

Superoxide dismutase is produced by the body to remove an oxygen radical known as the superoxide radical ($O_2^{\cdot -}$). which is created by the addition of an extra electron to the oxygen molecule. This situation

may occur in the body when electrons accidentally leak from the electron transport chain during respiration in mitochondria or may be intentionally produced from molecular oxygen by activated phagocytes during the killing of bacteria. Host defence cells such as macrophages, neutrophils, eosinophils and monocytes all depend on the action of oxygen radicals to kill ingested micro-organisms (Curnutte and Babior, 1987). There are, in fact, a number of species based on the oxygen atom or molecule. Those with unpaired electrons have been termed oxygen-derived free radicals. This group includes the superoxide radical and the hydroxyl radical (·OH). Atomic oxygen, also termed singlet oxygen ($O\cdot^{-}$), is itself considered an oxygen radical, although oxygen usually exists in the diatomic state. Those oxygen-containing molecules associated with this group, but with paired electrons, are termed reactive oxygen species (Halliwell, 1989a). Examples of the latter include hydrogen peroxide and hypochlorous acid. Hypochlorous acid is also produced by neutrophils, catalysed by the enzyme myeloperoxidase, for the killing of bacteria. The terminology described here will be used throughout this report.

While the use of oxygen radicals is a highly effective mechanism by which phagocytes are able to kill micro-organisms, they are also able to destroy host tissue. The removal of superoxide radical is achieved by the enzyme superoxide dismutase and the reaction by which this occurs results in the production of hydrogen peroxide and oxygen :

$$O_2\cdot^{-} + O_2\cdot^{-} + 2\,H^{+} \longrightarrow H_2O_2 + O_2$$

The reaction of oxygen radicals on host tissue and the ensuing tissue damage has been implicated as being either the basis of, or at least

strongly associated with, a number of human disease processes. Hydrogen peroxide itself is capable of cellular damage, causing such changes as damage to cellular DNA, membrane lipid peroxidation and cell death (Weitzman, 1985). The role of oxygen radicals and associated reactive oxygen species in tissue damage will be discussed in greater detail later in this review.

The enzyme superoxide dismutase is also able to remove hydrogen peroxide in conjunction with the blood enzymes, catalase and glutathione peroxidase. In the presence of transition metals such as iron or copper, however, the conversion of superoxide to hydrogen peroxide may be rapidly catalysed to cause the generation of another oxygen radical, the hydroxyl radical, in a reaction known as the Fenton reaction. Transition metals are able to exist in several valency states and so are able to donate or receive electrons in oxidation or reduction reactions.

2.3 The Hydroxyl Radical

The hydroxyl radical is considered one of the most reactive chemical entities known to science in that it is capable of reacting with virtually any molecule. Hydroxyl radicals have also been implicated in a number of human disease states and these will be reviewed later in this report.

Homolytic fission of the O-O bond in hydrogen peroxide will yield two hydroxyl radicals (·OH). Such homolysis can be achieved by both heat and ionising radiation, such as ultraviolet radiation (Halliwell, 1989a).

The simple addition of hydrogen peroxide to ferrous salt (Fe^{2+}) also forms hydroxyl radical. This was first observed by Fenton in 1894,

when he described the powerful oxidising properties of a mixture of hydrogen peroxide and ferrous salts. The classic Fenton reaction was conducted with the reagents at molar concentrations and in acidic media. Acidification was necessary to prevent the precipitation of a ferric salt. Haber and Weiss (quoted by Cohen, 1984) subsequently established that the oxidising chemical species in the Fenton reaction was the hydroxyl radical. These early inorganic chemical experiments were important because they helped establish the concept of free radical intermediates in chemical reaction mechanisms.

Haber and Weiss proposed that superoxide radical and hydrogen peroxide were capable of reacting directly to form the hydroxyl radical (the Haber-Weiss reaction). This reaction was therefore proposed in later years as the mechanism of hydroxyl radical generation from superoxide in biological systems. Although this reaction was thermodynamically possible, it was found that in biological systems they occur at rates that are too slow to be significant (Halliwell, 1978a). Subsequent investigations established that it was iron in the ferrous state that catalysed the production of hydroxyl radical from superoxide and hydrogen peroxide (Halliwell, 1978b). This reaction also appears to be subject to various modifying factors. Baker and Gebecki (1986), for example, found that hydroxyl radical production in biological systems was significantly affected by pH. Acidic conditions increased the yield of hydroxyl radicals in such systems.

The Fenton reaction therefore is :

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \cdot OH + OH^-$$

The products of the reaction are the ferric ion, the hydroxyl radical and the hydroxyl ion. From a strictly chemical view, cuprous, and indeed titanous ions, may also substitute for the ferrous ion since they are also transitional metal ions.

Fe^{3+} can further react :

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + O_2{\cdot}^- + H^+$$

More reactions are then possible :

$$\cdot OH + H_2O_2 \longrightarrow H_2O + H^+ + O_2^-$$

$$O_2{\cdot}^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2$$

$$\cdot OH + Fe^{2+} \longrightarrow Fe^{3+} + OH^-$$

Thus, a simple mixture of Fe^{2+} salt with hydrogen peroxide can provoke a series of oxygen radical reactions, the overall equation being :

$$2H_2O_2 + Fe^{2+} \longrightarrow 2\,H_2O + O_2 + Fe^{3+}$$

From the above discussion, it is clear that iron in the ferrous state is capable of causing the production of hydroxyl radicals from hydrogen peroxide. Hydrogen peroxide in human tissues, in disease states or as the result of traumatic injury to tissue, is produced by the action of superoxide dismutase on the superoxide radical which may have been produced by activated phagocytes. The question of the availability of iron for such a reaction has been the subject of intensive biochemical research in the last decade.

2.4 Availability of Iron in Biological Systems

The average adult male contains approximately 4 - 5 grams of iron, absorbs approximately one milligram per day from the diet and excretes the same amount. Females lose more iron through menstrual loss.

Two-thirds of the body's iron is found in haemoglobin, with smaller amounts in myoglobin, various enzymes, or in transport or storage proteins. Under normal conditions, iron is taken up from the gut and is transferred in plasma by transferrin which acts as a carrier protein (Guyton, 1976). Normally, transferrin in blood is only 30% loaded with iron, so that there is significant capacity for greater iron transport. Iron which is not required for the major consumers, haemoglobin and myoglobin, is stored intracellularly in either ferritin or haemosiderin. Haemosiderin is an insoluble product formed by the action of lysosomes on ferritin (Halliwell and Gutteridge, 1985b). Much discussion has been conducted in the biochemical literature as to the possible sources of iron in biological systems which may make it available for a Fenton reaction and thus able to catalyse the production of tissue-destructive hydroxyl radicals. From the above discussion, it can be seen that in normal body systems, there are a number of potential sources of iron for such a reaction. These will now be considered.

2.4.1. Haemoglobin.

Whole red blood cells contain SOD, catalase and glutathione peroxidase and can act as sinks for superoxide radical and hydrogen peroxide. In the presence of hydrogen peroxide, haemoglobin has been reported to release iron which can then take part in the Fenton reaction and generate hydroxyl radicals (Gutteridge, 1986). This reaction resulted in the destruction of DNA. In this study, it was shown that it was released iron, not an iron-haem complex, that resulted in the DNA damage. Methaemoglobin has also been reported to be release iron for similar reactions (Puppo and Halliwell, 1988). It has been proposed that

this mechanism may account for the damaging effects of free haemoglobin at sites of inflammation. At present it is considered that haemoglobin represents the most likely source of iron available for the Fenton reaction in body systems.

2.4.2. Transferrin.

Transferrin is a plasma protein that binds two moles of ferrous iron per mole of protein with a very high affinity at physiological pH. Since it only carries approximately 30% of its capacity in normal conditions, there is little free iron available in body fluids or tissues. This in itself is a bacteriostatic mechanism. In such conditions as haemochromatosis, the iron transport system is overloaded and transferrin is saturated with iron (Woolf, 1986). It is proposed that much of the tissue destruction that occurs in such iron overload diseases is mediated by iron-catalysed hydroxyl radical production. At a pH of six or less, iron becomes less tightly bound to transferrin. This may be of significance when considering iron availability in the presence of hydrogen peroxide in acidic media.

2.4.3. Ferritin

Iron can also be released from the ferritin molecule by hydrogen peroxide, superoxide radical and by organic oxides (Biemond et al., 1984; Gutteridge, 1985). Haemosiderin, the insoluble product of ferritin, also represents a potential source of iron but is considered a far less significant contributor to the *in vivo* generation of hydroxyl radicals (Halliwell and Gutteridge, 1985a). Haemosiderin is also produced by the action of cells of the reticulo-endothelial system on haemoglobin. As discussed earlier,

Grossman (1988) has suggested that haemosiderin, as a breakdown product of haemoglobin, contributes to tooth discoloration after trauma to teeth. However, because the discoloration occurs in non-vital teeth, which, by definition, have no blood supply, one must question the availability of the reticulo-endothelial cells which could act on haemoglobin to produce haemosiderin.

2.4.4. Intracellular iron pool.

This consists of iron bound to chelators of low molecular mass such as citrate, ATP and ADP. On an intracellular basis, this iron is capable of catalysing the formation of hydroxyl radicals from hydrogen peroxide.

2.5 Iron Chelates

Another significant phenomenon associated with the iron-catalysed production of hydroxyl radicals is the function of iron chelates. Halliwell (1978) demonstrated that various molecules which could chelate iron were able to reduce the ability of iron to contribute to the Fenton reaction. Thus, chelators such as diethylamine penta-acetic acid (DETAPAC) and desferri-oxamine were able to inhibit hydroxyl radical production in systems which were known to promote iron catalysis of the Fenton reaction. It should be noted here that any mechanism which prevents hydroxyl radical production will also prevent the chain reaction which produces other toxic species. Desferrioxamine is used therapeutically in iron overload diseases and its effect may be to reduce the possibility of iron-catalysed hydroxyl radical production. Conversely, a chelator which is used widely in the practice of endodontics, namely ethylenediamine tetra-acetic acid (EDTA), may in fact accelerate

hydroxyl radical production. Halliwell (1978) demonstrated that EDTA certainly had no inhibiting effect. According to Cohen (1984), a combination of iron and EDTA is exceptionally unstable and is capable of autoxidation (the rapid oxidation of ferrous ions or ferrous chelates by molecular oxygen) with the concurrent generation of hydroxyl radicals. Thus, the spontaneous autoxidation of iron-EDTA in phosphate buffer is complete in less than 30 seconds at neutral pH. The initial source of oxygen in the autoxidation of ferrous ions in an aqueous Fe^{2+}-EDTA solution is probably atmospheric oxygen, with subsequent electrophilic attack on water molecules. This produces hydroxyl radicals which can then attack substrate molecules such as salicylate :

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2{}^{\cdot -}$$

$$O_2{}^{\cdot -} + H_2O \longrightarrow {\cdot}OH + {}^{-}OOH$$

This, of course, is a simplification of a most complex series of chemical reactions which are not yet fully understood by the chemistry community. This applies to much of the discussion of oxygen radicals. Despite intensive research into the *in vivo* action of oxygen radicals, there are still large areas of uncertainty with the basic chemical reactions involving them.

The apparent influence of EDTA on hydroxyl radical production through iron autoxidation may hold implications for teeth discoloured by iron salts and which are exposed to concentrated solutions of EDTA during the course of dental treatment, including intracoronal bleaching.

3. HYDROXYL RADICALS AND TISSUE DAMAGE

3.1 General

Hydroxyl radicals have been described as one of the most reactive chemical entities known to science (Halliwell, 1989a). Whereas other reactive oxygen species such as superoxide radical and hydrogen peroxide may diffuse some way before reacting with tissue structures, hydroxyl radicals, by virtue of their extreme reactivity will react with the first molecules they meet. This includes virtually every molecule found in living cells such as sugars, amino acids, phospholipids, deoxyribosenucleic acid (DNA) bases and organic acids. Secondary radicals are then produced, causing a chain reaction of radical production. Thus, only tissue structures in the immediate vicinity of hydroxyl radical generation will be directly affected by the hydroxyl radical itself. The average radius of diffusion for hydroxyl radicals will therefore be quite small and has been estimated as being less than 100 nm (Slater, 1984), while the half-life of the hydroxyl radical in biological systems has been calculated as only a few microseconds.

Hydroxyl radicals exert their tissue-damaging effects on several levels. On a molecular level, there are three main types of reactions caused by hydroxyl radicals. These are :

1. Hydrogen atom abstraction. This appears to be a major mechanism facilitating lipid peroxidation of cell membranes and will be discussed in greater detail later.
2. Addition. The ability to add on to aromatic ring structures, such as purine and pyrimidine bases in DNA.

3. Electron transfer. Transfer of electrons from such moieties as the chloride ion :

$Cl^- + \cdot OH \longrightarrow Cl\cdot + OH^-$

On a cellular level, hydroxyl radicals may cause damage through :

1. Damage to DNA. Free hydroxyl radicals cause chain reactions which propagate through cellular DNA to cause strand breakage. Alterations to DNA bases, purine and pyrimidine, are also possible through the addition reactions discussed previously. This alteration to DNA may result in cellular mutations and has been postulated as a mechanism involved in carcinogenesis.

2. Cell membrane destruction. The most significant mechanism through which this process occurs is the process of lipid peroxidation. Hydroxyl radicals generated close to cell membranes attack fatty acid side chains of membrane phospholipids (Halliwell, 1989a). Fatty acid side chains with several double bonds, such as arachidonic acid, are attacked preferentially. The hydroxyl radical is able to abstract a hydrogen atom from one of the carbon atoms of the side chain and combines with it to form water molecules. The reaction leaves a carbon centred radical (- C· -) in the cell membrane, which usually rearranges to form a conjugated diene structure. If two radicals collide in the cell membrane, cross-linking of the fatty acid side chains would occur as the two unpaired electrons combine to form a covalent bond. Under normal physiological conditions, however, the most likely fate of the carbon-centred radical is to combine with oxygen to create a peroxyl radical. These radicals in turn also attack fatty acid side chains by abstracting hydrogen to produce other carbon-centred radicals as well as lipid

hydroperoxides. Lipid hydroperoxides in turn can further decompose to yield a range of highly cytotoxic products such as such as aldehydes. Peroxyl radicals and cytotoxic aldehydes are also capable of damaging membrane proteins leading to inactivation of various membrane receptors and membrane bound enzymes to cause protein cross-linking. One hydroxyl radical can result in the production of hundreds of other radicals being formed in this manner with ensuing cell membrane damage. The overall results of cell membrane lipid peroxidation can include loss of membrane fluidity, a fall in membrane potential, increase in permeability to ions, and eventual membrane rupture. With rupture of the cell membrane, cellular contents such as lysosomal hydrolytic enzymes are released, leading to interstitial tissue damage in the vicinity of the damaged cell.

Hydroxyl radicals have also been shown to have a destructive effect on the components of connective tissue. Greenwald and Moy (1980) demonstrated that hydroxyl radicals and superoxide radicals had the ability to dramatically reduce the viscosity of hyaluronic acid. These experiments implicated these oxygen radicals in the depolymerisation of hyaluronic acid, which is a major component of ground substance in connective tissues.

Curran et al. (1984) exposed purified Type I collagen to hydroxyl radicals which had been generated by the addition of ferrous iron salts to hydrogen peroxide and found that not only could hydroxyl radicals directly degrade soluble collagen but that, at relatively low levels, could potentiate the destructive role of proteinases by increasing the susceptibility of collagen to them. The authors also made the point that the destruction of extracellular matrix components of connective tissue,

such as collagen, generates peptides which are known to be chemotactic for inflammatory cells.

Rowley and Halliwell (1983) demonstrated that superoxide radical and hydrogen peroxide reacted in the presence of ferrous salts to form hydroxyl radicals, which in turn attacked hyaluronic acid. The authors also found that the reducing agent, ascorbic acid, was able to interact with hydrogen peroxide and ferrous salts to produce hydroxyl radicals independent of superoxide radical. They believed this was due to the reduction of ferric ions in the area to ferrous ions by the ascorbic acid which then enabled the ferrous ion catalysis of hydroxyl radical production from hydrogen peroxide.

Simon et al (1981) generated hydroxyl radicals through the enzymatic action of xanthine oxidase on acetaldehyde and demonstrated that human fibroblasts exposed to such a radical flux were killed. Agents that reduced the concentration of hydrogen peroxide in the area, and hence the generation of hydroxyl radicals, were able to reduce the degree of fibroblast cell destruction.

3.2 Role of Transition Metals in Tissue Damage Caused by Hydroxyl Radicals

As described in Section 2.2, transition metals, such as iron and copper are good promoters of hydroxyl radical production in the presence of hydrogen peroxide. As such, they are able to participate in the initiation of reactions like lipid peroxidation. Transition metal ions which are added to lipid systems already containing traces of peroxides are also able to decompose these peroxides into peroxyl and alkoxyl (organic oxygen) radicals that can themselves abstract hydrogen and perpetuate

the chain reactions which contribute to membrane lipid peroxidation (Halliwell, 1989b). Being more stable than oxygen radicals, and able to cross membranes, these alkoxy radicals can extend and prolong tissue damage. As described in the previous section, reducing agents such as ascorbic acid can accelerate these metal ion dependent peroxidation reactions because the reduced forms of the ion reacts more quickly than the oxidised form.

3.3 Cell Damage Caused By Hydrogen Peroxide

It is apparent that hydrogen peroxide is a normal product in oxidative metabolism as well as being produced in the phagocytosis and killing of bacteria. (Weitzman et al., 1985). In recent years, much research has been devoted to investigating the role of hydrogen peroxide itself in tissue destruction. Hydrogen peroxide has no unpaired electrons and therefore is not an oxygen radical. It can easily pass through cell membranes because it is uncharged (Halliwell and Gutteridge, 1985a). Exposure to concentrated doses of hydrogen peroxide will result in rapid cell death (Weitzman, 1985). Vercellotti et al. (1991) recently reported that the exposure of endothelial cells to a "bolus" of hydrogen peroxide could induce a range of cell defects and changes that culminated in cell death. These changes included activation of the hexose monophosphate shunt, oxidation of intracellular glutathione (a hydrogen peroxide scavenger), and fall in cellular ATP levels. These were followed by an increase in intracellular calcium and sodium ion levels, cytoskeletal alterations, cell membrane bleb formation, and finally cell lysis. DNA damage could be induced within seconds of exposure to hydrogen peroxide, resulting in stimulation of poly-adenosine diphosphate ribose polymerase, which

caused an immediate fall in nicotinamide adenine dinucleotide (NAD) levels and interference with ATP synthesis. Of interest in the study of Vercellotti et al. was that hydrogen peroxide could also induce cellular alterations short of cell lysis. The authors postulated that alterations may include enhanced glutathione flux, stimulation of the hexose monophosphate shunt and possibly a fall in cellular ATP. A more intense exposure of the cell to hydrogen peroxide could alter DNA structure with possible late effects on cell growth and repair. This is supported by the findings of Schruafstatter et al. (1988) who demonstrated that DNA exposed to levels of hydrogen peroxide at concentrations of 10^{-4} to 10^{-5} mol/l undergoes strand breakage.

Klein-Szanto and Slaga (1982) displayed that hydrogen peroxide caused weak but definite tumour-promoting activity in mouse skin, while Weitzman et al. (1986) found that when hydrogen peroxide alone was applied to the buccal mucosa of hamsters, it was able to induce pathological changes frequently associated with pre-neoplastic lesions and that, when combined with a known carcinogen, hydrogen peroxide was able to augment the carcinogenic effect.

3.4 Body Defence Mechanisms Against Oxygen Radical Destruction

As described previously, various enzymes function in the body to remove many of the oxygen-derived free radicals. Superoxide radical is detoxified by the enzyme superoxide dismutase to produce hydrogen peroxide. Hydrogen peroxide in turn is scavenged by enzymes such as catalase and glutathione peroxidase. Of these two, the latter probably exerts the most significant influence (Halliwell, 1989a). The removal of

both superoxide and hydrogen peroxide will decrease the generation of hydroxyl radicals.

Removal of the ferrous form of iron will also inhibit the production of hydroxyl radicals. The body is able to limit the presence of free ferrous iron through the binding of these ions to proteins such as transferrin, which transports iron, as well as to iron storage proteins such as ferritin. Since transferrin normally carries 30% its potential capacity of iron, in situations where free iron occurs in the body, transferrin is able to dramatically increase the amount of iron bound to it. This then limits the amount of free iron which can be used to catalyse the production of hydroxyl radicals from hydrogen peroxide.

Another means of limiting the cell damage initiated by oxygen radical attack on cell membranes with subsequent lipid peroxidation of the phospholipid bilayer is the action of anti-oxidants. Alpha-tocopherol, commonly called vitamin E, and ascorbic acid (vitamin C) are both capable of minimising the consequences of lipid peroxidation by acting as "chain breaking oxidants". Alpha-tocopherol is a lipid-soluble molecule that resides in the interior of the phospholipid membrane and which has a hydroxyl group which can easily lose a hydrogen atom. When peroxyl or alkoxyl radicals are produced after hydroxyl radical attack on fatty acid side chains (which initiates the lipid peroxidation chain reaction), alpha-tocopherol is preferentially attacked causing termination of the chain reaction (Halliwell, 1989a). There is some evidence to suggest that the tocopherol radical produced in this way can then migrate to the surface of the membrane, where it is converted by ascorbic acid back to alpha-tocopherol (Halliwell, 1989b). It is probable that anti-oxidants, like tocopherol, which are not potent reducing agents (like ascorbic acid) will

lack the undesirable action of reducing ferric ions to ferrous ions and thus making them available for the Fenton reaction (see Section 3.2).

3.5 Therapeutic Agents Opposing Oxygen Radical-Induced Tissue Damage

A number of disease states in the human body have been either ascribed to, or at least significantly associated with, the activity of oxygen radicals on tissue. The hydroxyl radical has been particularly heavily investigated, especially regarding its relationship to diseases such as rheumatoid arthritis, atherosclerosis and cancer. The role of hydroxyl radicals in iron-overload diseases, such as that occurring in thalassaemia, has also gained attention.

Much of the study carried out into the destruction of connective tissue components, such as collagen and hyaluronic acid, has been done using rheumatoid arthritis as the disease model. A recent study by Tiku et al. (1990) has supported the view that oxygen radicals play a direct role in the aetiology of cartilage matrix degradation in rheumatoid disease. The treatment of rheumatoid arthritis has employed a number of therapeutic agents. Betts and Cleland (1982) demonstrated that penicillamine as well as salicylate, gold sodium thiomalate and chloroquine, all inhibited the degradation of hyaluronic acid caused by hydroxyl radicals, which were generated either directly by the addition of ferrous salts to hydrogen peroxide or enzymatically by the addition of xanthine oxidase to hypoxanthine. Penicillamine, salicylate, gold sodium thiomalate and chloroquine are all therapeutic agents used in the treatment of rheumatoid arthritis. These agents appeared to have a

similar potency to known hydroxyl scavengers such as mannitol and benzoic acid. Carlin et al. (1985) studied the inhibitory effect of various anti-inflammatory drugs on the depolymerisation of hyaluronic acid by enzymatically-generated hydroxyl radicals and found that salicylic acid, acetyl-salicylic acid and gentisic acid (2,5-dihydroxybenzoic acid) effectively reduced the production of oxygen radicals, including hydroxyl radicals. Disease states involving iron-overload include haemochromatosis and thalassaemia. In the latter, iron overloading occurs due to the necessity for multiple blood transfusions. In both these conditions, iron-binding proteins such as transferrin and ferritin, are saturated and the excess iron is available to catalyse the production of hydroxyl radicals with resultant tissue damage. A drug which appears to have been quite successful in the treatment of such conditions is the chelating agent, desferrioxamine. This agent appears to be most effective in preventing iron-dependent hydroxyl radical production as well as being a powerful inhibitor of iron-dependent lipid peroxidation (Halliwell and Gutteridge, 1986). The drug appears to be safe in therapeutic doses of 50-60 mg/kg of body weight, but in higher doses has been associated with ocular abnormalities and auditory changes.

Two other areas of human tissue damage which may benefit from the use of iron-chelating agents such as deferrioxamine and anti-inflammatory agents, such as salicylic acid, are those of reperfusion injuries and traumatic (especially crushing) injuries.

In situations of ischaemic injury, such as after myocardial infarct, it has been shown that, provided the ischaemia itself does not cause irreversible damage, further tissue damage may actually occur through oxygen radical production when the ischaemic tissue is reperfused by

blood. Reperfusion injuries are now recognised as primarily oxygen radical-induced tissue damage (Braughler, 1987). If anti-oxidants are included in the re-oxygenation medium, tissue function is better preserved. The use of anti-oxidants in the preservation of organs which are being transplanted is also gaining increasing attention (Halliwell, 1989a).

During traumatic or crushing injuries, cells rupture and their contents, including intracellular iron, are released. In addition, bleeding in the area provides a potential source of iron-containing haemoglobin. If this iron is available to catalyse the production of hydroxyl radicals, then further tissue damage may occur through the various hydroxyl radical mechanisms described earlier. Animal studies using iron-chelating agents, such as the aminosteroid-based iron chelator U74006F, have demonstrated the prevention of catalysis of radical reactions in such situations (Hall and Yonkers, 1988).

Existing therapeutic agents including chelators like desferri-oxamine and anti-inflammatory agents such as salicylic acid may have application in various areas of dentistry, particularly in the treatment of traumatic injuries affecting teeth. The management of the exarticulated tooth, for example, very much depends on maintaining the vitality of periodontal ligament (PDL) cells. It is possible that further damage is done to these cells on replantation of the tooth during subsequent reperfusion of the tissue, mediated by oxygen radicals. Perhaps anti-oxidant or anti-inflammatory agents may be of benefit in preventing possible oxygen radical-induced damage to these tissues.

4. DETECTION OF HYDROXYL RADICALS

4.1 Methods Used in the Detection of Hydroxyl Radicals

Numerous methods have been employed in the detection of hydroxyl radicals over the years. Early research was conducted into hydroxyl radical generation in the context of inorganic chemistry. Different techniques evolved with the need to investigate and detect hydroxyl radicals generated in biological systems. Research into the production of hydroxyl radicals in human disease has required the development of techniques employing reagents which are less toxic to the analyst and to the experimental models themselves.

A list of techniques used to detect hydroxyl radicals includes:

1. Hydroxylation of phenols (aromatic hydroxides). Aromatic compounds react extremely well with hydroxyl radicals (Halliwell and Gutteridge, 1985b). The reaction products are identified by either gas-liquid chromatography, colourimetric reactions or by their fluorescence spectra. This group includes a technique whereby the hydroxylation of salicylic acid (a phenolic acid) is used to detect the presence of hydroxyl radicals. This is the technique employed in the present study and will be discussed in greater detail in the next section.

2. Conversion of methional (CH_3-S-CH_2-CH_2-CHO) and related compounds into ethylene gas (DiGuiseppi and Fridovich, 1984). Methional reacts with hydroxyl radicals to form ethylene gas which may be measured by gas-liquid chromatography (Halliwell and Gutteridge, 1985b).

3. Use of characteristic electron spin resonance signals, with or without spin traps. Electron spin resonance (ESR) detects the presence of unpaired electrons in the radical. An unpaired electron acts like a small magnet. When it is exposed to an external magnetic field, the electron can align itself such that it has one of two possible energy levels. If electromagnetic radiation of the correct energy is applied, it will be absorbed and used to move the electron into the higher energy level which produces an absorption spectrum. The number of lines in the ESR spectrum is called the hyperfine structure and the radical can be identified by this structure (Halliwell and Gutteridge, 1985b). Spin traps can also be used to identify hydroxyl radicals. In this technique, the reaction of the free radical with nitrones or nitroso-compounds (spin traps) results in the production of "long-lived" nitroxide free radicals which can be detected using conventional ESR spectrometry. Splitting (analysis) of the hyperfine structure then allows identification of the radical (Britigan, 1986).

4. Decarboxylation of radio-labelled benzoic acid. In this technique (Sagone et al, 1980; Winston and Cederbaum, 1982), benzoic acid with radio-labelled ^{14}C in the carboxyl group is attacked by hydroxyl radicals to release radioactive carbon dioxide, which is detected using a scintillation counter. The amount of radioactive carbon dioxide detected is considered a measure of the production of hydroxyl radicals and their subsequent reaction with radiolabelled benzoic acid.

5. Conversion of dimethylsulphoxide into formaldehyde by interaction with hydroxyl radicals.

6. Degradation of deoxyribose into products that form a colour with thiobutyric acid (TBA) (Halliwell and Gutteridge, 1985a)

Halliwell and Grootveld (1987) have suggested the possibility of detection of hydroxyl radicals *in vivo* by virtue of their reaction with phenylalanine. Hydroxyl radicals attack phenylalanine to produce ortho- , para- and meta-tyrosine. This approach appears to be less toxic to cells than salicylate and does not interfere with arachidonic acid metabolism.

Other hydroxyl radical detection techniques are becoming available which do not require the use of such toxic agents as benzene or the various nitroso compounds necessary for spin-trapping. These include assaying for the products of DNA oxidative damage after attack by hydroxyl radicals, ultra-violet measurement of diene conjugates (produced by hydroxyl radical attack on phospholipid membranes) as well as the detection of end products of cell membrane lipid peroxidation such as toxic aldehydes (Halliwell and Gutteridge, 1985b).

These techniques all have inherent limitations. Spin trapping, for example, requires the use of specialised equipment and is prone to artifacts (Richmond et al., 1981). Oxygen radicals other than hydroxyl radicals are capable of reactions with both tryptophan or methional.

4.2 Hydroxyl Radical Detection Using Hydroxylation of Salicylate

The hydroxylation of salicylate has gained increasing acceptance over the last decade as a very sensitive and relatively simple method of hydroxyl radical detection which employs less toxic compounds than

those techniques using benzene or the nitroso compounds used for spin-trapping.

Halliwell (1977) was one of the first to suggest that the hydroxylation of salicylate can be brought about by hydroxyl radicals generated from hydrogen peroxide in the presence of trace amounts of ferrous iron. Richmond et al. (1981) reported the measurement of hydroxyl radicals by the hydroxylation of salicylate and concluded that both superoxide radical and hydrogen peroxide interacted in the presence of iron salts to produce hydroxyl radicals. This study relied on the colorimetric detection of the reaction products after high performance liquid chromatographic (HPLC) separation, but the authors suggested that electrochemical detection (ECD) may have proved to be a more sensitive detection mechanism.

Grootveld and Halliwell (1986) detailed methods by which HPLC with ECD could be used to analyse the reaction products of salicylate after attack by hydroxyl radicals and thus detect the generation of hydroxyl radicals. In this study, hydrogen peroxide was mixed with a ferrous salt in phosphate buffer at pH 7.4 to produce hydroxyl radicals in the presence of salicylate. Three reaction products were detected and measured. These were 2,3-dihydroxybenzoate (2,3-DHB), 2,5-dihydroxybenzoate (2,5-DHB) and a minor product, pyrocatechol (1,2-dihydroxybenzene), in proportions of 49%, 40% and 11% respectively.

The study by Grootveld and Halliwell (1986) was perhaps encouraged by the findings of Cleland et al. (1985) who reported the presence of 2,5-DHB as a metabolite of salicylate in the plasma of patients with rheumatoid arthritis and who were taking anti-inflammatory doses of aspirin. From this, it was considered that the hydroxylation of

salicylate may have proved to be an effective assay for hydroxyl radical production occurring *in vivo*. In a recent article, however, it has been reported that, *in vitro*, the P450 cytochrome system is able to catalyse the NADPH-dependent hydroxylation of salicylate to 2,5-DHB in rat and rabbit hepatic microsomes (Halliwell et al., 1991). Since 2,3-DHB was reported not to be produced in this reaction, its presence is suggested as a better test of hydroxyl radical production.

Salicylate is reported to react with hydroxyl radicals with a rate constant of between 5×10^{-9} and 10^{-10} $M^{-1}s^{-1}$ (Hiller, 1983, as quoted in Grootveld and Halliwell, 1986). The reaction of hydroxyl radical with salicylate is shown in Figure 4.1 and is discussed in detail in Appendix 3.

Sagone et al. (1987) examined the attack on salicylate caused by the stimulation of granulocytes which produced a range of oxygen radicals. The experiments conducted in this study revealed that catalase (a hydrogen peroxide scavenger) had limited effect on the hydroxylation of salicylate, indicating that hydrogen peroxide had no direct effect on the salicylate. Direct hydroxylation of the salicylate by superoxide radical and hypochlorous ion was also excluded. It was concluded that it was the hydroxyl radical which was responsible for the hydroxylation of salicylate.

Davis et al. (1989) also proposed that activated granulocytes (neutrophils) represented a pathway for salicylate hydroxylation. Neutrophils were incubated in salicylate solution which was then separated by HPLC-ECD. Both 2,5-DHB and smaller quantities of 2,3-DHB were detected, suggesting that neutrophils generated hydroxyl radicals. This contrasts with the amounts of reaction products suggested by Grootveld and Halliwell (1986), who claimed that more 2,3-DHB was

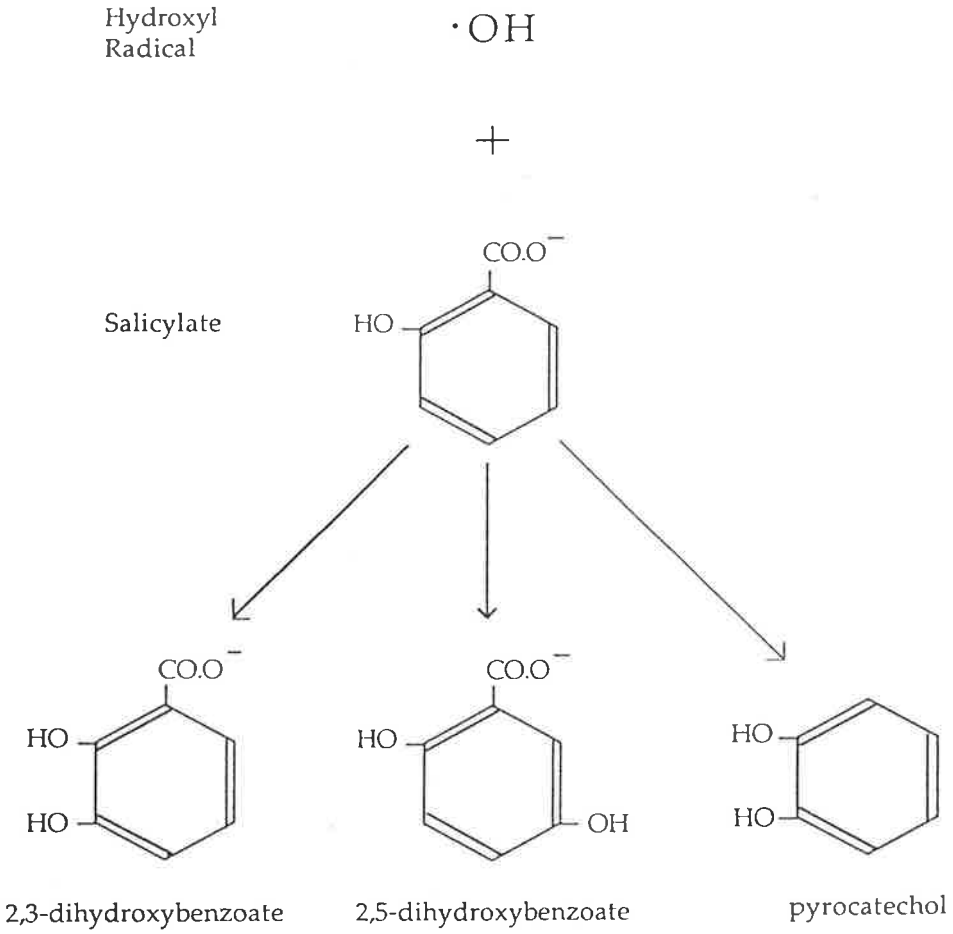

Figure 4.1 Reaction products of the attack by hydroxyl radicals on salicylate. The two major reaction products are 2,3-DHB and 2,5-DHB. Pyrocatechol is also produced as a minor reaction product (Grootveld and Halliwell, 1986).

produced than 2,5-DHB. The authors further proposed that, while the main anti-inflammatory effect of salicylates may be attributed to an inhibition of the cyclo-oxygenase pathway, it may also depend on the metabolism of salicylate at the site of inflammation and may actually serve to protect tissue from hydroxyl radical damage. In addition, the metabolites of salicylate themselves may be anti-inflammatory. Thus, although salicylate metabolism occurs mainly in the liver via microsomal pathways, it is possible that hydroxylation of salicylate can occur *in vivo* at local sites of inflammation. For example, 2,3-DHB is able to chelate iron, thus reducing its availability for hydroxyl radical production and participation in lipid peroxidation. It is also a moderately effective scavenger of hydrogen peroxide (Graziano et al., 1974). The reaction product 2,5-DHB is a potent inhibitor of prostaglandin synthesis (Holmes et al., 1985). Capelle et al. (1992), investigated the scavenging ability of salicylic acid and its hydroxylated metabolites on hydroxyl radicals and found that pyrocatechol was 6600 times more potent than 2,3-DHB and 2,5-DHB at scavenging this radical.

The use of salicylate hydroxylation as a measure of hydroxyl radical activity has served to demonstrate the link between the generation of this oxygen radical and various human disease states. Powell and Hall (1991) used HPLC-ECD detection of 2,3-DHB and 2,5-DHB in a perfusate containing salicylate as evidence of hydroxyl radical production in re-perfused ischaemic myocardium in rat hearts. The results indicated that salicylate could be used as a very sensitive probe for hydroxyl radical production in such a system. Das et al. (1991) perfused ischaemic heart muscle with salicylic acid during re-perfusion. Both 2,3-DHB and 2,5-DHB were detected using HPLC-ECD. From this, the authors concluded

that hydroxyl radicals were produced during heart muscle re-perfusion after ischaemia.

The technique of salicylate hydroxylation, while being sensitive and relatively simple, may also suffer from some limitations. Harkin et al. (1991) demonstrated that even brief (one second) exposures of salicylate solutions to ultra-violet light are sufficient to cause the production of hydroxyl radicals and that the same reaction products of the hydroxyl radical with salicylate (2,3-DHB and 2,5-DHB) are detectable by HPLC-ECD. The exposure of test solutions of salicylate may therefore be affected by exposure to ambient U.V radiation.

4.3 Theory of High Performance Liquid Chromatography (HPLC)

Chromatography is defined by Heftman (1975) as a method of analysis in which a flow of solvent liquid or gas 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.

The first chromatographic separations were described by the Egyptians as long ago as 1500 B.C in techniques employed for the separation of dyes in papyrus. In 1906, the Russian botanist Tswet coined the term chromatography and the process has been refined and improved to a point where it is now an important part of chemical and biochemical analysis.

Liquid chromatography, where solvent is passed over the sorptive medium in a cylinder or column, can be divided into :

1. Classical or conventional liquid chromatography (LC)

2. High performance liquid chromatography (HPLC) - sometimes also referred to as high pressure liquid chromatography. HPLC employs more sophisticated instruments, exhibits greater column efficiency and uses extremely sensitive detectors leading to higher resolution.

The basis of HPLC is the passing of a liquid (mobile phase) through a chromatographic column, packed with sorbent particles, along which solutes within the mobile phase are separated. These solute bands can be detected at the end of the column by a variety of means. Usually either electrochemical detection or ultra-violet spectrometry are employed. The form of HPLC used in this research project was reverse-phase chromatography. Reverse-phase chromatography separates molecules based upon their degree of hydrophobicity. Columns used in reverse-phase chromatography generally employ porous, modified silica particles as the sorbent support matrix within the column, but various synthetic polymers may also be used.

Separation of moieties in reverse-phased chromatography therefore occurs by differential hydrophobic interactions of the experimental sample (solute) with the hydrophobic functional groups along the column matrix. These functional groups may either bind weakly or strongly to solute molecules and this determines the rate at which the molecules pass along the column. Thus, sample molecules which are polar or only mildly hydrophobic will react only slightly with the functional groups along the column and their retention time on the column (elution time) will be less than that of hydrophobic molecules which will bind more strongly with column matrix functional groups and be held longer. As a

result, hydrophobic molecules will elute with a greater retention time. As molecules come off the column they can be detected, usually by either ultra-violet spectroscopy or by electro-chemical detection as peaks in the detector signal. In the present study, electro-chemical detection was employed.

4.4 Theory of Electro-Chemical Detection (ECD)

High performance liquid chromatography with electro-chemical detection (HPLC-ECD) may be considered to be the generation of an electrolysis current at a fixed point along the constantly flowing mobile phase employed in the HPLC apparatus. 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 (Kissinger, 1984). A positive potential will result in oxidation, while a negative potential will result in reduction of molecules. Solute zones (separated sample molecules) pass along this mobile phase and into a very low volume, thin-layer cell where the flow is forced into a thin film passing over the electrode which is maintained at a fixed potential, usually between 0.5 and 1.0 volts. If the potential is greater than that required for electrolysis of the solute, a measurable charge passes from the electrode to the molecule or *vice versa*. The resulting current is directly proportional to the concentration of the solute passing through the channel. This detection is represented in Figure 4.2.

The more positive the potential of the electrode, the more powerful an oxidising agent it will be. As the concentration of solute (sample molecule) rises and falls while passing through the thin layer cell, the electrolysis current proportionately follows this change. The resulting

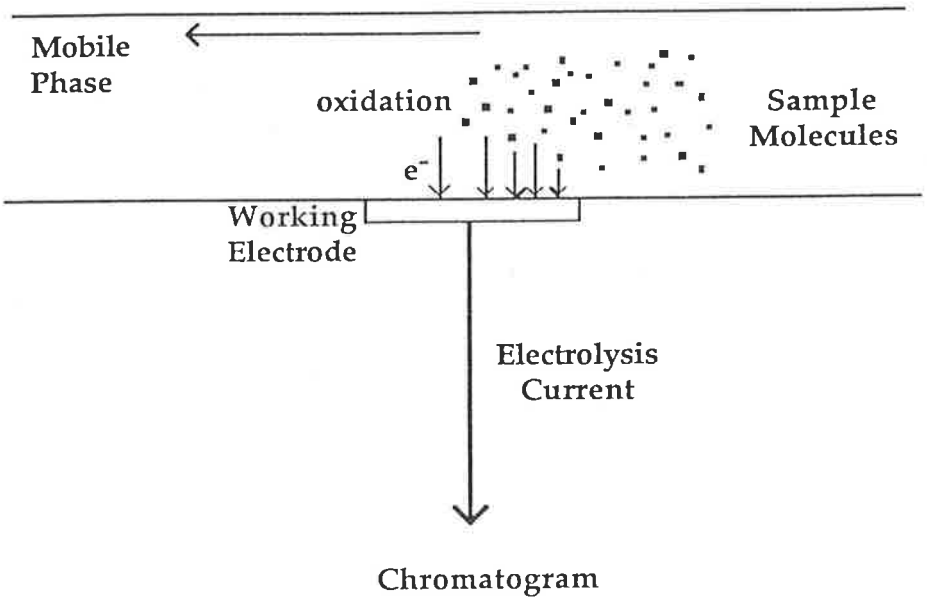

Figure 4.2 Schematic representation of electro-chemical detection of solute (sample molecules). As the sample molecules pass the working electrode they are oxidised and this generates a current which is directly proportional to the concentration of the the solute. The resulting current is then registered by the recorder as a peak on a chromatogram.

current, as a function of time, is amplified and sent as a 0-10mV signal to a recorder and a chromatogram is produced. Electro-chemical detectors usually consist essentially of two plastic insulator blocks made of acrylic which are tightly pressed around a slotted Teflon gasket to form a rectangular channel. A working electrode is inserted into the wall of one of the plastic blocks. Its composition is critical to the performance of the detector. Glassy (vitreous) carbon, carbon paste, mercury and amalgamated gold and platinum have all been used in this application. A glassy carbon working electrode was employed in the present study. Glassy carbon is a hard, amorphous carbon material which is capable of being polished to a mirror-like finish. When housed in the plastic block, the glassy carbon cell offers good solvent resistance, which is particularly useful with mobile phases which contain relatively large volumes of methanol. It is important to choose a solvent (mobile phase) that permits the electrode reaction to occur. The mobile phase must allow ionisation of the solute molecules to enable detection. It must also be sufficiently electrochemically inert at the electrode surface so that background current is negligible and will not interfere with the detection of oxidation current from sample molecules.

5. HYDROXYL RADICAL PRODUCTION IN ROOT-FILLED TEETH : A HYPOTHESIS

In the previous discussion, the causes of tooth discoloration in traumatised teeth were ascribed to the deposition of blood components which have been hydraulically forced into dentinal tubules. The deep discoloration which results is possibly due to haemoglobin or to breakdown products of haemoglobin. Iron, either bound within haemoglobin or free as an iron salt is considered to be a prime agent associated with this type of tooth discoloration. In an effort to remove this discoloration clinicians bleach these teeth after they have been root-filled. The bleaching agent used is usually 30% hydrogen peroxide employed either alone or in combination with sodium perborate. Given that sodium perborate breaks down to sodium metaborate and hydrogen peroxide, the common bleaching agent in routine bleaching procedures is hydrogen peroxide.

There is abundant evidence in the inorganic chemistry and biochemistry literature to demonstrate that hydrogen peroxide in the presence of ferrous iron is capable of generating hydroxyl radicals. Since iron salts are a likely agent in tooth discoloration after traumatic injury and that haemoglobin or its breakdown products are present in such stains, then it is likely that the co-ordinated ferrous iron in these compounds is available for the generation of hydroxyl radicals when these discolorations are treated with hydrogen peroxide.

This study proposes to test the hypothesis that hydroxyl radicals are generated in root-filled teeth which have been discoloured by blood and then bleached with hydrogen peroxide using a procedure analogous to clinical dental practice.

6. MATERIALS AND METHODS

6.1 Materials

Materials used in the canal preparation and root-filling phase of this project were those used commonly in clinical dental practice. The sources and components of these materials are detailed in Appendix 1. Chemicals and reagents used in the HPLC-ECD detection of hydroxyl radical phase were of analytical grade. Crystalline forms of homogentisic acid and 2,3-dihydroxybenzoate were stored as recommended (desiccated, in darkness and below 4^0C). The 2,5-dihydroxybenzoate and sodium salicylate were stored at room temperature in darkness. All aqueous solutions were made up with MilliQ water. Analytical grade 30% v/v hydrogen peroxide solution was used throughout the project. Hydrogen peroxide was stored in a light-tight polyethylene bottle at room temperature. In order to use the hydrogen peroxide, required amounts were poured into a clean beaker and then precisely apportioned by pipette. Unused hydrogen peroxide in the beaker was then discarded. The chemicals and materials used in this phase of the project and their respective sources are also detailed in Appendix 1.

6.2 Methods

Forty single-rooted premolar teeth, which were removed for orthodontic reasons, were extracted in a manner aimed at ensuring that little or no damage occurred to tooth surfaces, particularly in the cervical region. Forceps were applied to buccal and lingual surfaces of the tooth and, where possible, only on the enamel of the crown. Teeth used in these experiments were used with patient consent. After extraction, teeth

were stored in phosphate-buffered isotonic saline (pH 7.4) at 4^0 C until they were to be used. The time between extraction and use ranged between one and four weeks. Constraints of time and availability prevented the acquisition of greater numbers of teeth for use in this study.

Periodontal ligaments of experimental teeth were then removed by rubbing with sterile surgical gauze moistened with isotonic saline. The use of chemicals which have tissue solvent properties, such as sodium hypochlorite (see below) on the external root surface was avoided to ensure that there was no contamination of that surface by chemicals which subsequently may have affected the assay for hydroxyl radicals.

All teeth were then examined under a stereomicroscope (Wild Heerbrugg M400) at a magnification of x7.5 to determine the condition of the cementum layer at the cervical region. Teeth with discernible defects in cementum away from the cervical region were discarded. The remaining teeth were divided into two groups; those with visible cementum defects in the cervical region and those without. Artificial defects were then created mechanically on a number of the teeth such that half the experimental group (20 teeth) had cementum defects(natural and artificial) at the cervical region and half did not. No further distinction was made between artificial and natural defects subsequently. Artificial cementum defects were created mechanically using a No. 1 round tungsten carbide bur to a depth of 0.2 mm. Four circular defects were created in each of these teeth at the cemento-enamel junction; on the buccal, lingual, mesial and distal surfaces respectively.

Occlusal access cavities were then cut into the crowns of all experimental teeth, followed by pulpal extirpation using endodontic files and 1% sodium hypochlorite irrigant. This irrigant was used to enhance

removal of intracanal organic debris as well as that of the odontoblastic processes from the proximal parts of the dentinal tubules. Care was taken to avoid contact of the hypochlorite with the external root surface (see above). All traces of hypochlorite irrigant within the tooth were removed by copious rinsing with isotonic saline.

Once pulpectomies had been completed, teeth were again divided into two groups; one group to be experimentally discoloured by blood and one to remain undiscoloured. Thus four experimental groups were created :

1. Discoloured by blood, no cementum defect in the cervical region,
2. Discoloured by blood, cementum defect in the cervical region,
3. Undiscoloured, cementum defect in the cervical region, and
4. Undiscoloured, no cementum defect in the cervical region.

Each group contained 10 teeth.

Teeth were discoloured by blood using an adaptation of a technique described by Freccia and Peters (1982b). Teeth, with access cavities left open, were immersed in a suspension of packed red blood cells in a centrifuge vial. The packed red blood cells were obtained from the Red Cross Blood Bank and were from a stock of time-expired supplies. The vial, containing the tooth and packed red cells was then centrifuged at a speed of 10,000 r.p.m for 10 minutes three times per day for three days, with resuspension by shaking in between. During this period the teeth were stored in an incubator chamber at $37 \pm 0.5\ ^{0}C$.

Once visible discoloration had been achieved in the required number of teeth, the root canals of all experimental teeth were then prepared with endodontic files to a size 30 apical stop, with intracanal

irrigation employing 1% sodium hypochlorite. All teeth were then root-filled with gutta percha and AH26^{R} sealer cement, using a lateral condensation technique, to a level three millimetres below the cemento-enamel junction. A two millimetre thickness of Cavit^{R} was then placed over the root filling to provide a seal between the root filling and bleaching agent. Thus, one millimetre of root canal below the cemento-enamel junction would be exposed to the potential passage of hydrogen peroxide. The walls of the pulp chamber and the exposed root canal were then lightly cleaned with a No.2 round tungsten carbide bur. (The constituents of AH26 sealer cement and Cavit are detailed in Appendix 1.)

Each tooth was then set into a one millimetre thick wax sheet (modelling wax) at a level two millimetres above the cemento-enamel junction. This assembly was then set into a 3 ml (12 mm diameter x 32mm height) polycarbonate assay tube containing 1mM (throughout this thesis, M will refer to moles per litre) sodium salicylate solution, such that the tooth below the wax sheet was suspended in this solution and the tooth above the wax sheet was not exposed to the sodium salicylate solution. A seal was then created by placing a layer of sticky wax followed by two thicknesses of Ketac^{R} varnish where the tooth penetrated the wax sheet. The test solution of sodium salicylate was thus exposed to the cervical region of the tooth (see Figure 6.1).

Prior to bleaching, the pulp chambers of half the teeth in each of the four experimental groups had their pulp chambers flushed with 15.0%w/v ethylenediamine tetra-acetic acid (EDTA) solution. This was carried out to remove smear layer, a procedure analogous to clinical practice (see Section 1.6). All teeth were then subjected to a thermocatalytic intracoronal bleaching technique. In order to achieve this,

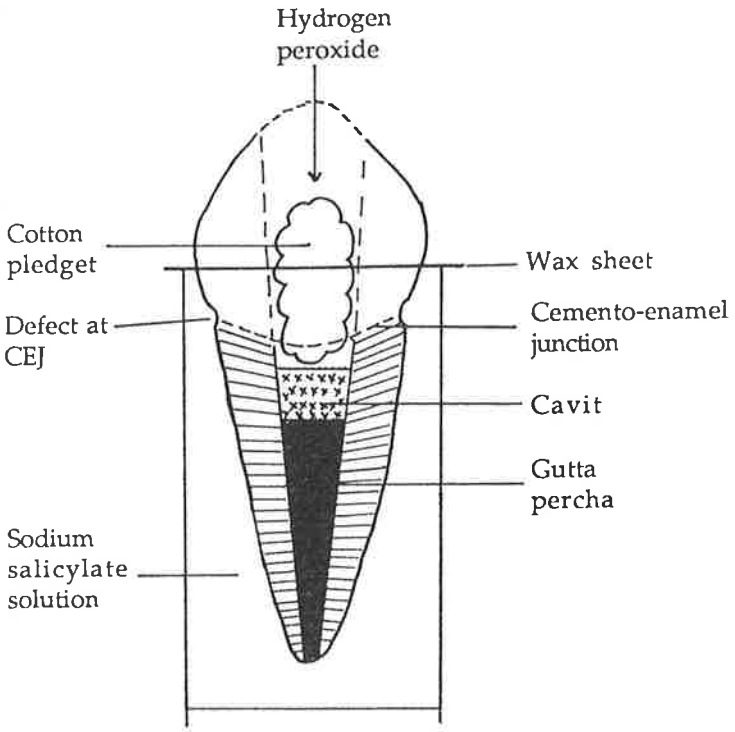

Figure 6.1 Tooth set into a wax sheet at a level 2 mm above the cemento-enamel junction. This structure was then set into a vial so that the root below the wax sheet was bathed in sodium salicylate solution. A thermocatalytic intracoronal bleaching procedure using 30% hydrogen peroxide was then carried out.

20 microlitres of 30%v/v hydrogen peroxide was pipetted into each pulp chamber which contained a small cotton pledget, such that the cotton pledget was saturated with hydrogen peroxide. This was then rapidly heated with a red-hot ball burnisher which had been heated over a blue flame. This process was repeated five times over a twenty minute period for each tooth. The red-hot ball burnisher was transferred immediately to the pulp chamber and was kept in contact with the cotton pledget, which was saturated with hydrogen peroxide, for periods of 10 seconds at a time. The negative control for this phase of the project was an undiscoloured tooth suspended in 1mM sodium salicylate solution. This control was carried out in a parallel series of experiments.

6.3 HPLC Detection of Hydroxyl Radical Production

Hydroxyl radical detection in this research project is an adaptation of techniques reported by Grootveld and Halliwell (1986), Wright and Priestley (1987) and Wright (1988). Hydroxyl radical generation was determined by the detection of reaction products of this radical with salicylate, using high performance liquid chromatography with electrochemical detection. Theoretical aspects of this technique have been discussed in Sections 4.2-4.4 above. To determine the presence of 2,3-DHB and 2,5-DHB (indicative of the generation of hydroxyl radicals) in the 1mM sodium salicylate solution, a 20 microlitre (µl) volume of the solution bathing the tooth roots from each of the experimental tooth samples was injected on to a reverse-phase HPLC column equipped with electrochemical detection. Prior to injection on to the column, a 20 µl volume of 1 µM homogentisic acid (HGA) was added to each sample as an internal standard. The HPLC unit consisted of an octadecylsilyl (C18)

μBondapak 5μm, 0.4 x 15 cm reverse phase column, with a Shimadzu LC-6A pump and a Shimadzu CR-4A recorder. The electrochemical detector used was a Bioanalytical Systems Inc. LC-4A detector with a RE-1 glassy carbon working electrode set at an oxidation potential of +0.80 volts against the Ag/AgCl reference electrode, with a sensitivity range of 5 nA per 10 mV of detector output to recorder. The oxidation potential of +0.80 volts had been shown by Wright (1988) to produce near maximal response with minimal noise in the chromatogram baseline. The mobile phase used in this system was composed of 70% aqueous phase (0.1 M KH_2PO_4 / 0.1mM EDTA) and 30%v/v methanol (pH 2.9) at a flow rate of 1.0 ml per minute. The mobile phase was de-oxygenated by constant sparging with helium. These chromatographic conditions were unchanged throughout the HPLC phase of the project and thus were used in the sensitivity assay, degradation study and performance of control experiments (detailed below). A flow diagram of this experimental design appears as Figure 6.2.

6.4 Statistical Method

Statistical analysis of the presence of reaction products formed as the result of hydroxyl radical generation was carried out using a three-way analysis of variance. This test was used to test the significance of associations between the production of hydroxyl radicals and :

1. Presence or absence of tooth discoloration
2. Presence or absence of defects in the cementum layer at the cemento-enamel junction
3. Whether or not the pulp chamber had been flushed with EDTA prior to bleaching

The amount of the reaction product 2,5-DHB, in picomoles, was used as the measure of hydroxyl radical production (see Section 4.2) in this statistical analysis. This was done because 2,5-DHB was the reaction product produced in greatest quantities, both in the experimental tooth samples and in the control experiments.

The choice of this reaction product as the measure of hydroxyl radical production is discussed again in Section 8.3.

6.5 Assay Sensitivity

In order to determine the limits of sensitivity of the detection system to both 2,3-DHB and 2,5-DHB, as well as of the internal standard, homogentisic acid, a range of between one and 800 picomoles of each of the three standards, dissolved in a constant volume of water, was injected on to the column. A standard curve of peak heights measured in nanoamps versus the amount injected was then plotted for each standard.

6.6 Stability of standards

Dihydroxybenzoates oxidise rapidly in both their solid forms and in solution. Due to the large number of experimental samples, the time before injection of these samples on to the HPLC column may have been up to six hours after bleaching, so it was important to ascertain the rate of degradation of the dihydroxybenzoates since this may affect the overall quantitation of hydroxyl radical production.

Twenty microlitre volumes of 2,3-DHB and 2,5-DHB as well as homogentisic acid, at a concentration of 10^{-6} M (equivalent to 20 pmol) were injected on to the column over a 27 hour period and their degradation curves at an ambient temperature of 20 ± 1^{0}C were plotted.

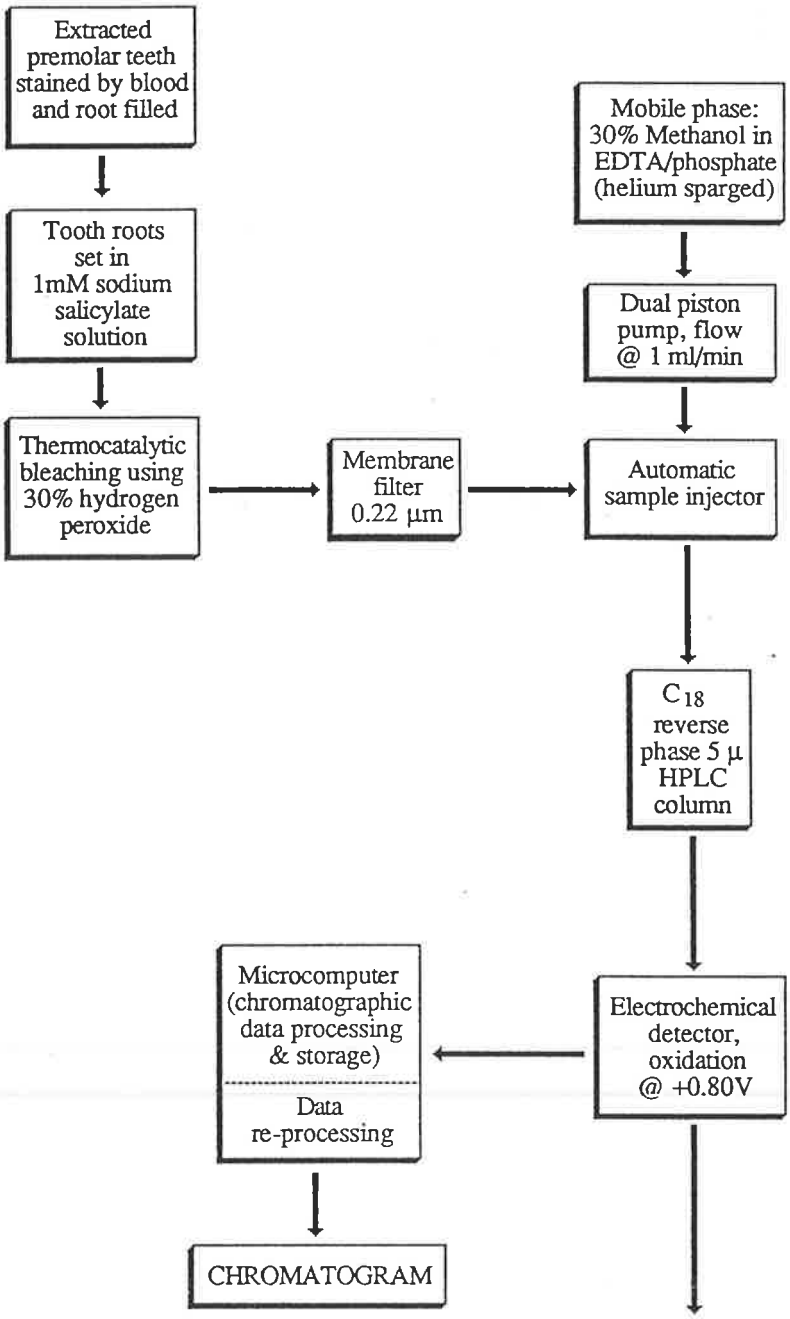

Figure 6.2 Isocratic HPLC method used to analyse hydroxylation products of salicylate caused by hydroxyl radical production in bleached root-filled teeth.

6.7 Control Experiments

A range of control experiments was conducted during the course of this study, but at a different time to the experimental phase detailed in Section 6.2. These control experiments may be categorised as follows:

1. Generation of hydroxyl radicals. Hydroxyl radicals were generated by the mixture of the following reagents in a 1 litre aqueous solution of 1mM sodium salicylate:
   a. The addition of 0.5ml 30%v/v hydrogen peroxide (equivalent to 6.45×10^{-3} moles) to 50μM (equivalent to 5×10^{-5} moles) concentration of ferrous sulphate.
   b. The addition of 2mM EDTA (equivalent to 2×10^{-3} moles) to a 500μM concentration of ferrous sulphate (equivalent to 5×10^{-4} moles). This reaction produces hydroxyl radicals by the autoxidation of ferrous iron in the presence of atmospheric oxygen.
   c. The addition of 2mM EDTA (equivalent to 5×10^{-4} moles) and 0.5 ml 30%v/v hydrogen peroxide (equivalent to 6.45×10^{-3} moles) to a 500 μM concentration of ferrous sulphate (equivalent to 5×10^{-4} moles).

A 20μl volume of each of the resulting solutions was then injected on to the column for electrochemical detection of reaction products.

Each different stage of the research project employed a freshly made mobile phase, the pH of which varied between 2.95 and 3.05. Different batches of mobile phase appeared to cause minor variations in the retention times of the standards (±1.1 minutes). Resulting chromatograms, therefore, would vary slightly among the various phases

of the project. For this reason, collateral runs of the mixed standards HGA, 2,3-DHB and 2,5-DHB in amounts of 20 pmol were injected on to the column with each fresh batch of mobile phase to more accurately ascertain the identity of reaction products.

2. Determination of the effect of concentration of ferrous iron on the generation of hydroxyl radical.

Ferrous sulphate in concentrations of 5 μM, 50 μM, 100 μM and 500 μM (in 1litre aqueous solutions containing 1mM sodium salicylate) were mixed with a 0.5ml volume of 30%v/v hydrogen peroxide solution. The concentrations of resulting 2,3-DHB and 2,5-DHB were then plotted over a one hour time course for each concentration of ferrous sulphate.

3. Various combinations of different final concentrations of reagents used in the research project made up in a 1l volume of MilliQ water:

a. ferrous sulphate : 500 μM (equivalent to 5×10^{-4} moles)
b. EDTA : 2mM (equivalent to 2×10^{-3} moles)
c. potassium dihydrogen phosphate : 0.1 M (equivalent to 0.1 moles)
d. sodium salicylate : 1 mM (equivalent to 1×10^{-3} moles)
e. hydrogen peroxide : 0.5 ml 30%v/v (equivalent 6.45×10^{-3} moles).

4. Determination of the identity of other products.

During the course of the experiment, it became evident that other reaction products besides 2,3-DHB and 2,5-DHB were being generated.

This was apparent in the experimental teeth as well as in control experiments. In the experimental tooth samples, a third reaction product was noted with an elution time between that of the internal standard (HGA) and that of 2,5-DHB. In Section 4.2, it was noted that a third minor reaction product of hydroxyl radicals with salicylate was pyrocatechol.

It was considered that pyrocatechol was most likely to be the third reaction product in these samples. In control experiments, it was also noted that a fourth substance was produced, eluting with the same retention time as the internal standard (HGA), even though HGA had not been included in the mixture.

In conditions of high radical flux, it is possible for further decarboxylation and/or hydroxylation of 2,3-DHB and 2,5-DHB to occur, although this is generally considered to be unlikely. Studies which have employed this technique of hydroxyl radical detection have not discussed such a phenomenon. It was decided, however, to inject on to the column potential products of DHB hydroxylation. The theoretical production of these molecules and their chemical structures is detailed in Appendix 3, and the possibility of their presence as well as the significance to experimental results is included in the discussion section.

Thus, the following were injected on to the column :

a. pyrocatechol (1,2-dihydroxybenzene) : 20 pmol
b. hydroquinone(1,4-dihydroxybenzene) :20pmol
c. resorcinol (1,3-dihydroxybenzene) : 20pmol, 200 pmol
d. phloroglucinol (1,3,5-trihydroxybenzene) : 20 pmol
e. pyrogallol (1,2,3-trihydroxybenzene) : 20 pmol

5. Effect of ultra-violet light upon sodium salicylate.

In order to investigate the effect of ultra-violet light on sodium salicylate and the potential effect this may have had on experimental results, 2 ml samples of 1mM sodium salicylate in clear glass HPLC injection vials were exposed to varying light conditions. These included sodium salicylate prepared in ambient light and then :

a. left in darkness for 12 hours,

b. exposed to ambient light (combined natural and fluorescent sources) for 10 minutes

c. exposed to ambient light for 12 hours, and

d. exposed to concentrated U.V light (Oliphant U.V lamp for chromatography, at a wavelength of 366 nm) for 10 minutes.

An assay was then conducted by HPLC-ECD for 2,3-DHB and 2,5-DHB as measures of hydroxyl radical production.

6. Effect of ultra-violet light on a combined mixture of sodium salicylate and hydrogen peroxide.

To determine the significance of interaction between sodium salicylate and hydrogen peroxide and to examine the modifying influence of ultra-violet light, 2 ml volumes of 1mM sodium salicylate were mixed with 0.5 ml volumes of 30%v/v hydrogen peroxide in darkness. These solutions were then exposed to the following light conditions :

a. Darkness for 10 minutes

b. U.V light (366 nm) for 10 minutes

c. Ambient light (natural with fluorescent) for 10 minutes.

d. Ambient light for 12 hours.

7. RESULTS

7.1 Standard Curves for HGA, 2,3-DHB and 2,5-DHB

A range of one to 800 picomoles of each of HGA, 2,3-DHB and 2,5-DHB were injected on to the HPLC-ECD apparatus. Figure 7.1 shows a sample chromatogram of a mixed solution of the standards, with 20 pmol of each standard injected on to the column.

From the range of injections, a series of standard curves for each of the three chemicals was produced. Figure 7.2 displays the linear relationship between the amount of standard injected on to the column and the recorder peak height, where the x-axis represents the amount of standard injected on to the column in picomoles and the y-axis represents the ECD electrolysis current generated in nanoamps. The ECD range was 5 nA per 10 mV of detector output.

From these data, regression analysis demonstrated a high degree of linear correlation between amounts injected and recorder peak heights :

2,5-DHB peak height (nA) = -0.24 + 0.17 x (pmols 2,5-DHB) $R^2 = 0.995$

2,3-DHB peak height (nA) = -0.68 + 0.14 x (pmols 2,3-DHB) $R^2 = 0.999$

HGA peak height (nA) = -0.66 + 0.12 x (pmols HGA) $R^2 = 0.997$

7.2 Limits of Sensitivity

Analysis of the standard curves for HGA 2,3-DHB, 2,5-DHB as well as chromatograms produced during the sensitivity assay showed the

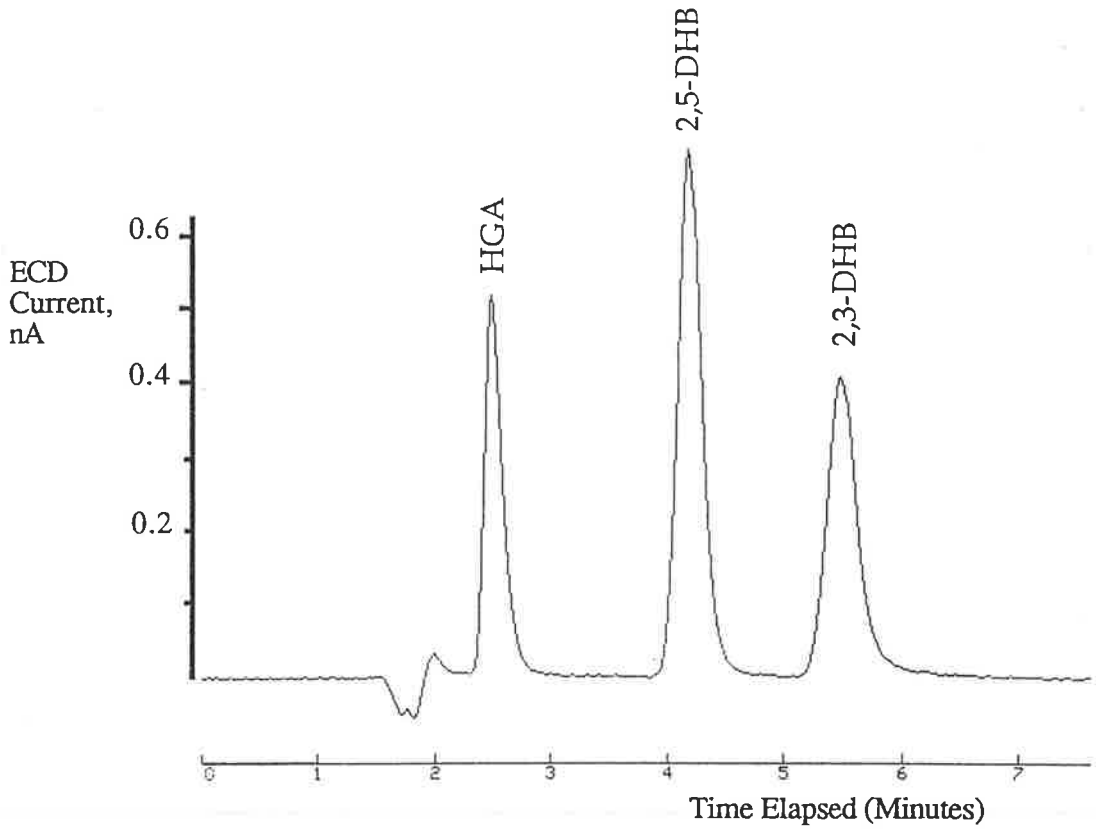

Figure 7.1 Sample chromatogram showing the standards HGA , 2,5-DHB and 2,3-DHB with 20 pmol of each injected on to the column and analysed by HPLC-ECD. The baseline fluctuations around 1.5-2.0 minutes are characteristic of the mobile phase used.

Standard Curves for HGA, 2,3-DHB and 2,5-DHB

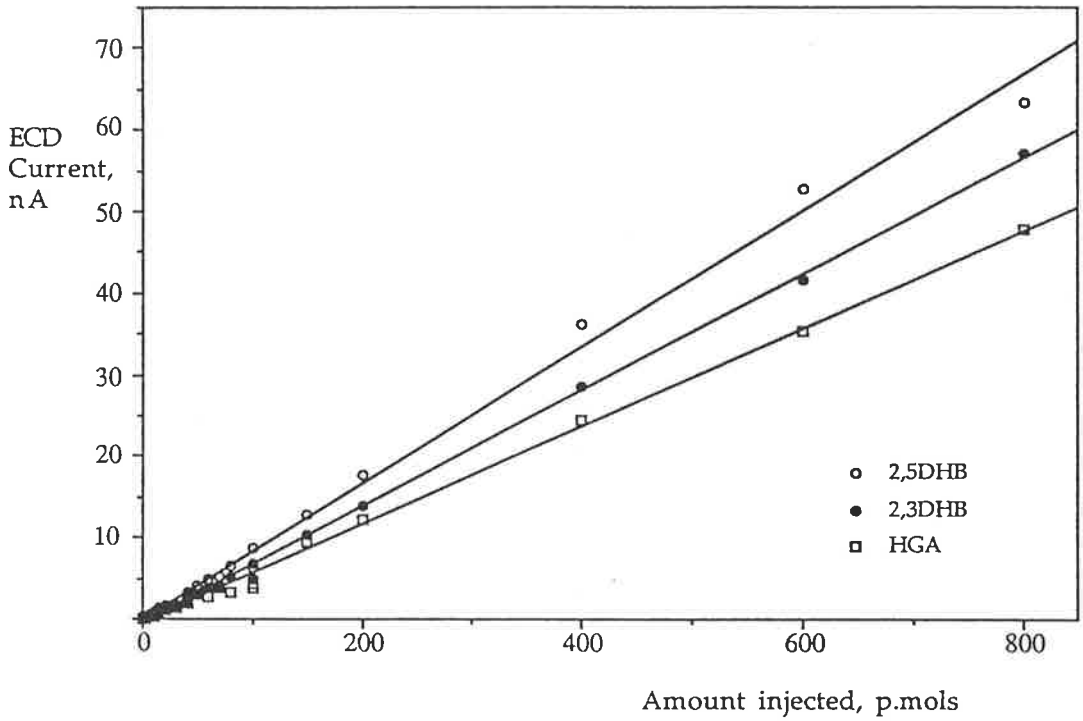

Figure 7.2 Standard curves for HGA, 2,3-DHB and 2,5-DHB with a range of 1-800 pmol in a volume of 20µl injected on to the column and analysed by HPLC-ECD.

practical limit of sensitivity in this detection system for them was one picomole. The signal to noise ratio at this amount of standard injected was 4 :1.

A sample chromatogram of these mixed standards with one picomole injected on to the column is shown in Figure 7.3.

7.3 Degradation of Standards

The stability of the standards HGA, 2,3-DHB and 2,5-DHB were analysed over a 27 hour period (from 6.00 pm to 9.00 pm the following day). This period of time was considered sufficient to monitor changes in the standards that could occur over the course of the experiment employing tooth samples, which was run over a period of approximately eight hours. Twenty microlitre volumes of sample were injected on to the column and the initial amount injected was 20 pmol. The standards 2,3-DHB and 2,5-DHB exhibited a low degree of degradation in comparison to that exhibited by HGA over the sample 27 hour period. A graph of the degradation curves for the three standards is shown in Figure 7.4, where the x-axis represents the time elapsed in hours and the y-axis represents the percentage remaining of the initial amount injected on to the column. Regression analysis for these exponential decay curves revealed the following correlations:

$$\% \text{ initial [2,3-DHB]} = 100 \times 10^{-1.03e-3 \times \text{hours}} \qquad R^2 = 0.805$$

$$\% \text{ initial [2,5-DHB]} = 98.7 \times 10^{-1.9e-3 \times \text{hours}} \qquad R^2 = 0.813$$

$$\% \text{ initial [HGA]} = 103 \times 10^{-1.74e-2 \times \text{hours}} \qquad R^2 = 0.979$$

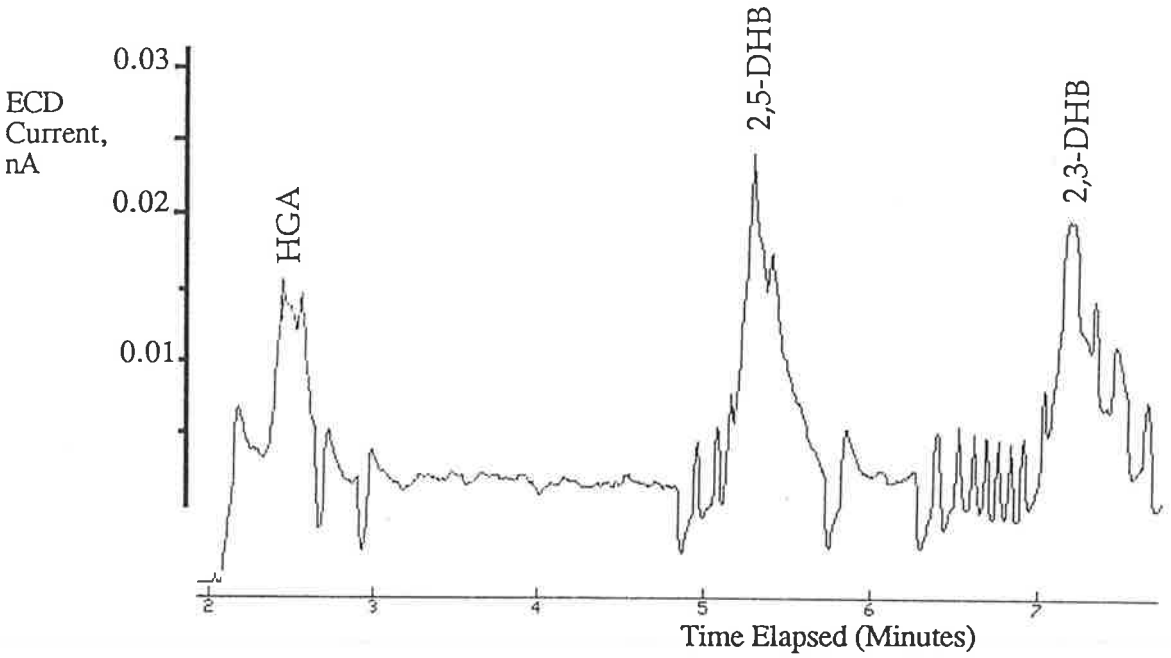

Figure 7.3 Sample chromatogram of the the standards HGA, 2,3-DHB and 2,5-DHB with one picomole of each, in a volume of 20 µl, injected on to the column and analysed by HPLC-ECD.

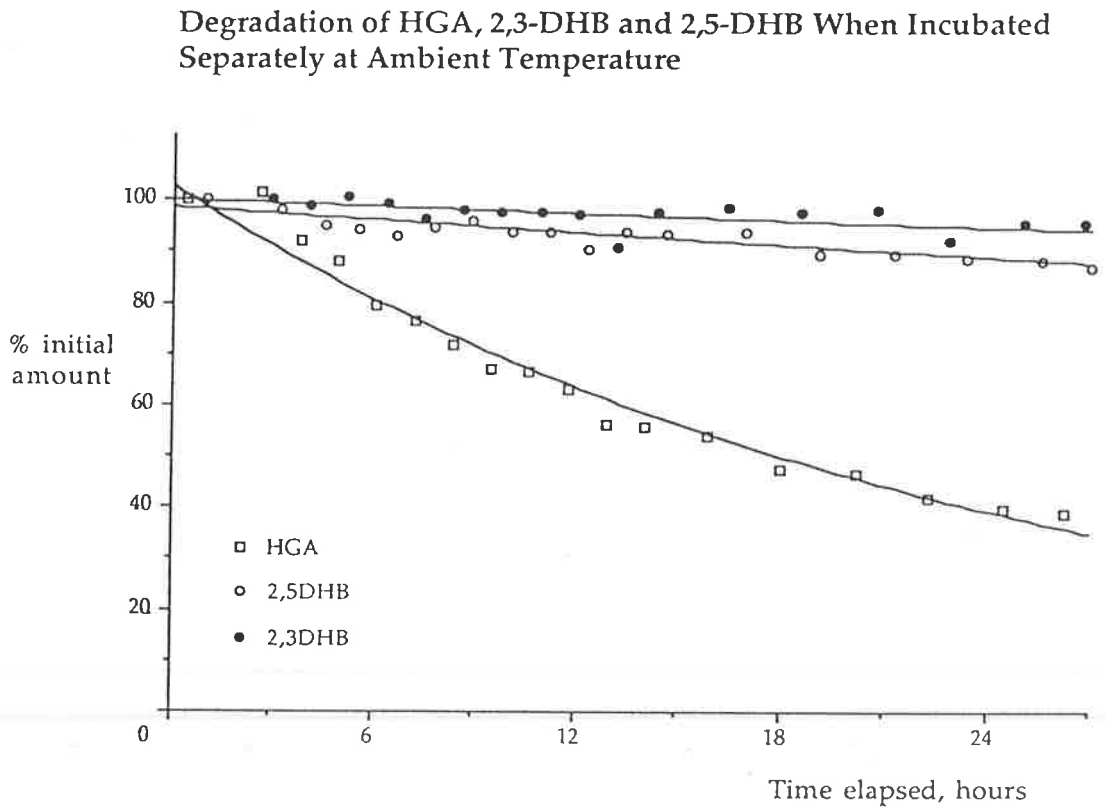

Figure 7.4 Degradation curves for 20 pmol of HGA, 2,3-DHB and 2,5-DHB over a 27 hour period. Analysis was made by HPLC-ECD.

Examination of the individual curves revealed that peak height values were apparently susceptible to short-term ambient temperature fluctuations. This appeared to be more a phenomenon reflecting changes in electrochemical detector sensitivity rather than changes in the actual standard being injected on to the column.

7.4 Control Generation of Hydroxyl Radicals

Hydroxyl radical fluxes were generated chemically by the addition of 50 μM ferrous sulphate to 0.5 ml of 30%v/v hydrogen peroxide, the autoxidation of 500 μM ferrous sulphate on the addition of 2mM EDTA and by the addition of 500 μM ferrous sulphate and 2mM EDTA to 0.5 ml 30% hydrogen peroxide. These reactions were all conducted in the presence of 1mM sodium salicylate in a one litre solution (see Section 6.7). Twenty microlitres of the resulting solution was then injected on to the column for electrochemical detection of reaction products.

On the addition of 0.5 ml 30% hydrogen peroxide to 50 μM ferrous sulphate in the presence of 1 mM sodium salicylate, three definite peaks were produced on the resulting chromatogram, which is shown as Figure 7.5. A fresh mobile phase was employed for each aspect of the project and it was noted that retention times for the standards exhibited minor fluctuations with different batches of mobile phase. A collateral injection of standards allowed confirmation of the identity of reaction products seen on resulting chromatograms.

Assuming that all of the reactants were able to

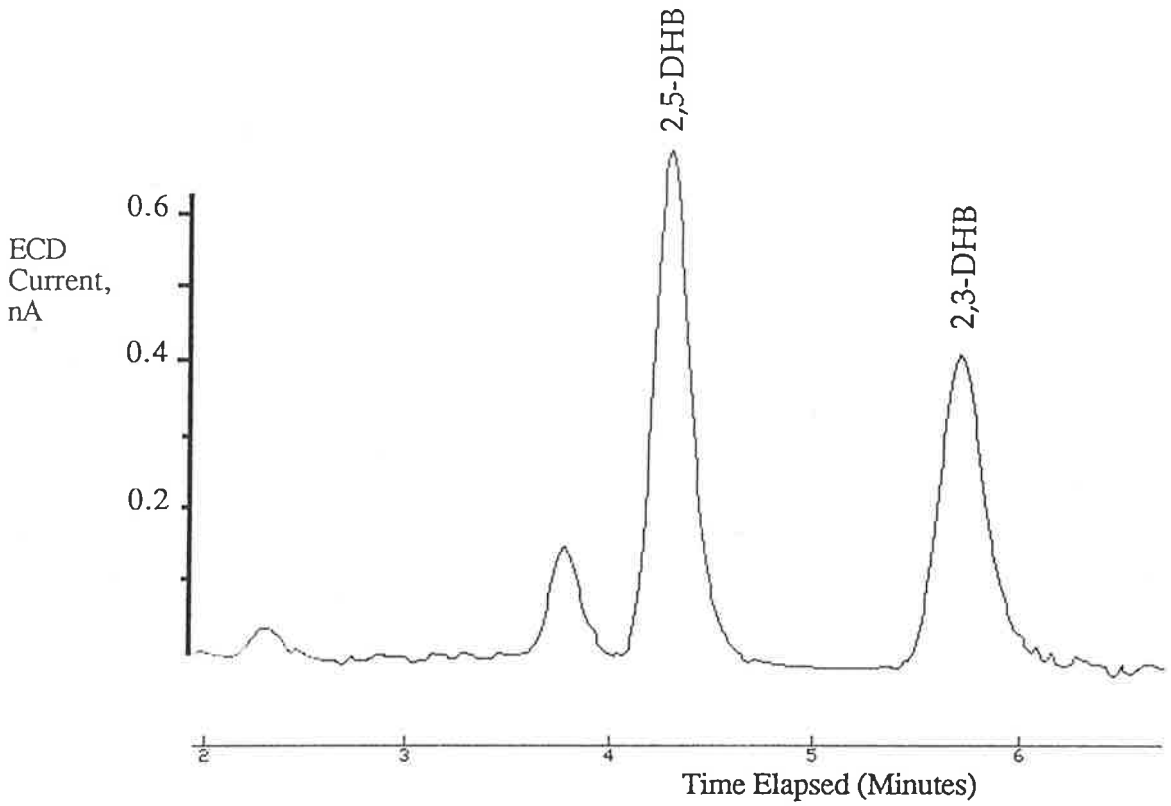

Figure 7.5 Sample chromatogram showing reaction products after the generation of hydroxyl radicals by the addition of ferrous sulphate to hydrogen peroxide in the presence of sodium salicylate. The small peak preceding 2,5-DHB is probably pyrocatechol (see Section 7.7)

react fully, it can be seen from the chromatogram that the products are present in the proportions of approximately 12%, 38% and 50% for pyrocatechol, 2,3-DHB and 2,5-DHB respectively. The identity of pyrocatechol was confirmed in a later control experiment (see Section 7.7).

The amounts of reaction product yielded from this generation of hydroxyl radicals was:

2,3-DHB : 21.4 pmol

2,5-DHB : 24.8 pmol

The ratio of 2,3-DHB to 2,5-DHB was therefore 1 : 1.2.

The generation of hydroxyl radicals by the autoxidation of ferrous iron was achieved by the addition of 2mM EDTA to a solution of 500 μM ferrous sulphate in the presence of a 1mM concentration of sodium salicylate. This was carried out in a one litre volume. In Section 2.5, the inherent instability of an iron-EDTA complex which resulted in rapid autoxidation of the ferrous iron was discussed. This phenomenon is supported by the findings of this phase of the research project. From the resulting chromatograms it was calculated that the percentages of 2,3-DHB and 2,5-DHB produced were 40.1% and 31.0% respectively. A third reaction product (probably pyrocatechol) accounted for a remaining 28.9% of reaction product. The chromatogram resulting from this addition is shown as Figure 7.6. The amount of reaction products produced from this generation of hydroxyl radicals was :

2,3-DHB : 476.3 pmol

2,5 DHB : 242.9 pmol

The ratio of 2,3-DHB to 2,5-DHB was therefore 1 : 0.51

The generation of hydroxyl radicals by the addition of 2 mM EDTA and 50 μM ferrous sulphate to 0.5 ml 30% hydrogen peroxide in the presence of 1mM sodium salicylate resulted in a greater yield of reaction products compared with that of the previous reactions. A sample chromatogram of this addition is shown as Figure 7.7.

This addition produced reaction products in the following amounts:

2,3-DHB : 490.4 pmol

2,5-DHB : 319.5 pmol

The ratio of 2,3-DHB to 2,5-DHB was 1 : 0.65

These products were produced in molar proportions of 33.7% and 33.3% for 2,3-DHB and 2,5-DHB respectively, with a third reaction product (probably pyrocatechol) present in the proportion of 33.0%.

7.5 Effect of Ferrous Ion Concentration on Hydroxyl Radical Generation

The amounts of both 2,3-DHB and 2,5-DHB that were produced by the reaction of 0.5 ml 30% hydrogen peroxide with ferrous sulphate in concentrations of 5 μM, 50 μM, 100 μM and 500 μM over a 60 minute period were plotted against peak height values gained from resulting chromatograms. Graphs were produced for both 2,3-DHB and 2,5-DHB where the x-axes represented time elapsed in minutes and the y-axes represented the ECD electrolysis current in nanoamps. Four distinct curves are shown on each graph, representing the different concentrations of ferrous sulphate employed. These graphs are shown as Figures 7.8 and 7.9 for 2,3-DHB and 2,5-DHB respectively. The graphs display the link between ferrous ion concentration available within the system and the amount of hydroxyl radicals produced, as determined by the

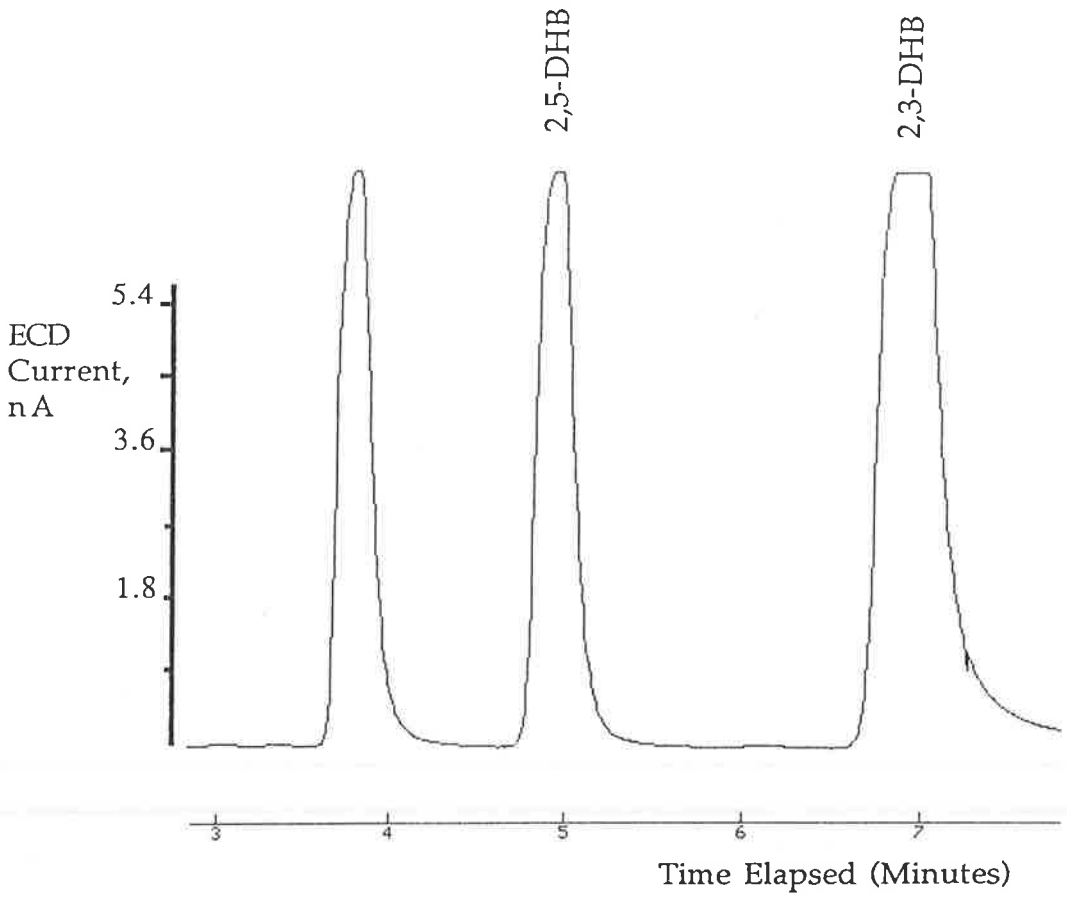

Figure 7.7 Chromatogram showing reaction products after the generation of hydroxyl radicals by the addition of 2 mmoles EDTA and 0.5 mmoles ferrous sulphate to 0.5 ml 30% hydrogen peroxide in the presence of 1mM sodium salicylate (in a 1 litre volume). The peak preceding that of 2,5-DHB is probably pyrocatechol (see Section 7.7).

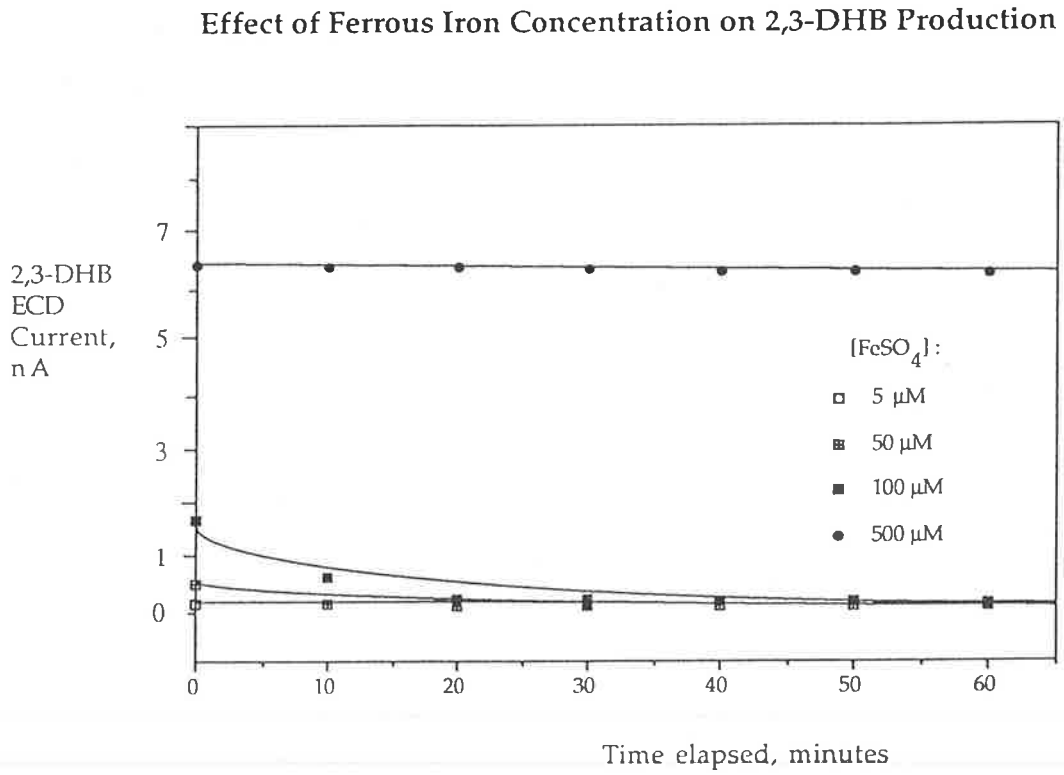

Figure 7.8 Effect of ferrous iron concentration on the production of 2,3-DHB (as a measure of hydroxyl radical generation).

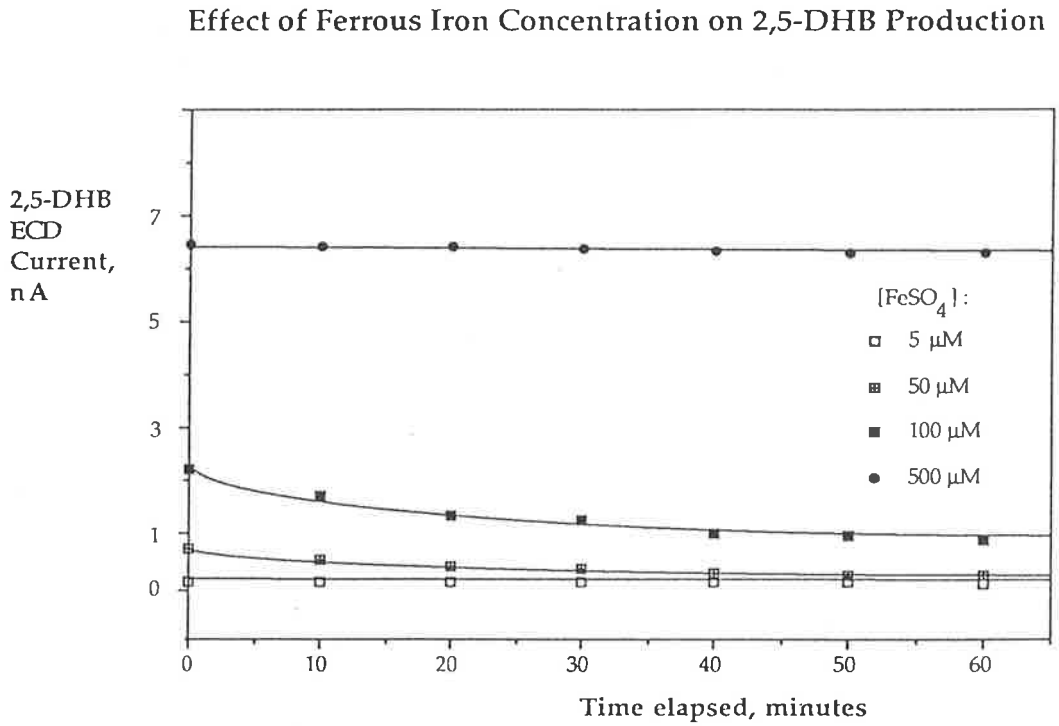

Figure 7.9 Effect of ferrous iron concentration on the production of 2,5-DHB (as a measure of hydroxyl radical generation).

amounts of reaction products measured. They also show that, invariably, hydroxyl radical production was complete by the time of the first sampling. The decrease in detectable levels may have been due to the rate at which the standards degraded.

7.6 Combined controls

A variety of combinations of the reagents employed in the HPLC phase of the research project were investigated for the types and amounts of reaction products. The combinations investigated and the amount of reaction products, measured as recorder peak height values in nanoamps are shown in Table 7.1. From these combinations, two other reaction products besides 2,3-DHB and 2,5-DHB were detected, particularly where KH_2PO_4 was included in the mixture and when there was a large production of hydroxyl radicals. The influence of the KH_2PO_4 would most likely have been to modify the pH of the reaction mixture. The first of these products was eluting with a retention time very similar to the internal standard, homogentisic acid (at 2.4 minutes), even though HGA had not been added to the mixture. The second was eluting between this first substance and 2,5-DHB (4.9 minutes) with a retention time of approximately 3.7 minutes. A sample chromatogram displaying these peaks is shown in Figure 7.10. The identity of these two extra substances is the subject of the results in the next section. It was apparent also that there was a minor reaction between hydrogen peroxide and the sodium salicylate itself to give reaction products which eluted at the same time as the standards. The significance of this will be considered in the Discussion section.

	Sodium Salicylate 1 mM	EDTA 2 mM	KH_2PO_4 0.1 M	$FeSO_4$ 500 µM	H_2O_2 0.5 ml 30% v/v	Amount 2,3-DHB, pmols/20 µl	Amount 2,5-DHB, pmols/20 µl	Comments
A	●	●		●		476.3 (6.43 nA)	242.9 (4.98 nA)	1. Peak at 2.4 mins: 0.12 nA 2. Peak at 3.7 mins : 4.64 nA
B	●	●	●	●		488.15 (6.59 nA)	313.2 (6.52 nA)	1. Peak at 2.4 mins : 1.9 nA 2. Peak at 3.7 mins : 6.59 nA
C	●			●	●	313.2 (6.42 nA)	215.0 (6.45 nA)	1. Peak at 1.7 mins : 1.2 nA 2. Peak at 2.3 mins : 6.48 nA 3. Peak at 3.7 mins : 5.2 nA
D	●		●	●	●	487.41 (6.58 nA)	320.5 (6.57 nA)	1. Peak at 1.62 mins : 0.21 nA 2. Peak at 2.4 mins : 0.66 nA 3. Peak at 3.7 mins : 6.47 nA
E	●	●		●	●	490.4 (6.6 nA)	319.5 (6.6 nA)	1. Peak at 1.6 mins : 0.21 nA 2. Peak at 2.3 mins : 0.38 nA 3. Peak at 3.7 mins : 6.48 nA
F	●	●	●	●	●	488.2 (6.59 nA)	322.0 (6.60 nA)	1. Peak at 1.6 mins : 0.32 nA 2. Peak at 2.3 mins : 1.90 nA 3. Peak at 3.7 mins : 6.59 nA
G	●				●	30.4 (0.41 nA)	31.7 (0.65 nA)	Peak at 1.7 mins : 0.17 nA
H	●					1.5 (0.01 nA)	2.92 (0.06 nA)	
I	●	●				Not detected	1.48 (0.02 nA)	
J					●			Peak at 1.7 mins : 0.34 nA
K	●	●	●			8.15 (0.18 nA)	8.78 (0.18 nA)	Peak at 3.8 mins: 0.08 nA

Table 7.1 Combined controls of the mixtures of various reagents used throughout the HPLC phase of the study, assessed for their ability to generate peaks with the retention times of 2,3-DHB and 2,5-DHB.

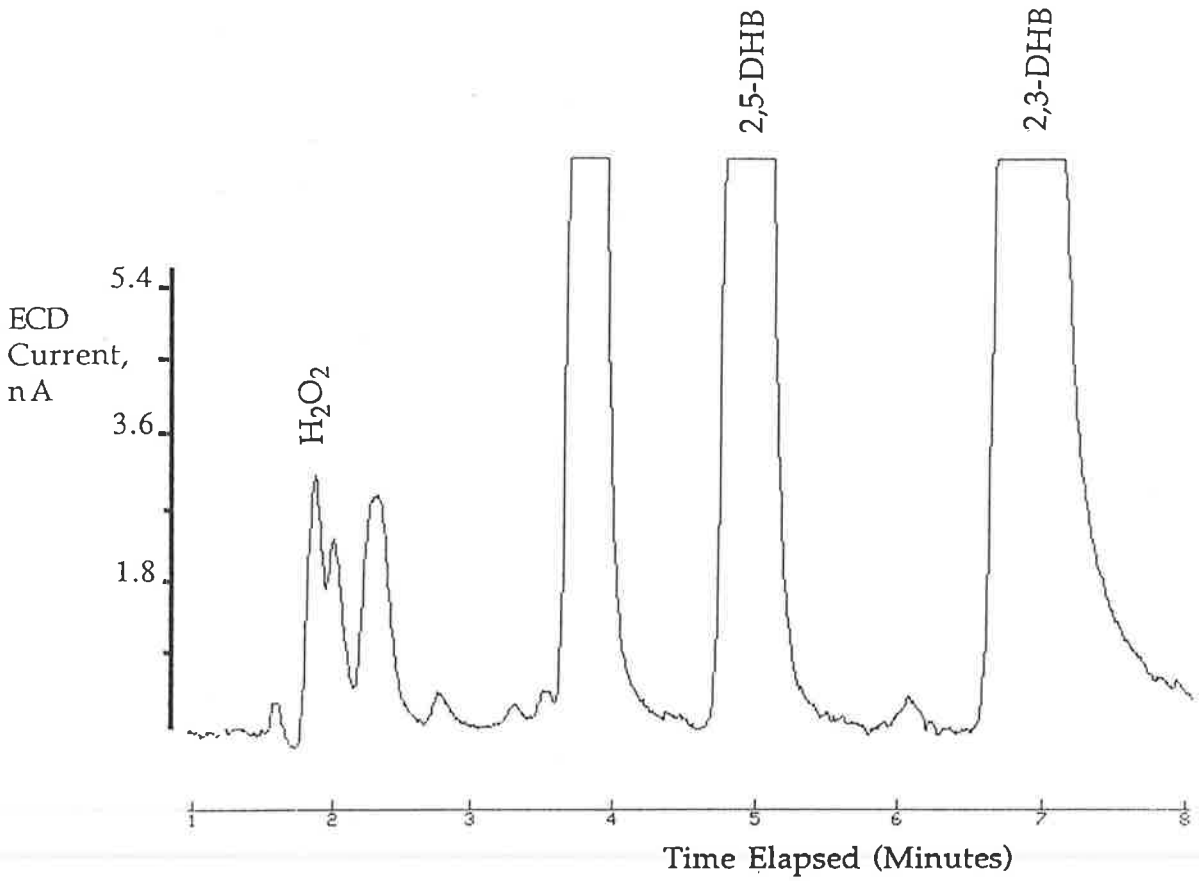

Figure 7.10 Chromatogram showing extra reaction product peaks at 2.4 minutes and 3.7 minutes in conditions of high hydroxyl radical flux. The probable nature of these peaks is discussed in Section 7.7.

7.7 Other Products of Control Experiments

In order to investigate the identity of the other reaction products noted in the control generation of high hydroxyl radical flux which appeared at retention times of 2.4 minutes and 3.7 minutes, the following were injected on to the column :

a. pyrocatechol : 20 pmol

b. hydroquinone : 20 pmol

c. resorcinol : 20 pmol, 200 pmol

d. pyrogallol : 20 pmol

e. phloroglucinol 20 pmol

From these results, it was evident that the third significant reaction product in the controlled generation of hydroxyl radicals, which was eluting with a retention time of approximately 3.7 minutes, was pyrocatechol. A chromatogram comparing this peak with those of the three standards, HGA, 2,3-DHB and 2,5-DHB is shown as Figure 7.11. Hydroquinone and pyrogallol were detected with an elution time of approximately 2.4 minutes, similar to that of the of the internal standard, HGA. Neither resorcinol nor phloroglucinol were detectable in the amounts injected, at least not within the 25 minute run time.

7.8 Effect of U.V Light on Sodium Salicylate

Two millilitre volumes of 1mM sodium salicylate in HPLC injection vials were exposed to varying degrees of ultra-violet light to determine the effect that ambient light may have had on sodium salicylate and so, potentially, on the experimental tooth samples. Analysis of these results revealed the detection of 2,3-DHB and 2,5-DHB. No significant difference was evident between amounts of these hydroxylated products

Sample	Amount, pmols	Elution Time, mins	Peak Height, nA	Molar electro-activity mA/mole
HGA	20	2.47	0.44	0.44
2,3-DHB	20	5.47	0.35	0.35
2,5-DHB	20	4.17	0.61	0.61
Pyrocatechol	20	3.62	0.58	0.58
Resorcinol	20	Not detected		
	200	Not detected		
Hydroquinone	20	2.46	0.63	0.63
Phloroglucinol	20	Not detected		
Pyrogallol	20	2.42	0.7	0.7

Table 7.2 Results of injection of possible hydroxylation products of salicylate in conditions of high hydroxyl radical flux. This control experiment also reinforced the identification of the third product as pyrocatechol. The ECD current per mole of substance is an indication of the specific electro-active sites within the molecular structure of each substance.

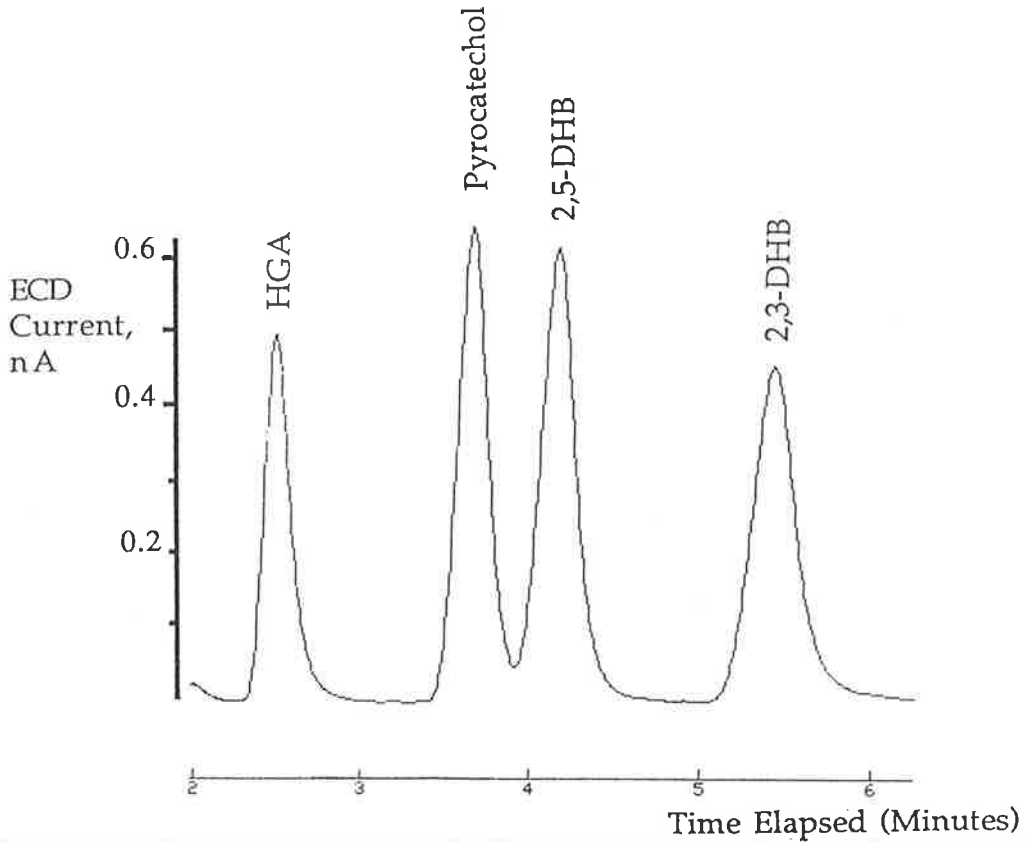

Figure 7.11 Chromatogram confirming the identity of pyrocatechol, which was injected with the standards HGA, 2,3-DHB and 2,5-DHB. Twenty picomoles of each substance was injected.

of sodium salicylate when stored in either darkness or in ambient light. When sodium salicylate was exposed to an intense source of ultra-violet light, however, there was a significant increase (approximately 15-fold) in the amounts of 2,3-DHB and 2,5-DHB, evidence of marked hydroxyl radical production. The third reaction product, pyrocatechol, was also produced in these samples with an increase in its production similar to that of 2,3-DHB and 2,5-DHB when exposed to U.V light. A graph displaying the results of this effect of ultra-violet light on sodium salicylate solution is shown as Figure 7.12.

7.9 Effect of U.V Light and Hydrogen Peroxide on Sodium Salicylate

Two millilitre volumes of 1 mM sodium salicylate solutions were mixed with 0.5 ml volumes of 30% hydrogen peroxide and exposed to various intensities of ultra-violet light ranging from darkness to intense U.V light for 10 minutes. In these samples the production of hydroxylated products of salicylate (2,3-DHB, 2,5-DHB and pyrocatechol) was also noted. There was no significant difference between the amount of reaction products produced in darkness and the amount produced, even after 12 hours, in ambient light. The overall levels detected were about three times greater than those in the absence of hydrogen peroxide (see Section 7.8 above) Again there was a significant increase (approximately 10-fold) in the amount of reaction products resulting from exposure to intense U.V light. A graph displaying these results is shown as Figure 7.13.

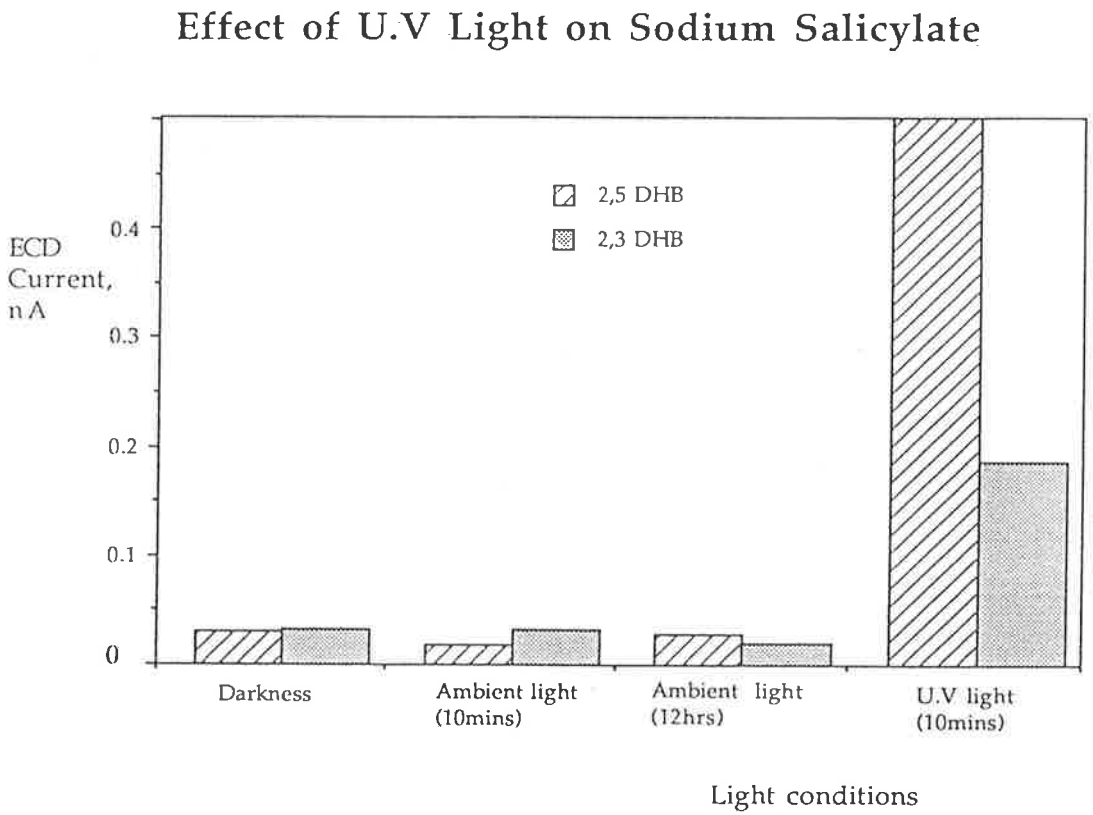

Figure 7.12 Effect of U.V light on sodium salicylate

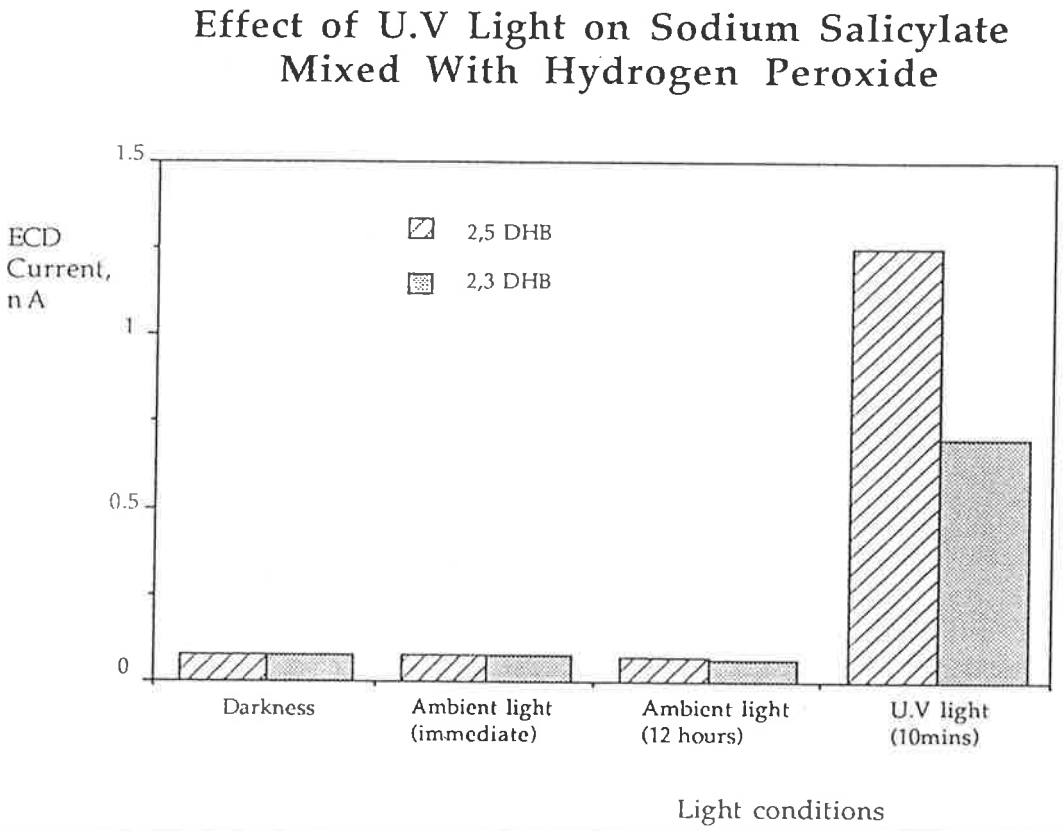

Figure 7.13 Effect of U.V light on hydrogen peroxide and sodium salicylate

7.10 Hydroxyl Radical Production in Bleached Root-Filled Teeth

A total of 40 samples from experimental teeth were analysed by HPLC-ECD over a period of 8 hours. The amount of reaction products of sodium salicylate with hydroxyl radicals detected in each experimental sample was calculated, in picomoles. The results for 2,5-DHB are presented in Table 7.3, while the results for 2,3-DHB and pyrocatechol are presented in Tables 7.5 and 7.6 respectively. As discussed in Section 6.4, the amounts of 2,5-DHB detected in samples was used in statistical analysis as the index of hydroxyl radical production because it was the reaction product found in greatest quantities.

Reaction products were detectable in a total of 25 (62.5%) of the samples and, of these, actual yields of 2,5-DHB varied from the detection limit of 1.0 pmol up to 21.0 pmol per 20 microlitre aliquot of sample. Yields of 2,3-DHB and pyrocatechol were lower at 0.3 to 20.2 pmol and 0.1 to 3.4 pmol respectively. Reaction products were detected in both discoloured and non-discoloured teeth, as well as in teeth both with and without defects at the cemento-enamel junction. No reaction products were detected in the samples from the negative control (undiscoloured, unbleached tooth in 1 mM sodium salicylate solution).

Three-way analysis of variance indicated that the presence of EDTA had no significant effect in any group on the proportion of teeth producing 2,5-DHB. There was, however, a consistent tendency in teeth producing 2,5-DHB, for the amount to be greater in the presence of EDTA. The mean increment varied from two-fold in controls to three-fold in discoloured teeth. Despite this trend, these differences were not statistically significant at the 5% level ($p=0.29$). A significant statistical relationship between the presence of tooth discoloration due to blood

components and the yield of hydroxyl radicals (p=0.039) was demonstrated in three-way analysis of variance (see Table 7.4). The overall trend was for higher yields of hydroxyl radicals to occur in the discoloured teeth. Basal levels of 2,5-DHB produced by control teeth (not discoloured and with no artificial defects) were either low, in 50% of teeth, or below detectable limits, in the remainder, and the presence of EDTA did not influence these figures. Overall, the level (with and without EDTA) of 2,5-DHB in this group was 0.7 ± 0.3 pmols per aliquot (n=10).

The presence of discoloration by blood dramatically increased the proportion of teeth producing detectable levels of 2,5-DHB to 100% (in teeth discoloured, but without the effect of defects) and significantly enhanced the mean yield to 5.6 ± 1.8 pmols per sample (n=10). Within this group, there was a trend for EDTA to give a further increment in 2,5-DHB yields (8.3 ± 3.3 pmols with EDTA *vs* 2.8 ± 0.3 pmols without, n=5 in each case) but this was not statistically significant (see Table 7.3).

The presence of defects did not enhance either the proportion of teeth producing detectable levels of 2,5-DHB or the yields in those teeth. The overall yield (with and without EDTA) was 0.5 ± 0.3 pmols per sample (n=10) which was not significantly different from the values(0.8 ± 0.2, n=10) for control teeth without artificial defects. There was, however, a significant interaction between the presence of discoloration and of defects (p=0.015), indicating that the highest yields of hydroxyl radicals occurred in teeth which were discoloured, but which did not have defects at the cemento-enamel junction. Thus, the presence of artificial defects in discoloured teeth greatly reduced the effect of discoloration, with only 60 of such samples producing 2,5-DHB, with

	DIS-COLOURED	DEFECT	EDTA	Amount of 2,5-DHB in sample, p.mols per 20 µl aliquot	Means ± S.E.	
a	●		●	6.3 21.0 6.5 6.3 1.6	8.3 ± 3.3	5.6 ± 1.8
b	●			3.3 3.0 1.8 2.8 3.0	2.8 ± 0.3	
c	●	●	●	0 0 2.7 0 1.5	0.8 ± 0.5	1.1 ± 0.3
d	●	●		2.5 1.8 1.5 0 1.1	1.4 ± 0.4	
e			●	0 1.3 2.1 1.5 0	1.0 ± 0.4	0.8 ± 0.2
f				1.0 0 1.1 1.0 0	0.6 ± 0.3	
g		●	●	0 0 2.1 2.9 0	1.0 ± 0.6	0.5 ± 0.3 (9)
h		●		0 0 10.3* 0 0	0.0 ± 0.0 (4)	

Table 7.3 Amounts of reaction product, 2,5-DHB assayed in experimental sample groups (n=5) as a measure of hydroxyl radical generation. Zero values indicate where the 2,5-DHB levels were below the nominal detection limit (1 pmol) of the assay.

* This aberrant value was estimated from a large HPLC peak which approximated with that of 2,5-DHB but was not accompanied by proportional peaks for 2,3-DHB and pyrocatechol. This value was not included in subsequent statistical calculations.

Source	n	Sum of Squares	Mean of Squares	F-test	P value
Discolouration (A)	20	47.24	47.24	4.65	0.039
Defect (B)	20	33.98	33.98	3.34	0.076
A x B	10	66.9	66.9	6.59	0.015
EDTA (C)	20	11.76	11.76	1.16	0.289
A x C	10	19.92	19.92	1.96	0.171
B x C	10	35.85	35.85	3.53	0.069
A x B x C	5	13.51	13.51	1.33	0.257
Error		324.84	10.15		

n= number of teeth in sample

Table 7.4 Three-way analysis of variance of hydroxyl radical generation under various conditions in the human tooth model based on the amounts of 2,5-DHB detected.
P = probability that the null hypothesis is correct using a 5% level of statistical significance

	DIS-COLOURED	DEFECT	EDTA	Amount of 2,3-DHB in sample, p.mols per 20 µl aliquot		
					Means ± S.E.	
a	●		●	0.9 20.3 0.9 6.9 0.5	5.9 ± 3.8	3.4 ± 2.0
b	●			0.7 0.9 0.8 1.4 0.6	0.9 ± 0.1	
c	●	●	●	0 0 1.0 0 0.6	0.3 ± 0.2	0.6 ± 0.2
d	●	●		1.7 0.8 1.2 0 0.4	0.8 ± 0.3	
e			●	0 0.5 0.8 0.9 0	0.4 ± 0.2	0.3 ± 0.1
f				0.3 0 0.5 0.4 0	0.2 ± 0.1	
g		●	●	0 0 0.8 0.8 0	0.3 ± 0.2	0.2 ± 0.1
h		●		0 0 0.8 0 0	0.2 ± 0.2	

Table 7.5 Amounts of reaction product, 2,3-DHB assayed in experimental sample groups (n=5) as a measure of hydroxyl radical generation. Zero values indicate where the 2,3-DHB levels were below the nominal detection limit (0.3 pmol) of the assay.

	DIS-COLOURED	DEFECT	EDTA	Amount of pyrocatechol in sample, p.mols per 20 µl aliquot	Means ± S.E.	
a	●		●	0.3 3.4 0.4 0.4 0.4	1.0 ± 0.6	0.7 ± 0.3
b	●			0.3 0.4 0.4 0.2 0.4	0.3 ± 0.0	
c	●	●	●	0 0 0 0 0	0.0 ± 0.0	0.1 ± 0.0
d	●	●		0.3 0.5 0.2 0 0	0.2 ± 0.1	
e			●	0 0 0.2 0.3 0	0.1 ± 0.0	0.1 ± 0.0
f				0 0 0.1 0.2 0	0.1 ± 0.0	
g		●	●	0 0 0.1 0.3 0	0.1 ± 0.0	0.0 ± 0.0
h		●		0 0 0.2 0 0	0.0 ± 0.0	

Table 7.6 Amounts of reaction product, pyrocatechol assayed in experimental sample groups (n=5) as a measure of hydroxyl radical generation. Zero values indicate where the pyrocatechol levels were below the nominal detection limit (0.1 pmol) of the assay.

even these teeth giving levels (1.1 ± 0.3 pmols, n=10) which were not significantly different from controls. As with other groups, the presence of EDTA did not affect the results (0.8 ± 0.5 pmols with EDTA *vs* 1.4 ± 0.4 without, n=5 in each case). The exact reason why the presence of artificial defects should completely negate the influence of blood discoloration on hydroxyl radical production is not readily apparent. Possible reasons are explored in Section 8.8.

A sample chromatogram of a discoloured tooth which had exhibited reaction products appears as Figure 7.14. Three distinctive peaks denoting reaction products were apparent. When compared to control injections these were concluded to be pyrocatechol (at 3.7 mins), 2,5-DHB (at 4.9 mins) and 2,3-DHB (at 6.8 mins). The internal standard (HGA) was evident with an elution time of 2.4 mins. A sample chromatogram of a discoloured tooth which had been flushed with EDTA appears as Figure 7.15 and can be compared with Figure 7.14 in which no EDTA had been used.

Three-way analysis of variance using the amounts of both 2,3-DHB and pyrocatechol produced, was also conducted. These tests revealed similar trends to those displayed by 2,5-DHB, although with a lower level of significance. Three-way analysis of variance tables for both 2,3-DHB and pyrocatechol are shown in Appendix 4 and Appendix 5 respectively.

In a total of five of the experimental samples, hydrogen peroxide peaks were detected on chromatograms. These peaks were seen in samples from two teeth without defects at the cemento-enamel junction and three teeth with defects at the cemento-enamel junction. The identity of these peaks was concluded by comparison with control injections of hydrogen peroxide (see Table 7.1). The yields of the reaction products

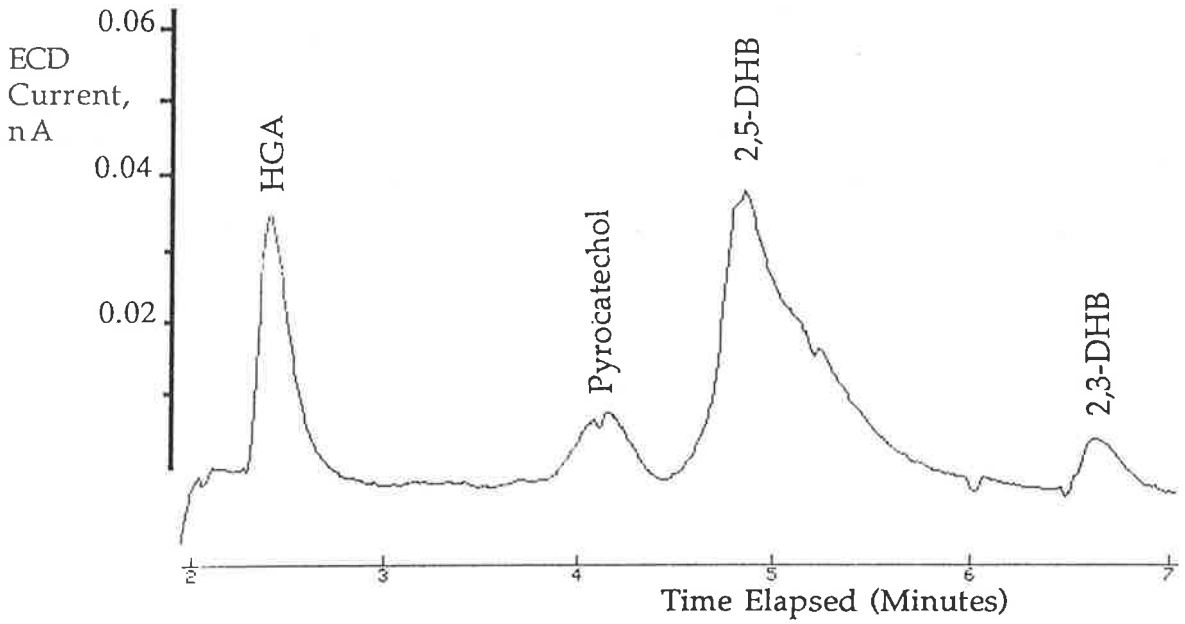

Figure 7.14 Chromatogram from an experimental tooth sample, where the tooth was discoloured and there was no defect at the CEJ. EDTA was not used to flush the pulp chamber prior to bleaching.

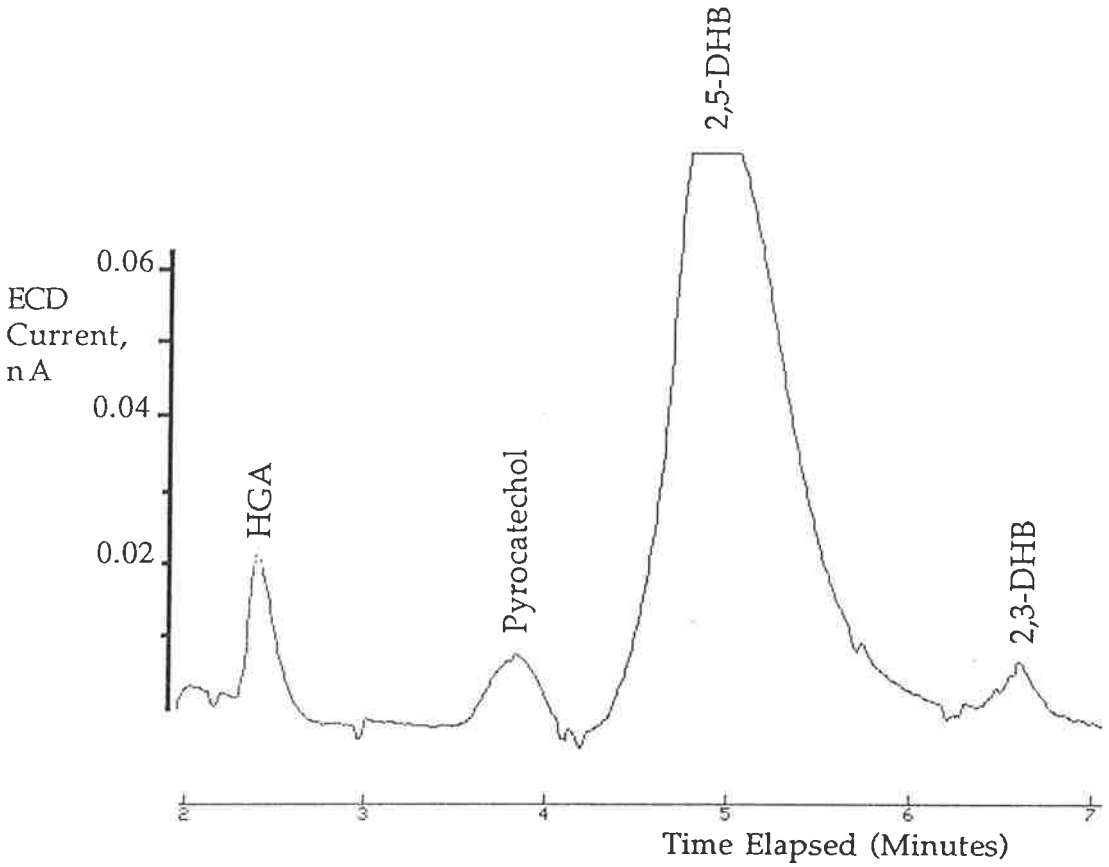

Figure 7.15 Chromatogram from an experimental tooth sample, where the tooth had been discoloured and there was no defect at the CEJ. EDTA had been used to flush the pulp chamber before bleaching.

2,3-DHB, 2,5-DHB or pyrocatechol in the presence of hydrogen peroxide peaks were not significantly different from the amounts of reaction products detected where no hydrogen peroxide peak was evident. A sample chromatogram displaying a hydrogen peroxide peak along with the other reaction products of salicylate hydroxylation is shown as Figure 7.16.

In the group of teeth which had been discoloured by blood, the mean ratio of the reaction products 2,5-DHB, 2,3-DHB and pyrocatechol was 1 : 0.5 : 0.09. In the non-discoloured tooth group, this proportion was calculated to be 1 : 0.3 : 0.08 for 2,5-DHB, 2,3-DHB and pyrocatechol respectively. The overall mean ratio of reaction products for the total sample group was 1 : 0.4 ± 0.06 : 0.09 ± 0.02 for 2,5-DHB, 2,3-DHB and pyrocatechol respectively. Thus, the ratio of reaction products was relatively constant in those samples where hydroxyl radical production was detected, regardless of whether discoloration was present or not. This ratio was also apparent in comparisons of teeth with or without defects created at the CEJ and in teeth with or without the introduction of EDTA. Detector peak heights for the internal standard (HGA) were examined and found to remain relatively constant over the course of the experiment. HGA peak heights, or rather the peak height detected at the retention time of 2.4 minutes, did not vary significantly with different amounts of reaction products found in the various categories of the experiment. This would suggest that there was no further hydroxylation of the reaction products (which would have also produced a peak at 2.4 minutes, representing pyrogallol) as may have occurred with the much higher fluxes of hydroxyl radical generated during the control experiments (for further discussion see Appendix 3).

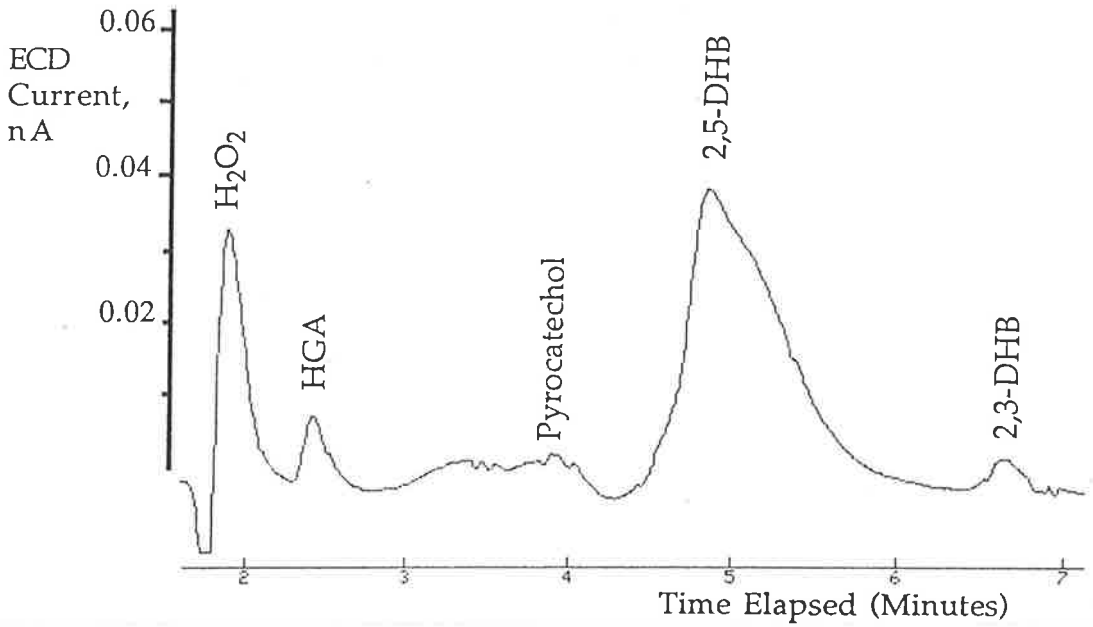

Figure7.16 Chromatogram showing salicylate hydroxylation products as well as a hydrogen peroxide peak (at 1.8 minutes).

8. DISCUSSION

8.1 Assay Sensitivity

A high linear correlation was evident for the standards HGA, 2,3-DHB and 2,5-DHB between amounts injected and recorder peak heights. With injections of relatively simple compounds, a high correlation could be expected and this indicates a good level of reproducibility of results in the detection system. A sensitivity level of one picomole of injected substance was found in this study and this accords well with the findings of Wright (1988), who also found a sensitivity of one picomole. Much of the experimental technique employed in this study has been adapted from the work of Wright and to obtain a similar level of sensitivity to some degree validates the results of the protocol employed here.

An aspect of this system which had the potential to create a degree of uncertainty was the variation of retention times for the standards with different batches of mobile phase. The point was made in the methods section that a fresh batch of mobile phase was made up for each phase of the project. Given that the overall period of the project was several months and given the multitude of samples injected on to the HPLC apparatus it was not possible to use the one batch of mobile phase. The pH of these different mobile phases only varied between 2.95 and 3.05. With each of these fresh mobile phases a run of 20 pmol of each of the mixed standards (HGA, 2,3-DHB and 2,5-DHB) was injected on to the column. This then allowed confirmation of the identity of reaction products in the various experiments and allowed for accurate standardised measurement of these reaction products. In the methods section these were termed collateral injections.

The electro-chemical detector proved to be a very accurate and sensitive instrument. Despite best attempts at keeping ambient temperature constant with air-conditioning there were minor fluctuations in recorder values depending on the time of day and, accordingly, ambient temperature. This may also have affected retention times. Consequently, most of the experimental runs were conducted in the early evening or overnight.

8.2 Degradation of Standards

Degradation curves for the three standards showed that the two dihydroxybenzoates were relatively stable over the 27 hour period of observation. Small, short-term variations in the recorder peak height values for these standards are apparent on their degradation curves and these probably reflect the variations in temperature which were discussed in the previous section. Overall, however, these standards remained relatively stable and this aspect of the project served to demonstrate that 2,3-DHB and 2,5-DHB, as reaction products of salicylate hydroxylation in the experimental tooth samples, could be measured reliably in the 8 hour period over which they were injected on to the column.

The internal standard HGA appeared to degrade quite rapidly so that after 8 hours there was approximately 75% of the initial amount remaining. Despite this degree of degradation in the control experiments, there did not appear to be a significant change in the amounts of HGA added to the experimental tooth samples, with these values remaining relatively constant in those samples. Further discussion on the suitability of HGA as the internal standard will be made later in this chapter.

8.3 Control Generation of Hydroxyl Radicals

Both the addition of ferrous sulphate to hydrogen peroxide and the autoxidation of ferrous iron by the addition of EDTA proved to be good positive controls for the generation of hydroxyl radicals, evidenced by the generation of reaction products from salicylate. This generation of hydroxyl radicals was quite rapid and appeared to be complete within five minutes. This observation is also supported by the addition of varying concentrations of ferrous sulphate to hydrogen peroxide. (See Section 7.5). In this experiment, no increase in the amount of reaction products were apparent after the first sample, which was injected on to the column within five minutes of mixing the reagents.

The detection of the reaction products in picomole amounts validates the adoption of this technique for detection of hydroxyl radicals in such a system. The two dihydroxybenzoates were identified by comparison with known standards. The third significant reaction product was detected with a retention time of approximately 3.7 minutes and its identification as pyrocatechol was the subject of a subsequent control experiment (Section 7.7). In this addition of 50 μM ferrous sulphate to 0.5 ml 30% hydrogen peroxide, the proportions of these reaction products was calculated to be 12%, 38% and 50% for pyrocatechol, 2,3-DHB and 2,5-DHB respectively. The results of this study thus indicate that there was a greater production of 2,5-DHB than 2,3-DHB. This contrasts with the proportions of reaction products reported by Grootveld and Halliwell (1986) as 11%, 49% and 40% for pyrocatechol, 2,3-DHB and 2,5-DHB respectively. While the present finding differs from the relative amounts suggested by Grootveld and Halliwell (1986), it is consistent with the findings of Wright (1988). From this simple addition of ferrous

sulphate to hydrogen peroxide in the present study, the ratio of 2,3-DHB to 2,5-DHB was found to be 1:1.2. This compares well with the findings of Wright (1988) who demonstrated a ratio of 1:1.5 in a chemically-generated system.

The controlled generation of hydroxyl radicals by the autoxidation of ferrous iron by EDTA and by the addition of both ferrous sulphate and EDTA to hydrogen peroxide resulted in the production of extremely large amounts of reaction products from salicylate with resulting chromatogram peak heights exceeding the maximum of the recorder range. These reactions displayed greater amounts of 2,3-DHB than 2,5-DHB, as was suggested by Grootveld and Halliwell (1986), but the difference between the two was only marginal. The findings of proportions of approximately 30% for each of these latter reaction products may actually reflect the data processor calculating values from peak heights which represent maximal values rather than true amounts of the reaction products.

Despite the suggestion by Halliwell (1991) that 2,3-DHB be used as the true indication of hydroxyl radical production (see Section 4.2), in this study 2,5-DHB was considered more appropriate as the measure of hydroxyl radical production in the statistical analysis of results because :

a. It was the most plentiful reaction product in this study
b. There was no likelihood of microsomal P450 cytochrome production of 2,5-DHB in this experimental system.

8.4 Effect of EDTA on Hydroxyl Radical Generation

In addition to EDTA participating in the autoxidation of ferrous iron to produce hydroxyl radicals, a number of the control experiments

showed that the addition of EDTA to a mixture of hydrogen peroxide and ferrous sulphate significantly increased the generation of hydroxyl radicals. This trend was noted also in the experimental tooth samples where EDTA had been used to flush the pulp chambers prior to the bleaching procedure. The significance of the latter will be discussed more fully in Section 8.8. The findings in this study that EDTA increases the yield of hydroxyl radicals supports previous studies. Halliwell (1978) demonstrated that EDTA potentiated the catalysis of hydroxyl radical production by iron. Maskos et al (1990) measured the yields of 2,3-DHB and 2,5-DHB from salicylate after generation of hydroxyl radicals in the presence of various oxidants. The addition of the complex ion Fe^{3+}-EDTA was found to increase the yield of 2,3-DHB and 2,5-DHB by a factor of approximately five. Park et al. (1987) found that the hydroxylation of sodium salicylate was maximised when the ratio of EDTA to ferrous iron was 1:2. In the control experiments of the present study, this ratio was only 1:0.25. Moorhouse et al (1985), on the basis of experimental evidence, suggested that in the presence of EDTA, hydroxyl radical production which had been catalysed by cobalt ions produced hydroxyl radicals which were formed "in free solution" and could then attack an aromatic (scavenger) molecule. This may have occurred either because when EDTA is present it prevents the cobalt ion from combining with other compounds such as phosphate buffer to form unreactive complexes, or by favourably altering the redox potential of the Co(II). They suggested that in the absence of EDTA, hydroxyl radicals acted in a 'site-specific' manner and were more difficult to intercept by hydroxyl radical scavengers. In this situation, the Co(II) probably exists bound to another compound, such as phosphate buffer. Any hydroxyl radicals formed

would then be directed towards the molecule to which the metal ion was bound. That is, the hydroxyl radical generation would be 'site-specific' and would, therefore, have more difficulty interacting with scavenger molecules such as salicylate. This situation may also apply to hydroxyl radical generation catalysed by iron.

In the control experiments carried out in this project the increase in reaction product yields may have been masked by the fact that reaction product peak heights were at the maximum of the recorder range, thus not allowing a true measurement of the increase in yields in the presence of EDTA. The trend to higher yields of hydroxyl radicals when EDTA was added to the reaction mixture, however, was certainly apparent.

As already discussed in Section 2.5, other agents such as DETAPAC and desferrioxamine are also strong chelators of iron and are able to effectively inhibit the generation of hydroxyl radicals by the reducing the amount of available iron. According to Marklund et al. (1983), most of the chelating agents have a low degree of specificity and consequently will chelate most polyvalent metal-ions such as Ca^{2+}, Mg^{2+} and Mn^{2+}. Given the apparent greater efficiency in reducing the amount of available iron for the Fenton reaction and the ability of agents such as DETAPAC also to chelate metal ions such as Ca^{2+}, perhaps further investigation into other chelating agents besides EDTA in the practice of intracoronal bleaching, and endodontics generally, is warranted.

8.5 Presence of Other Reaction Products

Many of the papers reviewed for this project, which used the technique of salicylate hydroxylation for the detection of hydroxyl radicals, considered only the reaction products 2,3-DHB and 2,5-DHB.

The reaction product, pyrocatechol, is seldom mentioned. In the study by Grootveld and Halliwell (1986), pyrocatechol was reported as a minor reaction product of salicylate hydroxylation by hydroxyl radical attack, constituting approximately 11% of the reaction products. Due to the fact that a third reaction product with a retention time of approximately 3.7 minutes was noted in both the control generations of hydroxyl radicals as well as in the experimental tooth samples, it was considered important to confirm that it was pyrocatechol which was being detected in these samples. The chromatogram shown in Figure 7.11 provides evidence that pyrocatechol was indeed the third reaction product. The possible products of subsequent dihydroxybenzoate hydroxylation which may have been produced as the result of high radical flux were analysed in the same control experiment. This was conducted to establish whether such products could interfere with the detection of either the reaction products or the internal standard. In the conditions associated with the experimental tooth samples, it is unlikely that the amounts of hydroxyl radicals being produced, particularly where sodium salicylate was present in excess by several orders of magnitude, could result in the further hydroxylation of the reaction products 2,3-DHB, 2,5-DHB and pyrocatechol. The theoretical production of these products of dihydroxybenzoate hydroxylation are considered in detail in Appendix 3. Pyrogallol was the only possible product of further DHB hydroxylation which was detected and this exhibited a retention time of 2.4 minutes, which was very similar to the internal standard, HGA. Hydroquinone was also detected with a retention time of 2.4 minutes, but, while it is a hydroxylation product of phenol, it is not considered to be a hydroxylation product of salicylate. In view of this, in conditions of

high hydroxyl radical flux, where it may be possible to further hydroxylate the DHB reaction products to pyrogallol, HGA may not be the ideal internal standard. This point may be further supported by the previous observation that HGA tends to degrade somewhat more rapidly than the reaction products 2,3-DHB and 2,5-DHB. It may be that hydroquinone could be more appropriate as an internal standard, particularly if its rate of degradation was less than that of HGA. As previously discussed, however, the recorder peak height values for the internal standard remained relatively constant over the course of the experimental tooth sample analysis, regardless of variations in the amounts of reaction products being detected. This would suggest that these further products of DHB hydroxylation were not being produced in this system where relatively small amounts of hydroxyl radicals were being generated and sodium salicylate was well in excess.

In the conditions created in this system, resorcinol and phloroglucinol were not detected over the 25 minute run time. In the study by Grootveld and Halliwell (1986), resorcinol was used as an internal standard and detected at approximately 16 minutes. This study, however, used a mobile phase of different composition, a different oxidation potential in the ECD and was also specifically designed to detect salicylate.

8.6 The Effect of Hydrogen Peroxide on Salicylate

Control experiments displayed an apparent minor reaction between hydrogen peroxide and sodium salicylate, resulting in the production of 2,3-DHB, 2,5-DHB and pyrocatechol. This obviously had the potential to affect the detection of hydroxyl radicals in both the control

and experimental tooth samples. Previous papers employing this technique of hydroxyl radical detection have not discussed this phenomenon in any detail. It was not discussed as a limitation in the techniques described by Grootveld and Halliwell (1986), Wright and Priestley (1985) or Wright (1988) from which the protocol for the present study was derived, nor has it been discussed in the reviewed articles where this technique was employed to detect hydroxyl radicals in certain biological systems. Halliwell (1977) reported that hydrogen peroxide did not hydroxylate salicylate in the absence of NADH or phenazine methosulphate. From this study, he concluded that while hydrogen peroxide was necessary in the process of salicylate hydroxylation, it did not hydroxylate salicylate on its own. Sagone et al. (1987) found that the hydrogen peroxide scavenger catalase only marginally inhibited the hydroxylation of salicylate by hydroxyl radicals from activated granulocytes, indicating that hydrogen peroxide had no direct effect on salicylate. Lunak et al (1987) investigated the methylene-blue hydroxylation of salicylic acid by hydrogen peroxide initiated by visible radiation and catalysed by ferric chloride. In this study, the main reaction product was found to be 2,3-DHB. In this photosensitive reaction, the first step was suggested as being the reduction of ferric iron to ferrous iron thus providing the latter for a Fenton reaction. In the absence of the ferric chloride, these authors reported, the reaction between hydrogen peroxide and salicylate proceeded extremely slowly if at all.

In the presence of hydrogen peroxide, sodium salicylate may also form a peroxysalicylic acid and water. This reaction will occur in slightly acidic conditions. Peroxysalicylic acid is considered to be quite unstable, however, and it is likely that the molecule would rapidly revert to

salicylic acid and hydrogen peroxide. Peroxysalicylic acid, however, is also one of the intermediate species formed in the complex chain of reactions that occurs during hydroxylation of salicylate by hydroxyl radicals. It is possible that the production of peroxysalicylic acid, albeit transient, may result in the production of limited amounts of 2,3-DHB, 2,5-DHB and pyrocatechol. This mechanism may thus also account for the apparent minor reaction seen between hydrogen peroxide and sodium salicylate in the control experiments.

To demonstrate the direct correlation between ferrous iron concentration and the generation of hydroxyl radicals various concentrations of ferrous sulphate were mixed with a constant amount of hydrogen peroxide. The graphs produced from these reactions clearly demonstrated that there was a direct link between the concentration of ferrous ions and the amount of reaction products resulting from hydroxyl radical generation. This control is supported by the findings in the experimental tooth samples that there was a greater production of hydroxyl radicals in discoloured teeth (a source of iron) as compared to the non-discoloured teeth. The significance of the results in experimental tooth samples will be considered in Section 8.7.

Another possibility accounting for the apparent production of reaction products from salicylate in the presence of hydrogen peroxide is the presence of trace amounts of iron, either in the MilliQ water from which solutions were made or, less likely, from the use of a stainless steel spatula used to apportion reagents during weighing. Hydroxyl radical production could have resulted from hydrogen peroxide reacting with this trace iron. Control experiments, however, established that there were no reaction products detected on the addition of EDTA to solutions of

hydrogen peroxide in the presence of sodium salicylate nor were any detected when only EDTA was added to sodium salicylate solution (see Table 7.1). In either case, if there had been trace amounts of iron in the water, the detection of reaction products after the generation of resulting hydroxyl radicals would have been expected.

In five of the experimental tooth samples, hydrogen peroxide was demonstrated to have passed through tooth structure into the sample solution. The identity of this peak was confirmed by comparing with a control chromatogram of hydrogen peroxide only. In these samples, the amounts of reaction products (2,3-DHB, 2,5-DHB and pyrocatechol) did not differ greatly from the other samples within that experimental group. This would indicate that while hydrogen peroxide was obviously present, there was no additional effect on the production of reaction products resulting from hydroxyl radical generation. The significance of this will be considered again in Section 8.8.

Thus, although there appears to have been a minor reaction between hydrogen peroxide and sodium salicylate in the control experiments, this does not appear to have been repeated in the experimental tooth samples. Previous studies which have investigated the hydroxylation of salicylate have agreed that such a reaction is insignificant if it occurs at all. In the experimental tooth samples of this research project the effect of hydrogen peroxide on sodium salicylate appears to be limited. This finding should dispel any uncertainties introduced by the findings in the control experiments as they may affect the bleaching phase of the project. There does, however, appear to be little work into this aspect of the technique and, given its apparently

increasing acceptance for the detection of hydroxyl radicals in various areas of biomedical research, it appears to warrant further investigation.

8.7 Effect of U.V Light on Hydroxyl Radical Production

The effect of ultra-violet light on hydroxyl radical production was investigated partly to determine the effect that ambient light may have had on tooth sample results given the findings of Harkin et al. (1991), and partly because ultra-violet light is employed in clinical dental practice to "activate" hydrogen peroxide in intra-coronal bleaching procedures. It was also considered important to determine whether U.V light may account for the apparent reaction between hydrogen peroxide and sodium salicylate, as discussed in the previous section. From the graphs presented in Figures 7.12 and 7.13 it is evident that exposure to intense U.V light causes significant increases in hydroxyl radical production with sodium salicylate alone or with mixtures of hydrogen peroxide and sodium salicylate when compared to solutions stored in either darkness or ambient light. Ambient light did appear to cause small amounts of hydroxyl radical production within sodium salicylate solution, but in insignificant levels. These levels did not appear to interfere with the experimental results. Reaction products were still detected in those samples which were mixed and stored in darkness. This possibly is due to the fact that achieving total darkness (an absence of U.V light) in such mixing procedures is difficult.

The finding of significantly greater amounts of hydroxyl radical formation from sodium salicylate alone when exposed to U.V light supports the findings of Harkin et al. (1991). Presumably, the U.V light is causing hydroxyl radical generation from the water molecules in this

aqueous solution. The yield of hydroxyl radicals is dramatically increased by the addition of hydrogen peroxide to the salicylate solution prior to irradiation with the U.V light. In this situation, not only is the splitting of the water molecules contributing to hydroxyl radical production but it is likely that the homolytic fission of hydrogen peroxide, as discussed in Section 2.3, is also adding to this generation of hydroxyl radicals.

The observation that hydroxyl radical production was dramatically increased after exposure to intense U.V light could also, in part, have demonstrated that trace amounts of iron were present in the water. Lunak et al (1984) examined the rate and yields of photo-initiated hydroxylation of sodium salicylate by hydrogen peroxide and found that there was a substantial increase when ferric iron was added to the system. They proposed that the U.V radiation used reduced the catalytically inactive Fe^{3+} to Fe^{2+}, thereby continuously producing catalyst for the Fenton reaction. Thus, it could also be possible that in the control experiments, ferric iron, which might be present in the water, does not react with EDTA to allow detection of reaction products of hydroxyl radical generation. When this solution is exposed to U.V light, however, this ferric iron would be reduced to ferrous iron and so would be available to catalyse the generation of hydroxyl radicals in the presence of hydrogen peroxide.

As discussed in Section 1.6, U.V light is also often employed in the "activation" of hydrogen peroxide during intra-coronal bleaching of root-filled discoloured teeth. In such teeth, not only is hydrogen peroxide present, but so, most likely, is a source of iron. Whether or not this iron is present in the ferric or ferrous state prior to bleaching, once the tooth has

been exposed to intense U.V light, this iron will be in the ferrous state and so able to catalyse hydroxyl radical production in the presence of hydrogen peroxide. The homolytic fission of hydrogen peroxide, by energy derived from the U.V radiation would probably augment this generation of hydroxyl radicals. In view of these factors, the bleaching of teeth using hydrogen peroxide activated by intense ultra-violet radiation should be approached with a degree of caution. The use of this form of activation certainly appears worthy of re-evaluation.

8.8 Hydroxyl Radical Production in Bleached Root-Filled Teeth

The reaction products of hydroxyl radical attack on sodium salicylate were detected in a total of 25 of the experimental tooth samples. These reaction products were detected in both discoloured teeth and non-discoloured teeth. Analysis of variance revealed, however, a statistically significant association between the presence of tooth discoloration by blood and the generation of hydroxyl radicals. Yields of hydroxyl radicals were greater in discoloured teeth than in non-discoloured teeth. From these results it can be reported that hydroxyl radicals are indeed generated in bleached root-filled teeth and this generation is significantly affected by the presence of discoloration caused by blood products. Given the greater yields in the presence of discoloration, it can also be deduced that ferrous iron is available for the catalysis of hydroxyl radical production. This, however, does not necessarily support the claim by authors such as Grossman, who suggest that tooth discoloration after trauma is due to breakdown products of haemoglobin, or to free iron salts within the dentine. As discussed previously, the most likely source of this available iron would be haemoglobin itself, which releases iron for the

Fenton reaction in the presence of hydrogen peroxide. With the concentration of hydrogen peroxide employed in this study, and indeed the concentration employed in clinical practice, this situation is quite possible. Given that hydroxyl radical reaction products were detected and that there was a significant contribution of tooth discoloration, there was probably a source of available iron within these teeth. Whether unbound iron accounted for tooth discoloration is a matter for further investigation.

In analysing the amounts of reaction products detected from experimental tooth samples, the diffusion of salicylate and of the reaction products themselves must be considered. In the experimental system designed for the present study to be successful, there would have to have been some diffusion by salicylate into tooth structure, followed by the escape out of the tooth by salicylate hydroxylation products so that they could then be detected. If not, all hydroxyl radical production would have occurred at the external surface of the tooth. It is most likely that the concentrations of the reagents necessary for hydroxyl radical production, namely hydrogen peroxide and iron (bound to haemoglobin) would decrease as the distance from the pulp chamber increased. Most hydroxyl radical production would therefore probably occur close to the pulp chamber. Given the dynamics of this system, it is unlikely that either all hydroxyl radical production would have occurred in the presence of salicylate or that all the reaction products of this reaction found their way back out into the external solution of salicylate to be detected. Thus, it is unlikely that all hydroxyl radical production could be detected in this system. That is, hydroxyl radical production in the experimental teeth was probably greater than that detected by the system employed in this

study. The generation of hydroxyl radicals close to the pulp chamber in itself may not be damaging to tissue, but, as discussed in previous sections, other longer-lasting oxygen radicals may also be formed by the action of hydroxyl radicals, which may add to the destructive effects of hydroxyl radicals produced closer to the external surface of the tooth.

Reaction products of hydroxyl radical activity were also detected with non-discoloured teeth. This phenomenon could be explained through a number of mechanisms. The possibility must be considered that iron, in a form that could be made available for the Fenton reaction was in the tooth prior to the discoloration phase of the protocol. Tooth extraction is itself a traumatic injury and it is quite possible that blood products in small quantities were forced up into dentinal tubules during this event, thus providing a source of iron without the tooth being obviously clinically discoloured. Thus, a free iron source may have existed in some of the non-discoloured teeth. The presence or absence of iron in experimental teeth was not established as it was considered beyond the scope of this project, however, such a determination would obviously be useful in confirming the iron-catalysed generation of hydroxyl radicals in these teeth.

Another possible reason for the detection of reaction products in non-discoloured teeth is that hydroxyl radical generation was occurring through simple homolytic fission as a result of heating by the red hot instrument. Such a situation would have, of course, occurred with all the experimental teeth to some extent, but the fact that the greatest hydroxyl radical production appeared to occur in discoloured teeth would support the argument that iron from the discoloration was the major catalytic agent for hydroxyl radical production.

A third possible reason for reaction products being detected in non-discoloured teeth is that hydrogen peroxide itself was reacting with the sodium salicylate as was noted in the control experiments. Here again, the fact that there were greater yields of hydroxyl radicals in discoloured teeth would indicate the proportionality between available iron and hydroxyl radical production. Hydrogen peroxide peaks were found in five of the experimental samples and in these samples there was no significant increment in the amounts of reaction products detected. It is unlikely, therefore, that the reaction of hydrogen peroxide itself with sodium salicylate could account for the detection of reaction products in non-discoloured teeth.

The finding of the hydrogen peroxide peaks was important because :

a. It demonstrated that hydrogen peroxide could diffuse right through tooth roots, thus confirming the findings reported by Rotstein et al. (1991).

b. It demonstrated that hydrogen peroxide was indeed capable of diffusing through tooth to a point where it could react with available iron and catalyse the Fenton reaction. The detection of reaction products thus confirms that hydroxyl radicals generated in this experimental system are able to be detected by the sodium salicylate solution in which the tooth root was bathed.

c. It demonstrated that in the experimental samples there was no significant effect of hydrogen peroxide on the sodium salicylate solution

The finding that reaction products were detected in more teeth without experimentally placed defects at the cemento-enamel junction than with defects might suggest that the cementum layer is unimportant in the diffusion of either hydrogen peroxide, hydroxyl radicals or sodium salicylate. Due to the small size of the hydrogen peroxide molecule, it would probably diffuse right through cementum given time. Rotstein et al. (1990) demonstrated significantly greater diffusion of hydrogen peroxide when there were cementum defects present. The presence of a cementum layer, therefore, was assumed to be significant for hydrogen peroxide. Hydroxyl radicals react with the first molecule they encounter so it is unlikely that they would themselves be able to penetrate the cementum layer. As discussed previously, sodium salicylate solution from the exterior may have been able to penetrate cementum (and dentine) but its hydroxylation products would also have to be able to diffuse back out into the main solution after attack by hydroxyl radicals in order to be detected. Thus, in teeth with defects, sodium salicylate may have entered tooth structure, reacted with hydroxyl radicals, but all the reaction products may not have been able to exit the tooth and be subsequently detected. A more likely explanation for the detection of reaction products in teeth without experimentally produced cementum defects, however, would be that there were defects already present in the cementum, due either to developmental defects, the extraction procedure, or a previous trauma which were not detected under the stereomicroscope. Since 10 teeth out of this group of 16 teeth had been experimentally discoloured, it could be argued that the discoloration was still the significant variable.

Control results in this study and the results of other previous studies (Maskos, 1990) have demonstrated that EDTA appears to increase the yield of hydroxyl radicals. This increased yield was also apparent in the experimental tooth samples, but there was no significant association between the use of EDTA and the generation or non-generation of hydroxyl radicals in bleached teeth. In those teeth where EDTA had been used to flush the pulp chamber prior to bleaching and hydroxyl radicals were generated, it appears that there were greater amounts of the reaction products 2,3-DHB, 2,5-DHB and pyrocatechol detected. In addition to the possibility that these hydroxyl radicals were formed " in free solution" (see Section 8.4) as suggested by Moorhouse et al. (1985), there must also be the possibility that any free iron in the teeth was autoxidised when the EDTA was introduced. However, given that most hydroxyl radical production was seen in discoloured teeth, the most likely source of iron for catalysing this production was haemoglobin, which released the iron after exposure to hydrogen peroxide. The possibility that autoxidation of free iron (that is, iron already in the tooth, not bound to haemoglobin) reacted with EDTA prior to the introduction of hydrogen peroxide must be considered, therefore, less likely. The clinical implications of this observation will be dealt with in the following section.

8.9 Implications For Clinical Dental Practice

The results of this study demonstrate that hydroxyl radicals are generated in bleached root-filled teeth. The generation of hydroxyl radicals was shown to be greater in teeth which had been discoloured by blood components. Since non-discoloured teeth are not usually bleached,

it is the group of discoloured teeth in the experimental group which are of most relevance to clinical dental practice. The incidence of invasive cervical resorption in bleached root-filled teeth has been suggested as being of the order of 6.9% (Friedman et al., 1988), although other workers suggest this figure may be lower (Heithersay et al., 1992). Normally, therefore, the body must be able to cope with both the agents employed during bleaching of teeth and the products of these agents after bleaching including, possibly, hydroxyl radicals. In some cases, however, where there may be defects in the tooth that allow the leakage of bleaching agents or their products, cervical periodontal tissue destruction occurs and there is an ensuing resorptive response against tooth structure. Both hydrogen peroxide and hydroxyl radicals have both been shown to cause cellular damage ranging from DNA alteration to cell death. There appears to be little information in the literature dealing specifically with the effect of hydrogen peroxide on the non-cellular components of connective tissue. Hydroxyl radicals have been shown to cause significant damage to connective tissue components collagen and hyaluronic acid. It may be that the combined influence of hydroxyl radicals and hydrogen peroxide is responsible for the degree of periodontal tissue destruction, both cellular and non-cellular, which can progress to invasive cervical root resorption.

The question arises as to whether it is clinically necessary to counteract agents such as hydroxyl radicals before they can institute the level of damage necessary to trigger such a resorptive response. At present, given the degree of toxicity of some agents, such as desferri-oxamine, it is probably not warranted to use such potent chemicals as a preventive agent for a relatively minor condition like invasive cervical

resorption. In this project, salicylate was used as the detector molecule for hydroxyl radicals, by virtue of its hydroxyl radical-scavenging ability. Salicylic acid is closely related to aspirin (acetyl-salicylic acid) which is widely used clinically as an anti-inflammatory agent. Its use in the scavenging of hydroxyl radicals during the bleaching of root-filled teeth, as well as a number of other areas of dentistry is worthy of further investigation, not only as a therapeutic agent but also perhaps as a marker for hydroxyl radical involvement in various pathological processes.

Another well-established hydroxyl radical scavenger, ascorbic acid, has the unfortunate additional effect of enhancing radical generation by reducing ferric iron to ferrous iron and thus making it available as a Fenton reaction catalyst. The anti-oxidant, α–tocopherol (see Section 3.4), which lacks this effect, may prove to be a good starting point for further research in this area.

The use of EDTA increased the yields of hydroxyl radicals in control samples and there was a trend to greater yields of hydroxyl radicals in teeth in which EDTA had been used to flush the pulp chamber prior to bleaching. This trend may have had increased statistical significance if there had been a larger number of teeth in the sample group. Flushing the pulp chamber with EDTA has been suggested as a means of removing smear layer prior to intra-coronal bleaching procedures. The use of EDTA in such a clinical procedure would appear to increase the risk of producing hydroxyl radicals in amounts that may contribute to the initiation of invasive cervical resorption. Given the finding by Casey et al. (1989) that there was no apparent difference to the success of bleaching if smear layer was present, perhaps the clinical use of

EDTA to remove smear layer prior to bleaching should now be reappraised.

EDTA is widely used in routine endodontic treatment as an intracanal irrigant. The use of EDTA solution concurrently with sodium hypochlorite solution has been found to be a highly successful antibacterial regimen (Byström and Sundqvist, 1985). It is considered that this is by virtue of the fact that EDTA removes the mineralised component of smear layer, thus allowing access by sodium hypochlorite solution to organic debris and to the bacteria infecting root canals and dentinal tubules. It is possible that EDTA in this application may also cause an increase in the production of oxygen radicals, possibly not including hydroxyl radicals, when combined with sodium hypochlorite. As discussed earlier, hypochlorous acid is a reactive oxygen species and a mixture with EDTA may result in an increased oxygen radical flux which effectively kills bacteria in the root canal. Such a possibility may also prove worthy of investigation.

Other iron-chelating agents besides EDTA were discussed in Section 2.5. The use of chelating agents such as DETAPAC, which can inhibit hydroxyl radical production by binding iron, yet also bind calcium, may be preferable to the use of EDTA, not only during bleaching, but also as a routine intracanal irrigant. Where EDTA appears to enhance hydroxyl radical production and to cause iron autoxidation, DETAPAC does not. Further research into alternative chelating agents is warranted.

Ultra-violet light was shown in this project to significantly increase the amounts of hydroxyl radicals generated. This generation could occur through simple homolytic fission of the hydrogen peroxide molecule. In

the presence of discoloration produced by blood components, this generation of hydroxyl radicals could be dramatically increased by virtue of the availability of iron for a Fenton reaction. Activation of hydrogen peroxide in the present study was achieved using an instrument heated to red heat. The degree of hydroxyl radical production comparing U.V light with heat is an area for further investigation. Clinically, given the degree to which there is an increase in hydroxyl radical production when hydrogen peroxide is exposed to U.V light, perhaps the use of this procedure should also be re-evaluated.

9. CONCLUSIONS

The aim of the present study was to test the hypothesis that hydroxyl radicals are generated when root-filled teeth, which have been discoloured by blood components, are bleached using 30% hydrogen peroxide. The detection of this highly reactive oxygen-derived free radical after bleaching could implicate it in the aetiology of the condition of invasive cervical root resorption which can occur subsequent to bleaching. Hydroxyl radicals have been shown to cause cell damage ranging from membrane lipid peroxidation, to DNA strand breakage to cell death. They have also been shown to degrade essential components of connective tissue such as collagen and hyaluronic acid.

Current thinking in the dental literature suggests that in teeth with a history of traumatic injury, subsequent deep discoloration is probably due to either breakdown products of blood components such as haemoglobin or to the production of various iron salts. Whether or not haemoglobin stays intact, this source of discoloration in such teeth represents a potential source of iron which, when exposed to hydrogen peroxide, can be made available for the Fenton reaction. The hydroxyl radicals which could be generated from such a reaction when these discoloured teeth are bleached with 30% hydrogen peroxide may account for periodontal tissue destruction in the cervical area of the tooth which may in turn progress to invasive cervical resorption.

Various methods are employed clinically in the bleaching of root-filled teeth. In the present study, a "thermocatalytic" method was employed where 30% hydrogen peroxide was rapidly heated with a red-hot metal instrument. This technique was used to bleach a total of 40

teeth, half of which had been experimentally discoloured by blood components (packed red blood cells). Two other experimental variables were also introduced into experimental group. These were the production of small defects in the cementum layer at the cemento-enamel junction, analogous with defects that may occur clinically after a traumatic injury to the tooth, and the flushing of the pulp chamber with EDTA, a procedure which is employed in clinical dental practice to remove smear layer on the pulp chamber walls. The technique employed to detect hydroxyl radicals was an adaptation of the techniques used by Grootveld and Halliwell (1986), Wright and Priestley (1985) and by Wright (1988). In this technique, sodium salicylate was used as a detector molecule and the detection of reaction products of hydroxyl radicals with salicylate (2,3-DHB and 2,5-DHB) using HPLC with electrochemical detection was to be considered as evidence of hydroxyl radical production.

Control experiments established that hydroxyl radicals could be detected with the technique at a high level of sensitivity. Three reaction products were detected, which were identified as 2,3-DHB, 2,5-DHB and pyrocatechol by comparison with known standards. A high degree of correlation was demonstrated between the amount of reaction product present and detector peak heights. Control experiments also established that hydroxyl radical yields were increased when EDTA was present in the reaction mixture. The exposure of hydrogen peroxide in the presence of sodium salicylate to intense ultra-violet light was also shown to increase significantly the amount of hydroxyl radicals when compared to darkness and to ambient light conditions.

Evidence of significant hydroxyl radical generation was found in 25 of the experimental tooth samples. Three reaction products were detected : 2,3-DHB, 2,5-DHB and pyrocatechol. A significant association was demonstrated between the production of hydroxyl radicals and the presence of discoloration produced by blood components. Yields of hydroxyl radicals were also calculated to be greater in these discoloured teeth. This suggested that the discoloration acted as a source of ferrous iron which could be made available for a Fenton reaction resulting in hydroxyl radical production. Hydroxyl radicals were also detected at a lower frequency and in lesser quantities in teeth which were not experimentally discoloured and the possible reasons for this have been discussed. It was also found that hydroxyl radical production was greater in teeth without defects created in the cementum layer at the cemento-enamel junction. Here again, greatest yields were detected in teeth which had been experimentally discoloured by blood. Several explanations have been proposed, the most likely explanation being that, in the majority of teeth, there were defects in the cementum layer which were not detected by stereomacroscopic examination. There was no statistically significant association demonstrated between the presence or absence of hydroxyl radicals and the use of EDTA to flush the pulp chamber prior to the bleaching procedure. However, the yields of hydroxyl radical were greatest in those teeth in which EDTA had been introduced. The reasons behind these observations have been discussed.

From the present study, therefore, it can be concluded that :

1. Hydroxyl radicals are generated in root-filled teeth which have been discoloured by blood and then bleached with 30% hydrogen peroxide.

2. The presence of EDTA appears to enhance, but not initiate, the production of hydroxyl radicals in root-filled teeth undergoing bleaching procedures.
3. Intense ultra-violet radiation causes significant generation of hydroxyl radicals in sodium salicylate solution alone. This production of hydroxyl radicals is further increased by the addition of hydrogen peroxide to the sodium salicylate solution. Exposure to ambient light, even for long periods did not enhance hydroxyl radical production.
4. The technique employed in this study is a sensitive and relatively simple method for the detection of hydroxyl radicals, although there are some aspects of the technique which deserve closer scrutiny. These include the effect of U.V radiation on salicylate and the interaction between hydrogen peroxide and salicylate. Some improvements to the methodology are proposed.

In the present study, hydroxyl radical generation has been demonstrated in root-filled teeth that have been discoloured by blood components and then bleached with 30% hydrogen peroxide. Given the reported tissue-damaging ability of the hydroxyl radical, the generation of this toxic chemical species may be one mechanism which underlies the periodontal tissue destruction and root resorption which can follow intracoronal bleaching of discoloured root-filled teeth.

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APPENDIX 1
SOURCES AND COMPONENTS OF CHEMICALS AND MATERIALS

Source	Components
AH26^{R} :	Epoxy resin
De Trey-Dentsply	bismuth oxide
Switzerland	hexamethylene tetramine
	titanium dioxide
	bis- phenol di-glycidyl ether
Modelling Wax^{R} :	Ceresin
Ainsworth Dental Co.	beeswax
Aust	carnauba wax
	synthetic resins
	synthetic waxes
Cavit^{R} :	Zinc oxide
Espe	calcium sulphate
W. Germany	zinc sulphate
	glycol acetate
	polyvinyl acetate
	polyvinyl chloride acetate
	triethanolamine
Gutta Percha :	Gutta percha (matrix)
Progress,	zinc oxide
Rudolph Gunz, Aust.	heavy metal sulphates
	plasticizers

Ketac^{R} Varnish : Espe, W.Germany	Synthetic resin in ethyl-acetate
Miltons solution^{R} : Proctor and Gamble, Aust.	Sodium hypochlorite : Available chlorine 1% with NaCl 16.5%

2,3-Dihydroxybenzoic acid (Analar): Sigma Chemical Co., USA

2,5-Dihydroxybenzoic acid (Analar) : Sigma Chemical Co., USA

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

Ferrous sulphate (Analar): BDH, Aust.

Homogentisic acid (Analar) : Sigma Chemical Co., USA

Hydrogen peroxide 30% v/v (Analar) : BDH, Aust.

Hydroquinone (Analar) : BDH, Aust.

Potassium dihydrogen phosphate (Analar): BDH, Aust.

Methanol (HPLC Grade) : Mallinckrodt , USA

Phloroglucinol(Analar) : Aldrich Chemical Co., USA

Pyrocatechol (Analar) : BDH, Aust.

Pyrogallol (Analar) : Aldrich Chemical Co., USA

Resorcinol (Analar) : BDH, Aust.

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

APPENDIX 2

ABBREVIATIONS AND TERMINOLOGY

This appendix explains abbreviations and symbols as well as terminology employed in the present study.

ADP :	adenosine diphosphate
ATP :	adenosine triphosphate
CEJ :	cemento-enamel junction. The junction at which, on the external tooth surface, the enamel covering the tooth crown meets the cementum covering the root.
DNA :	deoxyribosenucleic acid
DETAPAC :	diethylamine penta-acetic acid
2,3-DHB :	2,3- dihydroxybenzoate
2,5-DHB :	2,5- dihydroxybenzoate (also called gentisic acid)
ECD :	electro-chemical detection
EDTA :	ethylenediamine tetra-acetic acid
ESR :	electron spin resonance
Elution Time :	The time taken for a solute (sample molecule) to pass along the HPLC column after injection and be detected by the electro-chemical detector.
Gutta Percha:	The latex of various trees of the family *Sapotoceae*- essentially a polymerised hydrocarbon of the general formula $(C_5H_8)_n$ mixed with other resinous

substances. In dentistry, this material is used to fill the debrided canals of internally non-vital teeth.

HGA : homogentisic acid

HPLC : high performance liquid chromatography

Intracanal Irrigant : a solution used to flush the root canal during the process of canal debridement. As the canal is mechanically debrided by endodontic files, debris is flushed out by the irrigating solution.

Intracoronal Bleaching: the bleaching of a root-filled tooth by the insertion of bleaching agent into the pulp chamber.

MilliQ Water : double de-ionised water. All aqueous solutions in the present study were made up with this water.

NAD : nicotinamide adenine dinucleotide

NADP : nicotinamide dinucleotide phosphate

$\cdot OH$: hydroxyl radical

$O\cdot^-$: singlet oxygen

$O_2\cdot^-$: superoxide radical

Occlusal : pertaining to the masticatory surfaces of the teeth or to the plane in which they lie

Retention time : as for elution time

Root-filled tooth:	a tooth in which the root canal has been filled, usually by gutta-percha and a sealer cement, after the necrotic or non-viable pulp has been removed and the root canal has been chemomechanically debrided.
SOD :	superoxide dismutase
1,2,5-THB :	1,2,5-trihydroxybenzene
2,3,5-THB :	2,3,5-trihydroxybenzoate
U.V. :	ultra-violet
Vital tooth :	a tooth in which the pulp receives a viable blood supply. Generally, the tooth will respond positively to thermal or electric sensibility testing.
Vital bleaching :	the bleaching of a vital tooth by the application of bleaching agent to the external surfaces of the tooth.

APPENDIX 3

PRODUCTS OF SALICYLATE HYDROXYLATION

The attack by hydroxyl radicals on salicylate has been shown to result in the production of three significant reaction products; 2,3-DHB, 2,5-DHB and pyrocatechol (Grootveld and Halliwell, 1986). In conditions of high radical flux, however, where large amounts of these reaction products are generated, it is possible that a further interaction occurs between the reaction products themselves and the hydroxyl radical, particularly if there is continuous generation of hydroxyl radicals. The detection of extra peaks in chromatograms from control generation of hydroxyl radicals, where there was clearly a large production of hydroxyl radicals, appeared to confirm this possibility and thus the need to conduct further control experiments (see Sections 7.6 and 7.7). It was considered necessary to determine whether it was possible for these further reaction products to interfere with the measurement of either the internal standard, HGA (eluting at 2.4 minutes) or the measurement of 2,3-DHB, 2,5-DHB and pyrocatechol in the experimental samples. It should be noted that in the techniques used by previous authors, such as Wright (1988), the generation of hydroxyl radicals was terminated by the addition of agents such as phosphoric acid or trichloro-acetic acid. This was considered unnecessary in the present study because hydroxyl radical production was being conducted in a chemically generated system where production was virtually instantaneous and where there was not a continuous generation of hydroxyl radicals as would occur in an enzymatically generated system.

The attack by hydroxyl radicals on salicylate to produce 2,3-DHB and 2,5-DHB causes the addition of hydroxyl groups to either the 3'- or 5'-position on the aromatic ring. Maskos et al. (1990) investigated the mechanisms involved in the hydroxylation of salicylate by the Fenton reaction using gamma radiolysis. From their results the authors concluded that the aromatic ring structure was an ortho-para director during the electrophilic attack by the hydroxyl radical. That is, as a result of hydroxyl radical attack on the salicylate molecule, hydroxyl groups are directly added to either ortho- or para- positions. The meta-position is essentially deactivated. Although it is possible that hydroxyl groups are added to the meta- position in conditions of very high radical flux, the situation is very unlikely and would result in the production of insignificant amounts of such molecules. Thus, the production of 2,3-DHB and 2,5-DHB are the most likely because their production requires the addition of hydroxyl groups to either the ortho- or para- position of the salicylate molecule. The production of other possible products such as 2,4-DHB and 2,6-DHB are therefore unlikely because they would involve the addition of a hydroxyl group to a deactivated meta- position. A flow diagram of these products is shown as Figure A3.

The production of pyrocatechol has been suggested by Moorhouse et al. (1985) to be a decarboxylation reaction. It is more likely, however, to be a simple addition of a hydroxyl group then elimination of the carboxyl group on the salicylate molecule.

From a strictly stoichiometric viewpoint, it can be seen that the internal standard HGA with the structure shown in Figure A3 cannot be a product of salicylate hydroxylation due to attack by hydroxyl radicals.

As discussed earlier, in conditions of high radical flux, it may be possible for the first group of salicylate hydroxylation products to be further hydroxylated. Since it is unlikely that products such as 2,4-DHB and 2,6-DHB would be formed in the first stage, it is therefore very unlikely that their hydroxylation products would be present. Therefore it is unlikely that the products resorcinol (1,3-dihydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene) would be formed. In any event, these substances were not detected in the conditions of the control experiments in this study over the 25 minute run time. This was probably due to the HPLC conditions employed in the present study, which were quite different to those used by Grootveld and Halliwell (1986), who were able to detect resorcinol. While hydroquinone is a product of phenol hydroxylation (Moorhouse et al., 1895), it is not considered a possible product of salicylate hydroxylation. Thus, despite its retention time being similar to HGA, it is unlikely to have interfered with the experimental results in the present study.

The further attack by hydroxyl radicals on 2,3-DHB could yield pyrogallol (1,2,3 trihydroxybenzene) by the substitution of a hydroxyl group for the carboxyl group. Thus pyrogallol is a possible product of further DHB hydroxylation. Its retention time of 2.4 minutes suggests the possibility that it may have been one of the extra peaks noted in the control generation of high radical flux. As considered in the discussion section, with retention time very similar to that of the internal standard, this must raise some doubts about the suitability of HGA as the internal standard.

Another possible product of the hydroxylation of both 2,3-DHB and 2,5-DHB is 2,3,5-THB. This must also be considered an unlikely

product as the hydroxyl group would need to be added to a de-activated meta- position. Wright (1988) also considered the possibility of 2,3,5-THB production but was unable to detect it, despite using a number of methods. The further hydroxylation of 2,5-DHB may also result in the production of 1,2,5-THB with the substitution of a hydroxyl group for the carboxyl group. This compound was not tested in the control experiments because it is not commercially available and its custom manufacture apparently requires a series of complicated reactions.

The further hydroxylation of pyrocatechol is also unlikely because any hydroxyl group addition would have to occur at a meta- position.

The choice of HGA as the internal standard was made in accordance with the protocol used by Wright (1988). Considering its retention time is similar to that of pyrogallol, which may be generated in conditions of high radical flux, perhaps HGA may not have been the ideal internal standard. Grootveld and Halliwell (1986) reported two methods of hydroxyl radical detection using salicylate as the detector molecule. In the first method, the internal standard employed was 3,4-DHB, while in the second method, which was also designed to detect salicylate itself, resorcinol was used. Given the fact that it is unlikely that either resorcinol or 3,4-DHB would result from hydroxyl radical attack on salicylate, perhaps these compounds would have made better internal standards. However, since hydroquinone cannot be a product of salicylate hydroxylation, its use as an internal standard in this technique is worth investigating.

In conditions of high radical flux, it is possible that further hydroxylation of salicylate reaction products could occur. It must be considered, however, that in the experimental tooth samples, there did

not appear to be great amounts of reaction products generated, as compared to control generations. In addition, the concentration of the sodium salicylate solution, in which the tooth roots were bathed, ensured that salicylate was well in excess of the reaction products. The likelihood of further hydroxylation of 2,3-DHB, 2,5-DHB and pyrocatechol in the experimental tooth samples must therefore be considered to be extremely low and therefore not likely to have had any influence on the experimental results reported in this thesis.

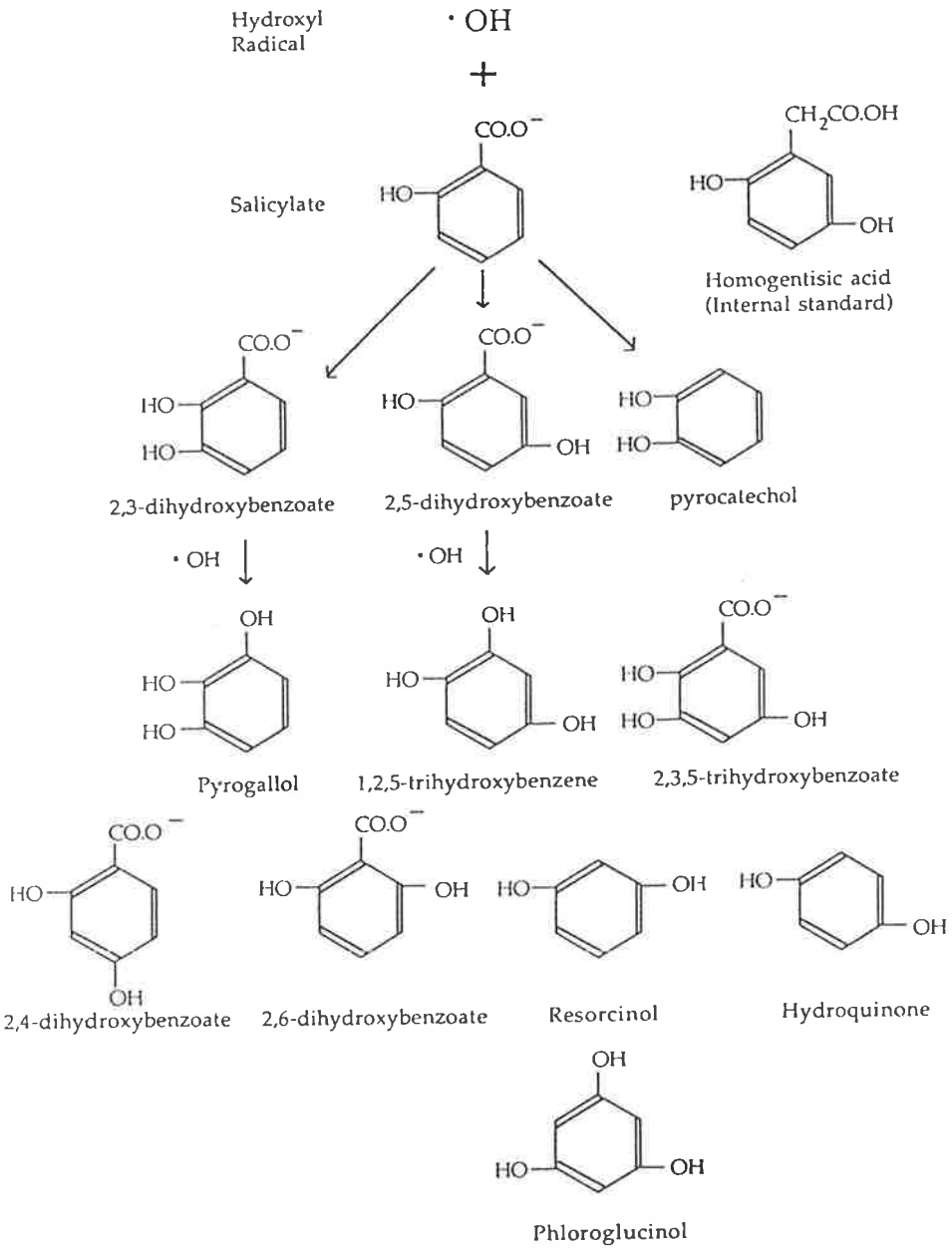

Figure A 3. Hydroxylation products of salicylate, 2,3-DHB and 2,5-DHB in conditions of high hydroxyl radical flux.

APPENDIX 4

STATISTICAL ANALYSIS OF AMOUNTS OF 2,3-DHB FROM EXPERIMENTAL TOOTH SAMPLES

Source	n	Sum of Squares	Mean of Squares	F-test	P value
Discolouration (A)	20	28.44	28.44	3.13	0.087
Defect (B)	20	20.72	20.72	2.28	0.141
A x B	10	18.13	18.13	1.99	0.168
EDTA (C)	20	14.73	14.73	1.62	0.212
A x C	10	10.87	10.87	1.20	0.282
B x C	10	19.14	19.14	2.11	0.157
A x B x C	5	18.62	18.62	2.05	0.162
Error		290.90	9.09		

n= number of teeth in sample

Three-way analysis of variance of hydroxyl radical generation under various conditions in the human tooth model based on the amounts of 2,3-DHB detected.

P = probability that the null hypothesis is correct using a 5% level of statistical significance

APPENDIX 5

STATISTICAL ANALYSIS OF AMOUNTS OF PYROCATECHOL FROM EXPERIMENTAL TOOTH SAMPLES

Source	n	Sum of Squares	Mean of Squares	F-test	P value
Discolouration (A)	20	1.03	1.03	4.39	0.044
Defect (B)	20	0.89	0.89	3.78	0.061
A x B	10	0.73	0.73	3.13	0.087
EDTA (C)	20	0.17	0.17	0.71	0.407
A x C	10	0.08	0.08	0.33	0.571
B x C	10	0.45	0.45	1.93	0.174
A x B x C	5	0.48	0.48	2.06	0.161
Error		290.90	9.09		

n= number of teeth in sample

Three-way analysis of variance of hydroxyl radical generation under various conditions in the human tooth model based on the amounts of pyrocatechol detected.

P = probability that the null hypothesis is correct using a 5% level of statistical significance