A Study of the Photolysis of *Porphyromonas gingivalis*

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DECLARATION

I hereby certify that the work embodied in this thesis is the result of my own investigation, except where otherwise stated. The human gingival fibroblasts were provided by Dr. Sajeda Meghji of the Oral and Maxillofacial Surgery Department of the Eastman Dental Institute. Scanning Electron Micrograph processing was carried out by Mrs Nicola Mordan of the Electron Microscopy Unit of the Eastman Dental Institute.

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ABBREVIATIONS

$AlPcS_2$	Aluminium disulphonated phthalocyanine
BM	Bacteroides Medium
CFU/ml	Colony forming units/ml
DABCO	1,4 – diazabicyclo[2.2.2]octane
EnX**p**	Endotoxin protein
FAA	Fastidious anaerobe agar
HeNe	Helium/Neon (gas laser)
J	Joules
kJ	Kilojoules
LAP	Lipid-associated proteins
M	Molar
mJ	Millijoules
mM	Millimolar
μM	Micromolar
nm	Nanometers
OM	Outer membrane
OM**p**	Outer membrane proteins
PEG	Polyethylene glycol
PIT	Pre-irradiation time
PM	Plasma membrane
PM**p**	Plasma membrane proteins
Po	Porins
PP**p**	periplasmic proteins
SAM	Surface associated material

SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TBO	Toluidine blue O
WC	Wilkins Chalgren

ABSTRACT

Porphyromonas gingivalis has been implicated in the development of periodontal diseases and the current treatments used are not entirely successful in eradicating periodontopathogens from the pocket. Photodynamic therapy has been successfully used in the treatment of a number of cancers and has been used *in vitro* to kill a variety of bacteria. Lethal photosensitisation of *Por. gingivalis* using toluidine blue O (TBO) with Helium/Neon (HeNe) laser light was compared to lethal photosensitisation using aluminium disulphonated phthalocyanine ($AlPcS_2$) with gallium aluminium arsenide (GaAs) laser light. It was found that there were substantial reductions in viable counts using TBO/HeNe laser light and when using $AlPcS_2$/GaAs laser light. Substantial kills were obtained when lethal photosensitisation was carried out under conditions most likely to be encountered in the periodontal pocket (presence of serum, increasing pH values, different *Por. gingivalis* strains and biofilm-grown bacteria). The involvement of singlet oxygen and hydroxyl radicals was investigated by carrying out lethal photosensitisation in the presence of the singlet oxygen enhancer, deuterium oxide. It was found that singlet oxygen and possibly hydroxyl radicals are involved in the killing of *Por. gingivalis*. Distribution studies using ^{3}H-TBO showed that most of the ^{3}H-TBO was bound to the outer membrane and SDS-PAGE analysis showed that there were alterations to the outer and plasma membrane proteins from cells sensitised with TBO and exposed to laser light. It was also found that there was DNA degradation as a result of lethal photosensitisation. The final part of the study involved conjugating antibody against surface components of *Por. gingivalis* with TBO and specifically to target *Por. gingivalis* to light-induced killing when in

the presence of commensal bacteria or host tissue. When sensitised with the antibody/TBO conjugate and exposed to laser light, there were no significant reductions in viable counts of *Streptococcus sanguis* or human gingival fibroblasts, whereas all of the *Por. gingivalis* present were killed.

In conclusion, *Por. gingivalis* can be killed effectively (100 %) when sensitised with 82 μM TBO at an energy dose of 4.4 J. Type II and possibly type I mechanisms are involved in the killing of *Por. gingivalis* and the outer and plasma membrane proteins and DNA are adversely affected by lethal photosensitisation. Damage to oral commensal organisms and oral host tissue can be avoided by using laser light in combination with TBO conjugated to antibody against *Por. gingivalis* surface components.

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CHAPTER ONE

INTRODUCTION

1.1 Oral flora in health and disease

The human oral cavity is a dynamic environment that supports over 350 bacterial species (Asikainen and Alaluusua, 1993). Unstimulated saliva has been reported to contain 150 million microorganisms per millilitre and there are about 100 billion bacteria per gram of wet gingival crevice debris as determined by direct microscopic count (Roth and Calmes, 1981). The oral microflora is large and diverse for two reasons: 1) Microorganisms, which are present in the environment (e.g. air, water and food), are continually introduced into the mouth. Some of these species colonise certain parts of the mouth and become a part of the oral microflora. However, most of the species which colonise the mouth are from other individuals as bacteria which are free living in nature can rarely survive inside the oral cavity without being eliminated by host defences or they may not be able to find a suitable environment for their needs; 2) Owing to the unique anatomy of the mouth, there is tremendous environmental variation within the oral cavity. There are two distinctly different types of tissue present inside the mouth. These are the mineralised tissue of the teeth and the epithelial tissue of the lips, cheeks, palate, gingiva and tongue. These tissues, together with saliva and the topography of the oral cavity, provide an excellent environment for the establishment of different microbial species. The bacteria that inhabit the oral cavity are generally not harmful to the host unless the host defence mechanisms falter (Jordan 1976; Wilson and Henderson, 1995; Scannapieco, 1994).

In the oral cavity, indigenous bacteria may be associated with two major oral diseases, caries and periodontal diseases (Marcotte and Lavoie, 1998). These diseases seem to appear following an imbalance in the oral flora that leads to

the emergence of potentially pathogenic bacteria. In order to define the processes involved in caries and periodontal diseases, it is necessary to understand the ecology of the oral cavity and to identify the factors responsible for the transition of the oral microbiota from a commensal to a pathogenic one. Marcotte and Lavoie (1998) stated that the regulatory forces influencing the oral ecosystem could be divided into three major categories: 1) host-related; 2) microbe-related; 3) external factors. The oral bacterial community appears to use coaggregation as a major mechanism for interbacterial adhesion and colonization of the host (Kolenbrander, 1995; Kolenbrander *et al.*, 1995). Survival of bacteria in the mouth is enhanced by dental plaque formation. Their continuance is associated with the bacteria's capacity not only to adhere and grow, but also to withstand oxygen, wide fluctuations in pH and carbohydrate concentration, and a diverse array of microbial interactions (Bowden and Hamilton, 1998).

We now know that there are over 200 different species present in dental plaque alone (Christersson *et al.*, 1991). Amongst these species are *Actinomyces*, *Bacterionema*, *Rothia* and *Leptotrichia,* which are exclusively found in the oral cavity, as they have never been isolated from any other habitat (Könönen *et al.*, 1994a and 1994b). 60-70 years ago, *Actinobacillus actinomycetemcomitans* and *Streptococcus mutans* were identified, and more recently, they were re-isolated and associated with dental pathology (Hardie, 1992; Höltta *et al.*, 1994). The supplemental oral flora consists of bacteria present in very low numbers, and if the environment changes, can become indigenous. For example in the case of *Lactobacillus spp.* when the pH of the mouth falls, these acid-tolerant bacteria can become numerically dominant and can be regarded

as being indigenous to carious lesions (Wolinsky, 1994).

1.2 Environmental selection

1.2.1 Anaerobiosis

A review by Loesche (1994) stated that the oxygen tension in the oral cavity was approximately 21 % but fell to 12-14 % when measurements were made over the tongue with the mouth closed. The oxygen tension in the periodontal pocket has been determined to be between 1 and 2 %. This indicates that the atmosphere over the dentogingival surfaces, and especially the subgingival plaque, is mainly anaerobic. The bacteria found in this area are anaerobes and those in the supragingival areas are facultative and microaerophilic as the oxygen tension here is anything from 1-20 %.

The bacterial flora can progress from a microaerophilic one to an anaerobic one with a corresponding decrease in the oxidation-reduction potential (Eh). Eh is the tendency for a medium or compound to oxidise or reduce a molecule by removing or adding electrons. Experiments carried out *in vivo* showed that newly-formed plaque had a Eh of +200 mV (the same as saliva) but became progressively negative until it eventually reached -141 mV as the plaque aged. This change in Eh of undisturbed plaque corresponds to a change from an entirely facultative flora to an anaerobic one where spirochetes can be detected (Gibbons and Vanhoute *et al.*, 1973).

1.2.2 Dietary constituents as a microbial nutrient source

Starches, proteins and lipids are not usually available to the oral microflora because they do not remain in the oral cavity for long enough to be broken

down and utilised (Loesche, 1994). However, utilisation of starches and proteins can occur if present in fibrous foods which permit retention between the teeth, or in sticky foods, retained in fissures, pits and contact points. Low molecular weight soluble carbohydrates such as sucrose and lactose are readily metabolised by oral microflora (Minah and Loesche, 1977). However, from the point of view of dental caries formation, the amount of time the food stays in the oral cavity is more important than its composition. The longer bacteria can have nutrients available, the more they can grow, the more acid is produced and the greater the plaque mass that accumulates (Loesche, 1994).

1.2.3 pH of the oral cavity

Bacteria produce organic acids which lower the pH, excrete reduced end products of metabolism that lower the Eh, produce hydrogen peroxide which oxidises some enzymes and membranes, and produce fatty acids and bacteriocins that inhibit the growth of other bacterial species (Oliveira *et al.*, 1998; Teanpaisan *et al.*, 1998). All of the above determine the microbial composition of any particular niche in the oral cavity. In an ordinary disease-free oral cavity, the bacteria in the oral flora grow best at pH 7.0, which is the pH of saliva. During eating, however, the pH of plaque can fall to 5.0. *In vitro* studies have demonstrated that when the pH of the growth medium is reduced from 7.0 to 5.0, *Streptococcus mutans* and *Lactobacillus casei* are capable of growth, which may explain the presence of these two organisms in cariogenic plaque and lesions (Svensater *et al.*, 1997; Fontana *et al.*, 1996).

1.2.4 Saliva

Saliva is made up of fluid from the major salivary glands (parotid, submandibular and sublingual), minor (accessory) glands and subgingival crevicular fluid (Edgar, 1998). Saliva also contains a high population of bacteria and food debris. Secretions from the major and minor salivary glands largely determine the composition of whole saliva (Edgar, 1998). Saliva is thought to have two general physiological functions. Firstly, it lubricates food for the passage through the digestive tract and initiates digestion via the action of the enzyme ptyalin. Saliva also provides protection to the hard and soft tissue owing to its lubricating and buffering properties. The buffering properties of saliva are essential for the protection of oral tissue from food and plaque. Stimulated saliva contains bicarbonate that causes an increase in salivary pH (normal pH is around 7.0 [Roth and Calmes, 1981]) which can markedly decrease the cariogenic potential of foods. Unstimulated saliva contains proteins and phosphate that also act as buffers, but to a much lesser extent than bicarbonate on stimulation (Edgar, 1998).

1.2.4.1 Antibacterial properties of saliva

Saliva provides mechanical cleansing and cleansing through the action of its antibacterial proteins such as lactoferrin and lysozyme (Li, 1998). Lysozyme (the action of which may be potentiated by anions), acts upon components of the bacterial cell wall causing lysis (Hamosh, 1998). Lactoferrin acts by removing iron from the saliva essential for bacterial growth (Schryvers *et al.*, 1998). Some bacteria however, can use lactoferrin as a source of iron. Sialoperoxidase oxidises salivary thiocyanate ion (SCN^-) to hypothiocyanate ($OSCN^-$). $OSCN^-$ is a potent antibacterial agent, which uses the hydrogen

peroxide produced by bacteria as an oxidant (Edgar, 1998). The main immunoglobulin present in saliva is IgA (Wilson, 1994). IgA causes the aggregation of bacteria and prevents their adhesion to oral tissues. Aggregation can also be caused by adhesins (pili) on the surface of the bacteria interacting with mucous glycoproteins (Newman and Nisengard, 1994). IgG may also play an important part in the lysis of bacteria in the gingival crevice by the activation of complement. This may also cause the opsonisation of the bacteria for phagocytosis by polymorphonuclear leukocytes (Edgar, 1998).

1.3 Relationship between saliva and plaque

Selective and successive adsorption of salivary glycoproteins to the tooth surface results in pellicle formation (Marsh and Bradshaw, 1995). Proteins such as glucosyl transferase from bacteria and IgG and albumin from gingival crevicular fluid are also present. Pellicle protects the teeth from chemical and mechanical offence, it can also, however, act as a substratum for the colonisation by bacteria (Gong and Herzberg, 1997). Plaque formation involves the incorporation of salivary proteins which are difficult to characterise as they are degraded in plaque (Edgar, 1998). It is thought they are incorporated by electrostatic bonds and hydrophobic interactions. More importantly, it is thought that the plaque is held together, not by these proteins, but by extracelluar glucans (Holt and Progulske, 1994). Microorganisms synthesise vast amounts of glucans from sucrose and form extracellular enzyme complexes such as glucosyltransferase (GTF). These extracelluar glucans, are insoluble and result in an increase in bacterial adhesion. *S. mutans* can bind these glucans resulting in aggregation of these organisms and also entrapment of other bacteria (Sanz and Newman, 1994).

1.3.1 Plaque

Dental plaque consists mainly of proliferating microorganisms, epithelial cells leukocytes and macrophages in an adherent intercellular matrix (Roth and Calmes, 1981). Bacteria make up approximately 70-80 % of plaque. In fact, plaque contains 1.7 x 10^{11} organisms per wet weight per gram (Sanz and Newman, 1994). There may be as many as 400 species in one site and there are microorganisms other than bacteria such as fungi, protozoa and viruses present (Sanz and Newman, 1994).

Teeth harbour a much larger microflora than soft tissue and are more susceptible to the effects of the microflora as teeth are non-generating and hence do not undergo continuous desquamation as do epithelial cells (Roth and Calmes, 1981). There is a quantitative and qualitative variation in the microflora that is characteristic of different tooth surfaces; oral bacteria can be isolated in differing proportions from different sites on the same tooth (Roth and Calmes, 1981). Microorganisms adsorb to teeth by the same mechanisms as when adsorbing to other oral surfaces. However, retentive areas of the teeth support a larger microbial burden than the exposed surfaces owing to less cleansing in these difficult-to-reach areas (Scheie *et al.*, 1984). The retentive areas are i) pits and fissures of the occlusal surfaces of the teeth; ii) interproximal areas; iii) gingival sulcus.

It has been found that *Streprococcus sanguis* and *S. mutans* are quintessentiqally tooth organisms whereas *S. mitis* is commonly found on the buccal mucosa. (Rosan, 1994). Filamentous organisms have been found to make up a small proportion of the fissure flora. A review by Roth and Calmes

(1981) stated that the differences in the microflora of occlusal fissures and other sites may be due to two factors: 1. Saliva probably plays a less significant role in supplying nutrients to resident bacteria because they often lie under food debris and undisturbed plaque. The bacteria in fissures may obtain nutrients from food debris embedded in plaque and other sources such as the plaque matrix or breakdown products of decomposing bacteria. Plaque from sites that rarely contain food debris may give rise to different sources of carbon and energy that may in turn allow different portions of the microflora to predominate. 2. The plaque present deep in pits and fissures is rarely disturbed. In this stagnant area, the microflora is able to establish a more stable ecosystem than would be possible in more accessible areas.

Although the interproximal areas have been shown to harbour mainly *S. mutans* compared to more exposed tooth surfaces (Scheie *et al.*, 1984), there seems to be no significant difference between interproximal plaque flora composition and that of buccal or lingual aspects of supragingival tooth surfaces (Roth and Calmes, 1981). In all these areas the early plaque deposits contain predominantly Gram-positive cocci and rods. As the plaque ages, there is a tendency for Gram-negative cocci and rods and filamentous microorganisms to be present (Listgarten, 1976).

The gingival sulcus harbours the most diverse microflora of any site in the oral cavity (Sutter, 1984). This is due to its retentive nature and also due to the unique physical and chemical environment of the sulcus. The subgingival microflora can be subdivided into two parts: i) plaque that is attached to the subgingival tooth surface, which in health is above the cementoepithelial

junction ii) the plaque which is not attached and instead is free-floating between the tooth and epithelial surfaces in the gingival sulcus (Sanz and Newman, 1994).

1.3.2 Subgingival plaque

Supragingival plaque directly and indirectly influences the establishment and relative proportions of subgingival micro-organisms (White, 1997). As the supragingival plaque matures and accumulates, inflammation develops which leads to oedema and enlargement of the gingival area. This causes an increase in the capacity of the subgingival area for bacteria to colonise. This enlarged space protects bacteria from normal oral cleansing. The environment in the gingival pocket is one where there is an increase in gingival crevicular fluid, desquamated epithelial cells, and bacterial end products, that influence the establishment of micro-organisms (Takata and Donath 1988). Many bacteria cannot directly adhere to tooth-surfaces and utilise supragingival bacteria (by binding to them) to colonise the subgingival area. Bacteria such as *Capnocytophaga* species bind selectively to root cementum and *Eikenella corrodens* binds selectively to epithelial cells demonstrating the ability specifically to colonise different subgingival areas (Yamazaki *et al.*, 1981; Dzink, *et al.* 1989). Once established in the subgingival area, gingival fluid proteins are the main nutrients for the bacteria. The bacteria are mainly fastidious anaerobes due to a low oxidation-reduction potential in this region. Under these conditions, the local environment and the host defence allow the various organisms to increase or decrease to a point where the resulting community can elicit pathology (Sanz and Newman, 1994).

Microscopic studies have provided information about subgingival plaque and separated it into tooth-associated, epithelium-associated and connective tissue-associated plaque (Sanz and Newman, 1994).

1.3.3 Tooth-associated subgingival plaque

The structure of tooth-associated subgingival plaque is very similar to that of supragingival plaque (Sanz and Newman, 1994). The bacteria present are densely-packed adjacent to the cuticular material covering the root surface. Close to the root surface the flora consists mainly of Gram-positive filamentous bacteria, although Gram-positive and Gram-negative rods and cocci are also present. The flora present herein is associated with calculus formation, root caries and root resorption in animal models (Sanz and Newman, 1994). The apical border of the tooth-associated plaque contains few filamentous organisms and is dominated by Gram-negative rods (Vrahopoulos *et al.*, 1992).

1.3.4 Epithelium-associated subgingival plaque

This type of plaque is in direct association with the gingival epithelium extending from the gingival margin to the junctional epithelium. The plaque contains mainly Gram-negative rods and cocci, flagellated bacteria and spirochaetes. As there is no definite intermicrobial matrix, the bacteria are loosely-adherent (Christersson *et al.*, 1991). The proportion of bacteria in the epithelium-associated plaque depends largely on the nature and activity of the periodontal condition. For example, in localised juvenile periodontitis and rapidly progressing periodontitis, the tooth-associated plaque contains mainly Gram-negative, motile organisms. It is thought that there may be a direct relationship between the bacteria in junctional epithelium and connective tissue-associated

bacteria within the gingival tissue (Roth and Calmes, 1981). Studies have shown that the soft tissue of the periodontal pocket contains distinct areas of bacterial accumulation. Other areas show a strong host response consisting of leukocytes and leukocyte-bacterial interactions. The fact that such distinct areas are present demonstrates that the pocket wall is constantly changing as a result of the interaction between the epithelium, epithelium-associated bacteria and host factors. The different environments may be important in the colonisation and growth of specific bacteria and allowing bacterial penetration into the tissues by some subgingival bacteria (Roth and Calmes, 1981).

1.3.5 Connective tissue-associated subgingival plaque

Studies have shown the presence of subgingival bacteria within the gingival connective tissue in various periodontal conditions. The presence of black-pigmented bacteria has been shown in acute ulcerative gingivitis, advanced periodontitis and localised juvenile periodontitis (Dahlen, 1993; Newman, 1984).

1.4 Periodontal diseases

Each tooth is composed of the crown, which projects beyond the gum and is covered by enamel, the cervical region and the root (Berkovitz and Moxham, 1981). The root is covered by cement and fixed into the alveolar bone of the maxilla (bone containing upper teeth) or mandible (jawbone) by the fibrous periodontal membrane forming a gomphosis (joint). The bulk of the tooth is formed of dentine, which has a cavity within it, which is filled by pulp. The pulp is a fibrous tissue containing nerves, blood vessels and lymphatics that pass into it via a foramen in the root apex (see figure 1.1).

The calcified dentine and cement arise from mesoderm (Berkovitz and Moxham, 1981). The cement is like bone with calcified lamellae and the dentine is harder, containing high amounts of organic material and a system of spiral tubules, which radiate outwards from the pulp cavity. Enamel, which is the third of the calcified tissues of the tooth, arises from the epithelium and is the hardest tissue of the body. It is heavily calcified, non-vital and lacks the capacity to regenerate in response to injury (Berkovitz and Moxham, 1981).

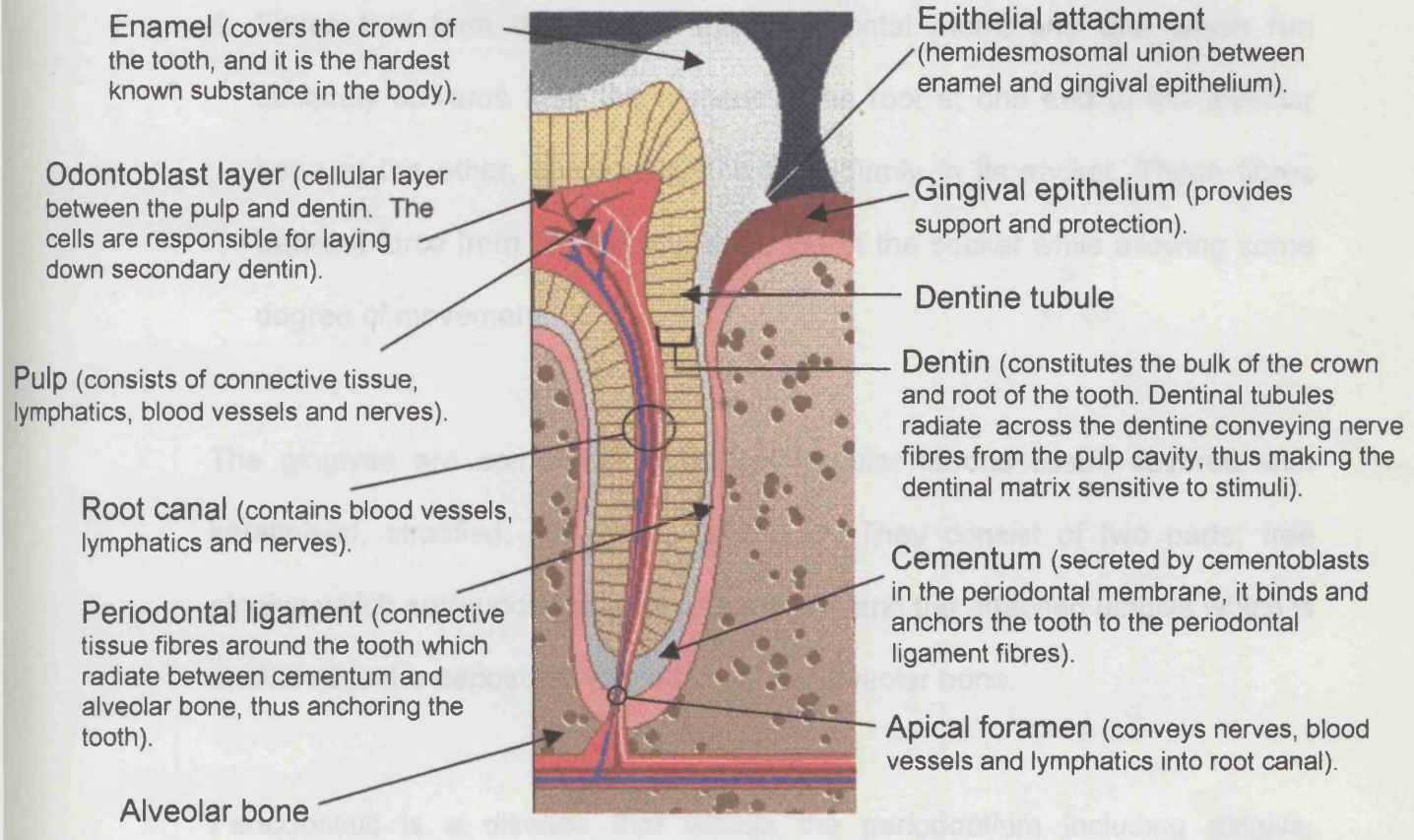


Figure 1.1 Diagrammatic representation of a tooth (adapted from Rateitschak).

The periodontal membrane consists of strong collagen fibres attached to the cement of the root. It is radiolucent, and the fibres can be divided into four groups depending upon the direction in which they run:

1. Fibres passing from the cervical regions of the teeth into the overlying gum are the gingival fibres. These hold the gum close to the cervical region of the

teeth.

2. The fibres that pass from the buccal and lingual cervical regions to the bone of the socket rim, which resist tilting movements of the teeth, are the alveolar crest fibres.
3. Fibres which pass from the mesial and distal cervical are
   i) trans-septal fibres and connect the necks of adjacent teeth
   ii) cervical fibres which pass to the inner part of the sockets just below their rims.
4. Fibres that form the bulk of the periodontal membrane and which run obliquely upwards from the cement of the root at one end to the alveolar bone at the other, suspending the tooth firmly in its socket. These fibres transmit force from the tooth to the bone of the socket while allowing some degree of movement.

The gingivae are composed of dense vascular fibrous tissue covered with keratinised, stratified, squamous epithelium. They consist of two parts: free gingiva which surrounds the neck of the tooth, and the attached gingiva which is anchored to the periostium (covering) of the alveolar bone.

Periodontitis is a disease that affects the periodontium including gingiva, gingival attachment, periodontal ligament, cementum and supporting alveolar bone (Meghji *et al.*, 1992). The diagram below (fig. 1.2) shows the difference between the structure of the periodontium in health and disease.

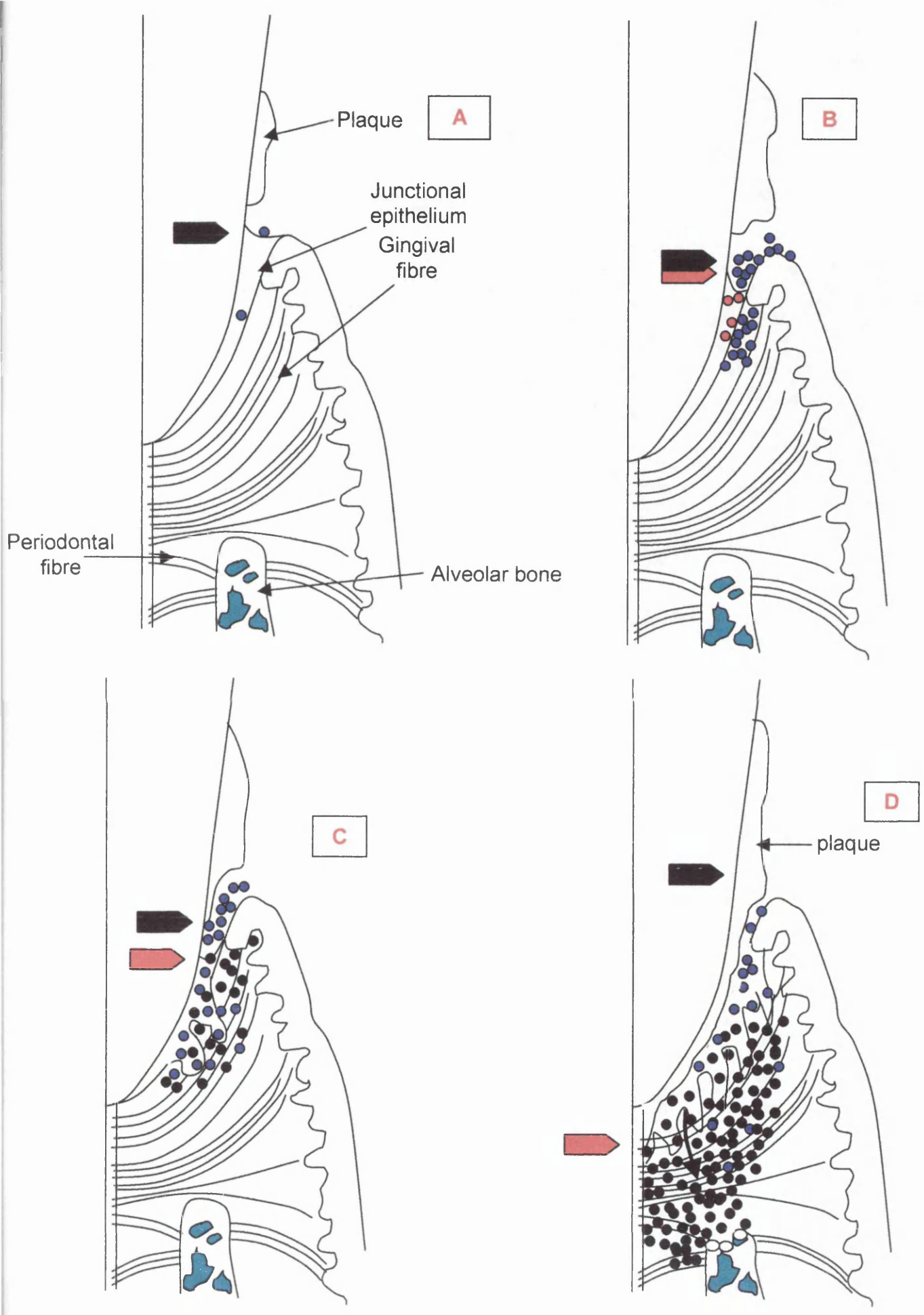

Plaque
A
Junctional epithelium
Gingival fibre
Periodontal fibre
Alveolar bone
B
C
D
plaque

Figure 1.2 (Page 33) Gingiva in health and disease. **A.** Healthy gingiva. Absence of plaque or very little accumulation. Normal junctional epithelium (JE). Minimum sulcular depth (black arrow). **B.** Initial / early gingivitis. Early plaque accumulation eliciting a gingival inflammatory response. In the initial lesion there is increased transmission of polymorphonuclear leukocytes (PMNs; blue dots) through the JE. As the early lesion develops, the PMNs create a wall against the plaque bacteria within the slightly deepened sulcus (red arrow). A lymphocytic infiltrate (black dots) is observed in the subepithelial tissues. **C.** Established lesion. The gingival sulcus may be displaced apically (loss of epithelial attachment) as a consequence of the advancing front of accumulating plaque resulting in the formation of a gingival pocket (distance between red and black arrows). There is no true loss of connective tissue attachment. **D.** Periodontitis. The most important histological differences between gingivitis and periodontitis are bone resorption, apical proliferation and ulceration of the JE (red arrow indicates base of pocket), and progressive loss of connective tissue attachment. In acute (active) phases there may be bacterial invasion of the tissue, with resultant abscess formation. (Figure adapted from Rateitschak, 1989).

There are different forms of periodontitis, and the microflora associated with each is quite difficult to assess, as obtaining samples of the microflora representing the disease, without contamination by organisms from other areas, is difficult (Socransky and Haffajee, 1994). The disease classification is based on clinical, host and environmental factors (Schluger *et al.*, 1990). The most common form of periodontitis is adult periodontitis (AP) which occurs worldwide and affects approximately 75 % of the American population (Rateitschak *et al.*,

1985). Sex, socio-economic factors, race, and the method of detection all contribute to the reported prevalence. The less common periodontal diseases which include periodontal abscesses, prepubertal periodontitis, herpatic gingivostomatitis, juvenile periodontitis, rapidly progressing periodontitis and drug or systemic-induced gingival conditions, account for less than 10 % of all periodontal diseases (Slots and Rams, 1990).

Occasionally it may be difficult to distinguish the various forms of periodontitis from one another. For example, AP in older patients with compromised immune systems may take on the characteristics of aggressive periodontal disease. In such cases, AP may exhibit more frequent periods of disease activity and a pocket flora similar to rapidly progressive periodontitis (RPP). These shifts may also occur between localised juvenile periodontitis (LJP) and RPP. All forms of periodontitis exhibit a clinical course with destructive (active) and quiescent periods (Christersson *et al.*, 1991).

The disease can range from slight localized early periodontitis (type I) to severe advanced periodontitis (type IV [Schluger *et al.*, 1990]).

Type I: Inflammation of the gingiva, characterized clinically by gingival hyperplasia, oedema, gingival pocket formation, pocket depth less than 4 mm and no bone loss. Treatment: Prophylaxis (cleaning) and continued plaque removal and control at home on a daily basis. Prognosis: Most disease changes are reversible at this stage with a good prognosis for retention of the teeth.

Type II: Progression of gingival inflammation into the surrounding bone crest and early bone loss resulting in pocket formation (4-6 mm). Treatment: Non-surgical pocket and root debridement to control infection, re-evaluation at 3-4

months of infected areas and the need for further care (i.e. non- surgical maintenance or surgical intervention). Continued plaque removal and control at home on a daily basis. Prognosis: Fair. There have been irreversible changes most of which can be treated with non-surgical maintenance. However, each individual is different and sometimes surgical correction is necessary.

Type III: A more advanced state of the previous condition, with increased destruction of periodontal structures associated with moderate-to-deep pockets (5-8 mm), moderate severe bone loss and tooth mobility. Treatment: Non-surgical pocket and root debridement to control infection, re-evaluation at 3-4 months of infected areas and the need for further care (non-surgical maintenance or surgical intervention). Continued plaque removal and control at home on a daily basis. Prognosis: Fair to guarded. Irreversible changes have occurred that are not as responsive to non-surgical management. For long term retention of teeth, surgery may be necessary.

Type IV: Further progression of periodontitis with severe destruction of the periodontal structures, increased pocket depth, usually greater than 7-8 mm with increased tooth mobility. Treatment: Initial treatment is non-surgical pocket and root debridement to control infection, and re-evaluation at 3 months of the need for further care of the infected areas. The need for surgery is almost certain. Prognosis: guarded to poor.

Some of the different forms of the disease and the associated bacteria are listed in the table below (Table 1.1), and Table 1.2 summarises the symptoms and manifestations of these diseases. There are over 350 bacterial species that can be found in the periodontal pocket (Slots and Rams, 1990) and there are a variety of other micro-organisms present e.g. *Entamoeba*, *Trichomonads* and

Candida. The microflora at the start of the disease is mainly Gram-positive rods and cocci. The pathogenic potential of the particular bacteria within the plaque varies from individual to individual and from one site to another within the mouth. When certain bacteria within the plaque increase to significant levels, and/or produce virulence factors, the "balanced" environment shifts towards one that is conducive to the development of the disease (Christersson *et al.*, 1991). Disease can also occur when the host is immunocompromised (Horning and Cohen, 1995).

Black-pigmented Gram-negative anaerobes have been associated with periodontal disease and tooth loss since they were first isolated by Burdon in 1928 (Dahlén, 1993). The most common bacteria found in periodontal disease are: *Porphyromonas gingivalis, Prevotella intermedia, Bacteroides forsythus, Campylobacter rectus* (formally *Wolinella recta*), and *Actinobacillus actinomycetemcomitans* (Sutter, 1984; Teanpaisan *et al.*, 1998; Hinode *et al.*, 1998).

Adult Periodontitis	**Localised Juvenile Periodontitis**	**Prepubertal Periodontitis**	**Rapidly Progressing Periodontitis**	**HIV-associated periodontitis**
A. actinomycetemcomitans (A.a) *Por. gingivalis* *Prev. intermedia* *Fusobacterium spp.* *C. recta* *T. denticola* *Enteric/Pseudomonas rods* *Streptococcus spp.* *Actinomyces spp.*	*A.a* *Capnocytophaga spp(?)* *Prev. intermedia* *C. recta*	Specific bacteria not demonstrated, but associated with: *A.a* *Prev. intermedia* *E. corrodens* *Fusobacterium spp.* *Por. gingivalis*	*Por. gingivalis* *B. Intermedia* *A.a* *F. nucleatum* Spirochetes	*A.a* *Por. gingivalis* *Prev. intermedia* *Fusobacterium spp.* *Peptostreptococcus. Micros* *C. recta* *T. denticola* *Enteric rods /Pseudomonads* *Candida*

TABLE 1.1 Bacteria involved in periodontal diseases

	Adult periodontitis	Rapidly progressing periodontitis	Localised juvenile periodontitis	Prepubertal periodontitis
ge	Begins at 30-40 yrs. Gradual development from pre-existing gingivitis	Immediately after puberty, diagnosed at 20-30 yrs, effects mainly ♀	12-20 yrs, more frequent in ♀	Occurs immediately after eruption of primary teeth. More frequent in ♀.
ocalisation	Irregular distribution. Severe destruction in primarily molar areas, but also occurs in anterior segments.	All teeth	Incisors and/or first molars in maxilla and mandible. Later, other teeth are involved.	Generalised form: usually all deciduous teeth are involved, permanent teeth may not be effected. Localised form: Limited numbers of teeth are involved.
anifestations	There are varying degrees of inflammation in the gingivae, shrinkage in some areas, fibrotic manifestations in others. There are lengthy intervals between aggressive and submissive stages. Tooth loss occurs in much later years, if not at all.	Severity and distribution of attachment loss varies considerably. During the aggressive phase there is severe alveolar bone loss, gingival bleeding with acutely inflamed proliferative gingival tissue.	There are normal appearing gingivae and very little supragingival plaque. There is localised alveolar bone loss, though this is discrete so that the distal aspect of a second bicuspid may have no bone loss, while the mesial aspect of a first molar may have several millimetres loss.	Generalised form: gingivae are intensely red with proliferation and cleft formation. There is rapid destruction of alveolar bone and in some cases root resorption. There may be skin and upper repiratory infections, and middle ear infections. Localised form: Minimal gingival inflammation occurs.

Table 1.2 Summary of periodontal diseases

1.4.1 Factors associated with the development of periodontal diseases

1.4.1.1 Age

Ageing is a consistent risk factor for periodontitis (Ismail and Lewis, 1994). However, loss of periodontal attachment and alveolar bone support with age is not inevitable (Burt, 1994). With efficient elimination of plaque and calculus, there is no clinically detectable loss of attachment (Axelsson, 1993). This status can only be achieved in highly-motivated patients who receive regular and meticulous professional care.

1.4.1.2 Smoking

An association between cigarette smoking and periodontitis has been confirmed in both cross-sectional and longitudinal studies (Bergstrom and Eliasson, 1987a and 1987b; Zambon *et al.*, 1996). Use of chewing tobacco has been associated with localised loss of periodontal attachment and oral leukoplakia but not with severe periodontal destruction (Rivera-Hidalgo 1986, Bergstrom and Eliasson, 1987b).

1.4.1.3 Diabetes

Diabetic patients (both type I and type II) are more likely to develop periodontal disease, and the periodontitis is more likely to be severe. Diabetes influences the progression and severity of periodontitis through changes in the small blood vessels, decreased collagen formation, and impairment of the host's defence mechanisms. Furthermore, complications associated with diabetes, such as impaired wound healing, can affect the patient's response to periodontal therapies (Mattson *et al.*, 1998).

1.4.1.4 Host status

Factors such as immune status and stress greatly influence the periodontal pathology (Haffajee and Socransky, 1994). For example, HIV-positive patients can either have HIV-gingivitis or HIV-periodontitis. Patients that are undergoing chemotherapy or psychotherapy can also have periodontal diseases associated with their illness. Hereditary, genetic and developmental factors also greatly influence the state of periodontal tissue and the recognition and detection of contributory factors are important in the assessment of treatment and maintenance of the periodontal disease (Genco, 1996).

1.5 Role of *Por. gingivalis* in periodontitis

The Koch-Henle criteria for identifying the microorganisms responsible for a disease have been modified in the present diagnosis of the etiological relationship of bacteria to different periodontal diseases (by Socransky, 1977):

1. The organism thought to be involved in the development of the disease should be present in high numbers. It should be infrequently found in healthy patients, or completely absent.
2. Elimination or suppression of the organism by treatment should reverse or reduce the disease.
3. There should be an increase in the host's immune response to the organism.
4. Infecting an animal model with the organism should lead to some of the characteristics of the disease (e.g. inflammation, connective tissue disruption and bone loss).
5. The bacteria should have pathogenic or virulence factors.

One of the main reasons *Por. gingivalis* has been implicated as an oral pathogen because of its frequent association with periodontitis derived from experimental animal models (Katz *et al.*, 1996; Blanchard *et al.*, 1991).

Por. gingivalis (formerly known as *Bacteroides gingivalis*) is an anaerobic, non-fermenting Gram-negative short rod that also may appear as cocci, depending upon the haemin content of the media it is cultured in, thus, making the bacteria a pleomorph (Shah, 1993). The shape of the bacterium is dictated by the growth of the cell wall, and surface protein expression can be increased or decreased depending upon the amount of haemin present in the culture medium (Bramanti and Holt, 1992; Genco 1995). This implies that an alteration in the culture medium-haemin content causes an increase or decrease in the expression of cell wall components leading a change the overall shape of the bacteria. In 1921, Oliver and Wherry first isolated *Porphyromonas* from the oral cavity and mistakenly recorded the source of the black-brown pigmentation as melanin. It is now known, however, that the pigmentation is due to protoheme. Protoheme is derived from the breakdown of haemoglobin by hydrolytic enzymes. In the periodontal pocket, haemin is a major component of gingival crevicular fluid. However, it is not available to the bacteria, as it is complexed by the plasma proteins, albumin and hemopexin. It is thought that proteases (cystein-proteinase and gingivain) are involved in the breakdown of haem-containing components of the host releasing haem which can be bound by the cell components prior to uptake (Grenier *et al.*, 1993; Smalley *et al.*, 1991).

Table 1.1 shows that *Por. gingivalis* occurs in almost all forms of periodontal disease, and therefore is one of the most important pathogens involved in

periodontitis. The major oral ecological niche for this species appears to be the subgingival plaque, though Van Winklehoff (1988) and others Zambon *et al.*, 1981; Dahlén *et al.*, 1992) found *Por. gingivalis* to occur in other sites such as tonsil, lateral border of the tongue and buccal mucosa. That *Por. gingivalis* is involved in periodontitis was partially deduced from the fact that it has rarely been found in the gingival sulci associated with healthy periodontal tissue, but it is found in high prevalence as part of the oral flora in subgingival sites associated with periodontitis (Slots, 1977; Christersson *et al.*, 1991; Socaransky and Haffajee, 1994). A prerequisite of *Por. gingivalis*, as with other periodontopathogenic bacteria, is to adhere to oral surfaces before the establishment of an infection. Fimbriae, haemagglutinating factors, vesicles and hydrophobic properties are components of *Por. gingivalis,* which are responsible for its attachment to tooth surfaces, periodontal tissue and other oral bacteria (Okamoto *et al.*, 1998).

The ultrastructure of a Gram-negative bacterium is shown in figure 1.3. In *Por. gingivalis*, the outer membrane proteins have been implicated in haem binding and can vary in their distribution and quantity depending on the levels of haem in the culture medium (Marsh *et al.*, 1993). Furthermore, Smalley *et al.* (1993), found that reductions in iron levels leads to an increase in the formation of vesicles which are involved in haemagglutination. Haemagglutinins are expressed on the surface of the bacteria in association with fimbriae (fimbrial adhesins), non-fimbrial surface components and soluble substances released into the medium (Mouton and Chandad, 1993). Haemagglutinins include those adhesins that attach to and agglutinate red blood cells. This hemagglutination activity together with trypsin-like activity distinguishes *Por. gingivalis* from other

Porphyromonas species (Okamoto, 1998).

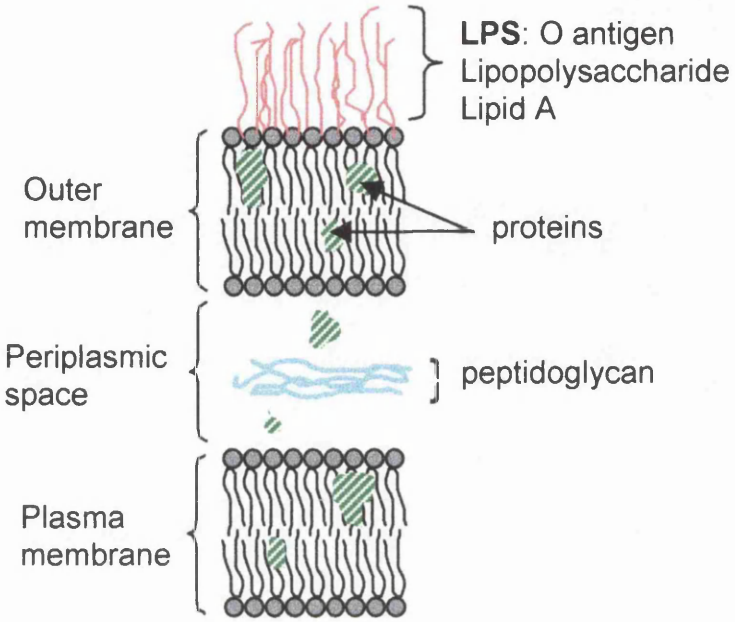


Figure 1.3 Diagrammatic representation of the cell wall of a Gram-negative bacterium.

The structure of the cell wall has been described on numerous occasions and the following is a summary. The outer leaflet of the outer membrane serves as a molecular sieve, excluding molecules with a molecular mass of ~700 and above. Molecules that are in excess of a molecular weight of 700 can enter the cell via porin proteins. The core polysaccharide of lipopolysaccharide (LPS) joins lipid A to the O-antigen and consists of the unique molecule, KDO (keto-3-deoxy-octanoate) and a seven-carbon heptose sugar. The LPS protects the cell from the action of antibodies and complement. It is also a bacteriophage receptor and is one of the major endotoxic molecules of Gram-negative bacteria. It is therefore, considered an important virulence factor of Gram-negative bacteria. The plasma membrane is often referred to as a unit membrane, and consists of ~70 % protein. The plasma membrane is the most

important component of the cells (both prokaryotic and eukaryotic). The cell would not survive without a functional plasma membrane as it is the site of catabolic and anabolic metabolism and regulates the transport of molecules into and out of the cell.

1.5.1 Adhesion and tissue invasion

Adherence to host cells is an essential step in the initiation of most infectious diseases (Murakami *et al.*, 1993). *Por. gingivalis*, once inside the oral cavity, comes into contact with epithelial cells (Sandros *et al.*, 1993), polymorphonuclear leukocytes (PMN), fibroblasts and lymphocytes which it may adhere to via LPS (Wilson, 1993).

It is well known that *Por. gingivalis* fimbriae may be involved in the adherence to host cells (Tokuda *et al.*, 1996; Kontani *et al.*, 1996; Lee *et al.*, 1991; Naito *et al.*, 1993; Nakayama *et al.*, 1996; and Njoroge *et al.* 1997). Many investigators have tried to elucidate what part, if any, of the fimbria is responsible for adhesion. Hamada *et al.* (1994) put forward the first direct genetic evidence demonstrating that the FimA protein of *Por. gingivalis* is essential for the interaction of the organism with human gingival tissue cells, through a function(s) encoded by the *fimA* gene. Naito *et al.*, (1993) showed from their work that: 1) fimbriae play an important role in colonisation through their hydrophobic activity; 2) fimbriae of non-invasive strains are associated with the major adhesin for attachment to gingival tissue, whereas fimbriae of invasive strains are weakly involved in adherence; and 3) there is no correlation between colonisation and the invasiveness of *Por. gingivalis.* Weinberg *et al.* (1997) showed that *Por. gingivalis* could invade normal human gingival epithelial cells

(NHGEC) by involvement of fimbriae. They suggested that adhesin-receptor interactions, such as fimbriae binding to a 48-kDa NHGEC surface receptor, may trigger activation of eukaryotic proteins involved in signal transduction and/or provoke the generation of surface *Por. gingivalis* molecules required for internalisation.

A review by Wilson (1995) of the effects of LPS from *Por. gingivalis*, *Prev. intermedia* and *Fusobacterium nucleatum* suggested that it has a marked effect on most types of cell found in the periodontal tissues including macrophages, lymphocytes, fibroblasts and osteoblasts. Fibroblasts and macrophages respond to oral LPS by secreting a range of cytokines, and other molecules, which can cause inflammation, immunomodulation and which can adversely affect tissue. Lymphocytes are stimulated by LPS to produce a wide range of antibodies with different specificities, hence provoking the inflammatory response. By its actions on bone cells, LPS can stimulate bone resorption and inhibit bone formation, resulting in erosion of the tooth-supporting alveolar bone. It is also thought that the attachment of the gingival junctional epithelium to cementum is inhibited by *Por. gingivalis* LPS (Slomiany *et al.*, 1991). The bone resorbing activity of fimbriae was shown by Kawata *et al.* (1994). Their study demonstrated the possibility that the fimbriae may stimulate bone resorption via the generation of inflammatory cytokines.

Por. gingivalis W50 fimbriae and haemagglutination factors have been shown to be involved not only in adhesion but also penetration of epithelial cells (Sandros *et al.,* 1993; Lamont *et al.,* 1992, 1995; Du *et al.*, 1997; Weinberg *et al.*, 1997). Lamont *et al.* (1995) found that the invasion was independent of the growth

phase as they found that *Por. gingivalis* was capable of replicating within the epithelial cells.

1.6 Treatments currently used for periodontal diseases

Control of environmental and local factors is the major basis of current periodontal treatment. Plaque and calculus evoke the majority of pathological changes in the periodontium and therapy is aimed at first identifying and removing these aetiological factors, and then correcting the defects the pathogens have created. The therapy for nearly all the periodontal diseases involves oral hygiene and educating the patient, scaling, root planing, chemotherapy, periodontal surgery and maintenance therapy (Tonetti *et al.*, 1998; Wong *et al.*, 1998).

1.6.1 Preventative measures for periodontitis.

Prevention of gingivitis and periodontitis is achieved primarily by controlling plaque and calculus around the teeth. Non-specific control of plaque and calculus requires their frequent removal from all accessible tooth surfaces, which consequently disturbs dental plaque ecology. Although this approach is most widely used, the use of antimicrobial agents is also on the increase (Killoy *et al.*, 1998). The accumulation of dental plaque leads to the development of gingivitis (Löe *et al.*, 1983), Theilade (1996) showed that gingivitis develops in healthy adults after 10 to 21 days in the absence of personal plaque removal. This study, while providing strong evidence for recommending at least daily brushing, does not address the long-term consequence of plaque accumulation and the different reactions among patients to qualitative and quantitative changes in dental plaque.

1.6.1.1 Professional care

The most common form of professional preventive care for gingivitis and periodontitis is scaling and polishing of teeth (Christersson *et al.*, 1991). Scaling removes calculus and bacteria either above the gingiva or inside the gingival crevice or pocket. After scaling, an abrasive is applied via a small rubber cup or a brush to remove stains and plaque and to smooth the scaled areas. In another preventive procedure, root planing, calculus is removed from the root surface using scalers, with or without surgical exposure of the root by opening a gingival flap (Waite and Strahan, 1990). The choice of procedure is determined by the depth of the periodontal pocket and accessibility of the area (Lindhe, 1983).

1.6.1.2 Antimicrobial prophylaxis

It has been shown that oral rinses containing chlorhexidine (0.12% or 2%), an antibacterial agent, are effective in reducing supragingival plaque and gingivitis (Anderson *et al.*, 1997). Side effects associated with chlorhexidine (from 0.1-0.2 %) use include increased calculus formation, alteration in taste sensation and staining of teeth (al-Tannir and Goodman, 1994).

1.6.1.3 Treatments currently under investigation

The goal of all periodontal treatment is to reduce the bacterial count in colonized areas and thereby reduce the infection (Lindhe, 1983). Traditional non-surgical treatments consist of tooth and pocket debridement with scaling and root planing procedures. Following this initial treatment regime, the affected areas are re-evaluated for further treatment. If active infection is still present the patient would have one of two options: re-scale and try maintenance therapy, or

to try surgery to eliminate the defective tissue surrounding the teeth (Lindhe, 1983). The surgery is primarily a means of creating a more cleansable area around the tooth so that one could adequately remove the bacterial plaque. A number of studies have shown that there may be another option to eliminate this type of infection: site-specific delivery of an antibiotic using a biocompatible, flexible copolymer tetracycline fibre referred to as Actisite (Latner, 1998; Valentine, 1994; Mombelli *et al.*, 1997; Litch *et al.*, 1996; Mombelli *et al.*, 1996; Goodson, 1996). A small fibre is placed in an infected pocket and Actisite releases high local concentrations of drug with low systemic absorption. The patient receives 10-15 times the concentration of antibiotic at the infected site with non-detectable blood concentrations as compared to systemic use of the antibiotic. This therapy has been found to be effective if used as an adjunct to scaling and root planing in certain patients at sites that do not respond to conventional therapy (Greenstein, 1995).

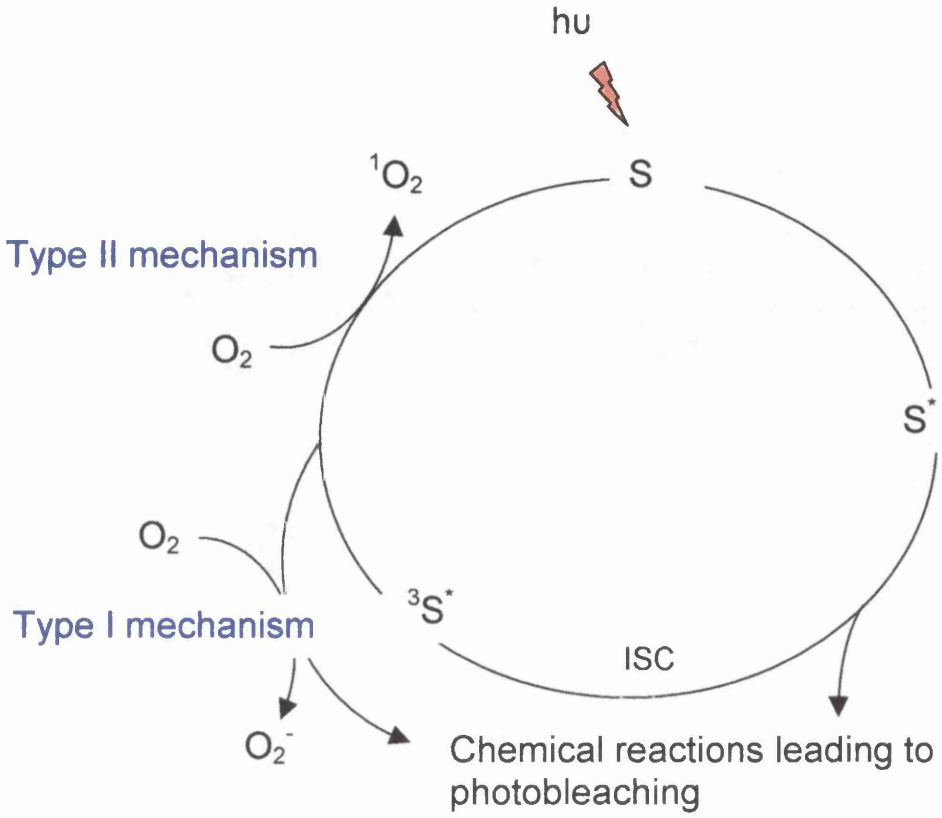
The discovery of a variety of naturally-occurring antimicrobial peptides opens a new dimension for antibiotic treatment. Magainins are small cationic peptides recently isolated from frog skin (Westerhoff *et al.*, 1995), and which are thought to possess antimicrobial activity by virtue of their ability to selectively disrupt bacterial membranes and are thought to cause minimal damage to host tissue. Other antimicrobial peptides, such as protegrins, α- and β-defensins isolated from porcine leukocytes (Miyasaki *et al.*, 1997 and 1998) and epithelial cells (Krisanaprakornkit *et al.*, 1998), respectively have been found to be effective against Gram-negative, facultative periodontopathogenic bacteria including *Capnocytophaga spp*, *A. actinomycetemcomitans* and *Por. gingivalis.*

1.7 Periodontal disease - disadvantages of current treatments

Orally-administered antimicrobial agents for the treatment of periodontal diseases often lead to ecological disturbances in the normal oral and intestinal microflora, which may lead to the overgrowth of potentially pathogenic microorganisms which may spread from one host to another (Edlund *et al.*, 1996). Furthermore, the use of antimicrobial agents also promotes the emergence of bacterial drug resistance, both in the periodontal pocket and in the normal oral (Sandham, 1994) and intestinal microflora (Edlund *et al.*, 1996). Topical administration of antimicrobial agents in the periodontal pockets causes restricted disturbances in the intestinal microflora, although there is a substantial risk of the development of resistance at the site of application. Other treatments of periodontitis such as the mechanical removal of dental plaque from the gingival margin, and surgery, are costly to the NHS and may be uncomfortable for the patient (Sullivan and Neish, 1997). No treatment has yet been found for periodontitis that can completely eradicate pathogenic bacteria from the infection site; hence there is a need to investigate either alternative treatments, or treatments that can be used in conjunction with the current methods.

1.8 Photodynamic therapy

A potential treatment for oral infections is photodynamic therapy (PDT). This works on the basis that a photosensitiser is applied to the infection and the area is then exposed to light of an appropriate wavelength, thus causing cell death due to the action of cytotoxic species.



S = ground state sensitiser

S^* = excited state sensitiser

$^3S^*$ = excited triplet state sensitiser

$^1O_2$ = singlet oxygen

ISC = intersystem crossing

After absorbing light the sensitiser is converted from a stable ground state to the singlet state, which undergoes intersystem crossing to a triplet excited state. Interaction of the triplet state sensitiser returns the sensitiser to its ground state and generates singlet oxygen. The singlet state can in turn be quenched by polypeptides (PP) or any other cellular component:

$^1O_2$ + PP → Pr1

The ground state sensitiser can quench the singlet oxygen

$^1O_2$ + S → Pr2

The triplet state sensitiser is quenched by a polypeptide

$^1S^*$ + PP → Pr3

And the sensitiser binds to the polypeptide

S + PP → (S-PP)

(After Michaeli and Feitelson, 1995).

Pr = product.

It is thought that singlet oxygen produced during the type II process is responsible for damage to amino acids (histidine, tryptophan and methionine) and to DNA bases (mainly guanine). It is thought that hydroxyl radicals are also involved in cellular damage, but to a lesser extent (Dougherty *et al.*, 1998).

A type I process can involve the abstraction of a hydrogen from the sensitiser to produce free radicals or electron transfer resulting in the formation of radicals or radical ions. These radicals or ions can then react with oxygen to yield superoxide radical anion which at low pH values can protonate to form the reactive $HO^{\cdot}_2$ radical.

Work on PDT seems to have started at the turn of the century. A review by Hayata and Konaka (1990), stated that in 1900, Raab showed that some dyes on their own were not harmful to cells, but became lethal when in the presence of oxygen and strong light (Burns, 1997). Another finding was the peroxidation of organic compounds in the presence of hydrogen peroxide and sodium

hypochlorite, which revealed the presence of singlet oxygen. It has been shown that singlet oxygen plays a role in organic-peroxidative reactions and photooxidation phenomena (Ravanat *et al.*, 1998; Kamat *et al.*, 1998).

1.8.1 PDT of cancers

The use of PDT dates back to India, 1400 BC, where Hindus treated vitiligenous skin and psoriasis using dye compounds in combination with sunlight (Burns, 1997). In Europe, the first indication that there was potential for the combination of dye and light leading to cellular death, was through the observation made by Raab in 1900. He found that, in the presence of acridine, paramecium died when exposed to light. Since Raab's work, it has been shown by von Tappeiner and Jesionik ([1905] reviewed by Burns, 1997) that skin cancers could be treated with eosin and sunlight and that in addition to light and dye, oxygen is required, hence the process was termed photodynamic action Von Tappeiner further proposed that this may have a therapeutic application. Between 1900-1950, there were several indications for the use of PDT to treat neoplastic diseases (Kessel, 1990). The photosensitiser predominantly used was hematoporphyrin (Hp) which was derived from blood by extracting haem (iron protoporphyrin IX) and then reacting it with hydrogen bromide and acetic acid and then decompsing the hydrogen bromide adduct with water (Milgrom and MacRobert, 1998). In 1960, Schwartz and Lipson (Dougherty *et al.*, 1998) developed hematoporphyrin derivative (HpD) which is a mixture of porphyrin monomers and oligomers derived from Hp. In 1975-76, through work carried out by Dougherty, HpD was indicated as having great potential for tumour eradication. This photosensitiser was the first to be used in clinical trials as HpD-photofrin I and HpD-photofrin II (the purified form) and these were referred

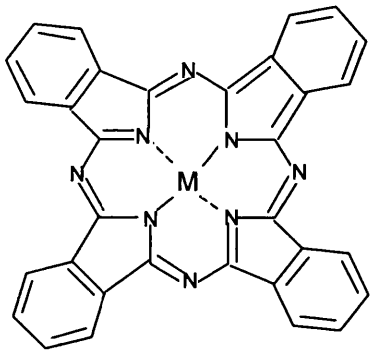
to as the 'first generation photosensitisers' (Dougherty *et al.*, 1998). Both forms of the photosensitiser have been shown to be successful when used *in vivo* (Carruth, 1998). However, HpD consists of a complex mixture of porphyrins, the composition of which varies with different preparations and storage times and there is a tendency for such complexes to form aggregates, which are known to reduce the biological efficiency of the photosensitiser (Bonnett, 1998). An additional complication is that patients receiving treatment with HpD can suffer from photosensitive reactions to daylight as a consequence of the dye accumulating in the skin (McCullough *et al.*, 1983; Berenbaum *et al.*, 1986; Messman *et al.*, 1997). Because of such disadvantages, a 'second generation' of photosensitisers was developed to fit the following criteria (Milgrom and MacRobert, 1998): i) there should be no toxicity in the dark. ii) The photosensitisers should be selective for tumour tissue and be cleared quickly from tissue. iii) They should be synthesised easily. iv) The quantum yield of singlet oxygen should be high. The greater the yield, the more damage singlet oxygen will incur to the tumour. v) They should be stable and pure. vi) As tissue penetration of laser light is greatest at the red end of the spectrum, the absorption of the photosensitiser should be greatest at the corresponding wavelength. Red light penetrates tissue well as haemoglobin – the main chromophore in tissue – absorbs weakly beyond 600 nm, so light can penetrate further at 630 nm compared to, for example, 550 nm. Berenbaum *et al.* (1986) synthesised isomers of 5, 10, 15, 20-tetrakis(*o, m* or *p*-hydroxyphenyl) porphyrin (o, m, p- THPP) and the corresponding chlorin o,m,p-THPC. It was found that m-THPC had the most potential as a photosensitiser against neoplastic diseases. It has since been tested *in vitro* (Hornung *et al.*, 1997) and *in vivo* (Peng *et al.*, 1995; Morlet *et al.*, 1995). It is now prepared commercially by

The two families of dyes that have been studied most with respect to PDT of cancers are the phthalocyanines and porphyrins. They are, to some extent, selectively taken up and retained in tumour cells (Moan, 1987). The lipophylic dyes (3THPP, PII, HP diethers $TPPS_1$ [tetraphenyl porphine (tetra) sulfonate] and $AlPcS_1$) are retained in the mitochondrial, nuclear and plasma and endoplasmic reticulum membranes. The hydrophophilic dyes ($TPPS_{2o}$, $TPPS_{2a}$, $TPPS_4$, $AlPcS_{2a}$ and $AlPcS_4$) localise in the lysosomes. Porphyrins have been shown to be immensely promising photosensitisers. Endogenous porphyrins are produced during haem synthesis in cells. 5-amino laevulinic acid (ALA) is the penultimate step in the production of protoporphyrin IX, which has been shown to be an efficient photosensitiser in the treatment of cancers. The treatment modality is such that the patient is administered a large dose of ALA orally, intravenously or topically (Kennedy *et al.*, 1992) and there is a temporary accumulation of protoporphyrin IX in rapidly proliferating cells. Due to its accumulation in neoplastic cells, together with the fact that it is fluorescent, it has been used in the diagnosis of tumours (Kreigmair *et al.*, 1996) and more recently in the lethal photosensitisation of bacteria (Orenstein *et al.*, 1998).

Interest in phthalocyanines started in 1985 when it was reported that some of these compounds are efficient photosensitisers in mammalian cells and have advantages over HpD. Ben-Hur (1993) described two advantages of phthalocyanines over HpD. Firstly, the extinction coefficient of phthalocyanines in the red is around 50 times greater then HpD, therefore allowing more efficient photon utilisation. Secondly, the absorption peak of phthalocyanines is red

shifted by about 50 nm compared to HpD. The efficient wavelength for phthalocyanines (680 nm) penetrates 50 % deeper into tissues than the wavelength required to activate HpD.

Phthalocyanines can be regarded as azoporphyrins containing a ring system made up of four isoindoles linked by nitrogen atoms:



Various groups can be added to the outer rings of the isoindole unit; if the valency of the central metal ion is greater than 2, it binds various axial ligands. All of these changes of the Phthalocyanine skeleton can profoundly affect its photobiology (Ben-Hur, 1993). The nature of the central ion has an effect on the fluorescence and heavy metal ions increase the intersystem crossing to the triplet state. The triplet lifetimes of H_2Pc, ZnPc, AlPc, CuPc and CrPc are 170, 245, 500, 0.065 and 0.02 μs, respectively. Due to the limited lifetime of Cu and Cr phthalocyanines, CuPc and CrPc are not used as photosensitisers.

1.8.2 Application of PDT to infectious diseases

Work on the lethal photosensitisation of bacteria began around the early 1960's. In 1966 Macmillan *et al.* studied the lethal photosensitisation of bacteria, yeast and algae using toluidine blue O (TBO) as a photosensitiser and light from a gas laser (21-30 mw). They found that 99 % kills were obtained with all of the bacteria tested after 30 min exposure except *Chromobacterium vioaceum*. They found that exposing the heavily-pigmented bacteria (cultured after 3 days growth) to laser light after sensitisation led to fewer kills compared to when the lesser pigmented bacteria were used (cultured after one day's growth). They tested the effect of laser light alone on pigmented bacteria as it was thought endogenous photosensitisers may be as effective in killing the bacteria as exogenous ones. They found that none of the pigmented bacteria were killed when exposed to laser light. They suggested two reasons for this; firstly, there is minimum absorption of the pigment at the wavelenghth of light used. Secondly, the organisms tested (*Chromobacterium vioaceum* and *Rhodoospirillum rubrum*) contained carotenoids which have been shown to protect against photosensitisation in other photosynthetic bacteria. It has also been demonstrated using *Sarcina lutea*, that the carotenoids present in the membrane afford protection to the cytotoxic species produced during lethal photosensitisation using TBO in combination with laser light. This however does not seem to be the case when Psoralen is used as a photosensitiser (Bellin *et al.*, 1969). This suggests that TBO may act on the cell membrane. The diagram below shows the possible binding sites of some photosensitisers.

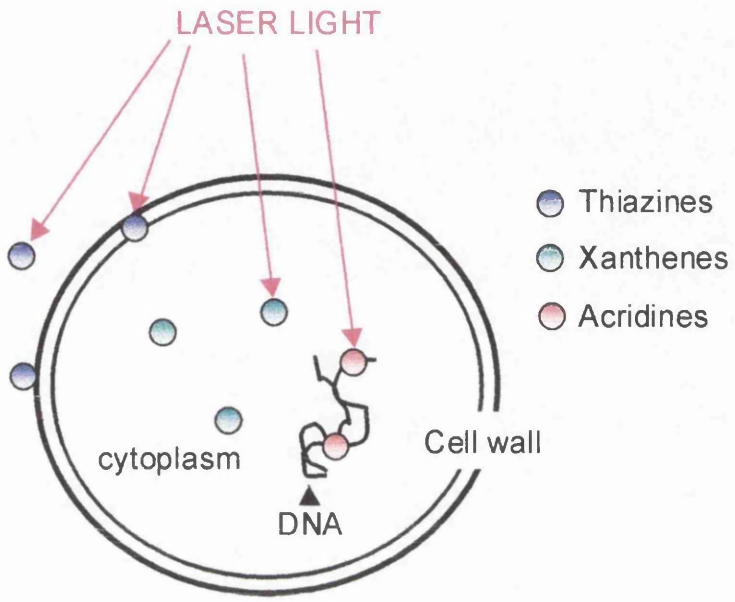
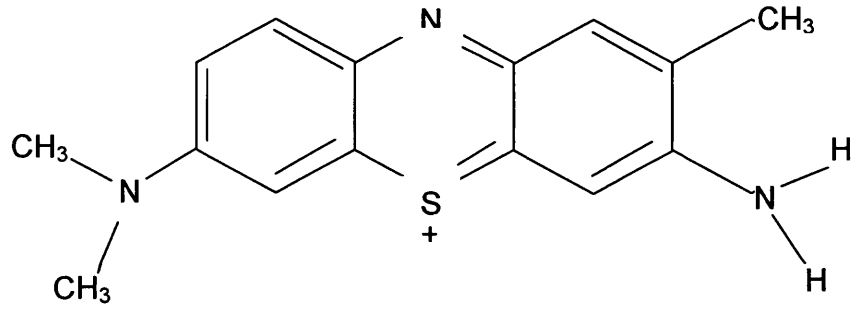


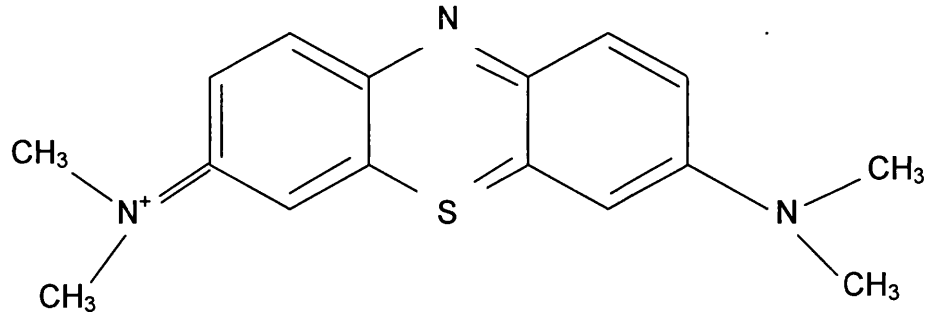
Figure 1.4 Localisation of different photosensitisers in the bacterial cell (Adapted from Ito, 1978).

Other groups such as Bellin *et al.* (1969) studied the lethal photosensitisation of *E. coli* using another thiazine dye, methylene blue. They also used Rose Bengal and Erythrosine B, Eosine Y, Neutral Red, Acridine Orange, Crystal Violet, Pyrorine Y and Rhodamine 6G. They found that cationic dyes act as sensitisers when at an alkaline pH and anionic dyes act at pH values slightly lower than their pK. They also demonstrated that the presence of oxygen was required to successfully kill *E. coli* when using any of the sensitisers. However, Macmillan *et al.* (1966) stated that the requirement for oxygen for successful killing is questionable. They suggested that in the absence of oxygen a lack of killing may be due the reduction of the dye (TBO) to the leuco form by dehydrogenases. Though they did not see a reduction (to a colourless form)

during their experiments, they postulated that it may be due to reduction occurring at the site of action. In fact it has been shown more recently (Bongard *et al.* 1995) that thiazine dyes can be reduced by endothelial cells causing the overall properties of the photosensitisers to change. The photoproperties of a photosensitiser are not straightforward because the heterogeneous nature of the biological systems can greatly affect these properties (MacRobert *et al.*, 1989). For example, the pH of the system (Pottier *et al.*, 1975) and the presence of protein (Phillips, 1997) can cause a change in the cyotoxic yield and increase in photosensitiser aggregation, respectively.



Toluidine blue O



Methylene blue

Figure 1.5 The thiazine group of dyes includes toluidine blue O (TBO), thionin and azures A, B and C, which are all derivatives of methylene blue. They are members of the quinone-amine group.

These dyes absorb at light wavelenghths between 550-700 nm (Tuite and Kelly, 1993) which is the reason for their characteristic blue colour. TBO has been extensively used in the staining of various cells such as ganglion cells (Weinberg, 1995). TBO has also been used in combination with other dyes for the analysis of various structures within articular cartilage. For example, it has been used in combination with malachite green for the staining of lipids, and in combination with Cuprolininic blue for the staining of proteoglycans (Guerra *et al.*, 1996; Shepard and Mitchell, 1976). Both TBO and MB are used in the diagnosis of cancerous tissue, and it was found by Bellin *et al.* (1969) that tumour cells were susceptible to killing by light when treated with methylene blue.

Much work has been carried out showing the effects of porphyrins and laser light on bacteria. Nitzan *et al.* (1983, 1989, 1994, 1995 and 1998) have published several studies on the effect of HpD, DP (deutoporphyrin), HP (haematoporhphyrin) and PP (protoporphyrin IX) on aerobic and anaerobic organisms. They demonstrated that Gram-positive bacteria, yeasts and mycoplasma but not Gram-negative bacteria are killed by porphyrins. Gram-negative aerobes are only killed if the membrane is disrupted with polymyxin nonapeptide (PMNP) as the porphyrins have to bind to the plasma membrane in order to exert any deleterious effects on exposure to light. They found that in addition to inhibition of growth there was alteration in protein, DNA and RNA synthesis. There was also perturbation of cell wall synthesis. Interestingly, they found that when the membrane of Gram-negative aerobic bacteria was disrupted using PMNP, lethal photosensitisation caused a decrease in viable counts, however, no effect was seen when PMNP was used on Gram-negative

anaerobes.

DP has been used in combination with PMNP in order to inactivate the aerobic Gram-negative organism, *Acinetobacter baumanii.* This microorganism is of special interest as it is a multi-drug resistant bacterium mainly acquired in hospitals (Nitzan *et al.*, 1998). Other porhpyrins (HpD, HP, PP) however do not have any effect on this bacterium. Current investigation into alternative photosensitisers has included tetra-methylpyridyl porphine (TMPyP). This sensitiser has been shown to kill this bacterium when used in combination with PMNP.

Table 1.3 below shows the various photosensitisers tested *in vitro* and *in vivo* to date.

1.8.3 Mechanism of lethal photosensitisation

Por. gingivalis, like all Gram-negative bacteria, has a negative charge on its surface (Shah, 1993) and hence may take up positively-charged photosensitiser more readily than negatively-charged ones. Membrane photosensitisation can lead to a decrease in plasma membrane potential, inhibition of molecular transport across the membrane, and both inhibition and activation of membrane associated enzymes (Kochever *et al.*, 1996). Cellular responses induced by plasma membrane photosensitisation include apoptosis (Agarwal *et al.*, 1991) and expression of early response genes (Luna *et al.*, 1994).

Table 1.3 Therapeutic applications of a range of photosensitisers

Photosensitiser	Application	Wavelength of light (nm)	References
Haematoporphyrin and derivative (HpD)	Cancers and *in vitro* lethal photosensitisation of bacteria.	630	Patrice *et al.*, 1990; Bown, 1993; Millson *et al.*, 1996; Kussovski *et al.*, 1995.
Protoporphyrin IX	Actinic keratosis, basal cell carcinoma. Undergoing clinical trials. *In vitro* for lethal photosensitisation of bacteria.	635	Svanberg *et al.*, 1994; Marcus *et al.*, 1996; Tope *et al.*, 1998; Henry *et al.*, 1995.
m-THPC	Cancers of the head and neck, upper aero-digestive tract, prostate, pancreas. Undergoing phase III trials.	652	Chang *et al.*, 1996; Grosjean *et al.*, 1996; Mikvy *et. al.*, 1997; Kübler *et al.*, 1999.
Disulphonated aluminium phthalocyanine	Skin, breast and oropharyngeal cancers clinical trials. *In vitro* lethal photosensitisation of bacteria.	675	Peng and Moan, 1995; Burns *et al.*, 1995; Griffiths *et al.*, 1997;
Lutetium texaphyrin	Cancers and atheromatous plaques. Undergoing clinical trials.	633	Woodburn *et al.*, 1996; Hammer-Wilson *et al.*, 1999; Ivy *et al.*, 1999.

The effect of lethal photosensitisation on membranes involves the intermolecular photodynamic cross-linking of membrane proteins (Shen *et al.*, 1996). This in turn involves the interaction of singlet oxygen with photo-oxidisable amino acid residues of L-His, Cys and Trp to form reactive species that interact non-photochemically with either a normal or reactive group in another protein to form a cross-link (Shen *et al.*, 1996). Shen *et al.* showed that the most reactive amino acid is histidine and the least reactive is lysine. The fact that only a few amino acids are prone to photo-oxidation may suggest that only those proteins containing a predominance of these target amino acid residues are adversely affected physicochemically by cytotoxic species. Girotti (1980) investigated the effect of lethal photosensitisation on membrane proteins. He showed that on exposure of human erythrocyte ghosts (pH 8, 10°C) to visible light in the presence of methylene blue (MB), there was a rapid loss of bands correlating to spectrin (an actin-binding plasma membrane protein) and the appearance of high molecular weight cross-linked derivatives seen on SDS-PAGE.

It has been well documented that guanine is a target of lethal photosensitisation (Tuite and Kelly, 1993). The damage caused to guanine as a result of lethal photosensitisation has been shown to be dependent on the type of photosensitiser used. Kvam *et al.* (1994) showed that with photosensitisers which bind to DNA, such as MB, there is an increase in the formation of guanidine breakdown products (mainly 8-oxo-dG) compared to when a non-binding photosensitiser is used (TSPP) on exposure to light of the appropriate wavelength.

There is no evidence as yet to show whether a type I or type II mechanism is involved in bacterial cell death. Using furocoumanins, Dall'Acqua and Martelli (1991) showed that type I and type II reactions lead to lipid peroxidation and cross-linking between proteins. They also suggested that a type III mechanism takes place where photoreactions occur between furocoumanins and unsaturated fatty acids.

1.8.4 Current applications of photodynamic therapy

PDT has been extensively used in the search for the treatment of various cancers (Dougherty, 1984). For example work is being carried out on the treatment of gastric carcinomas (Sibile *et al.*, 1995), bladder cancers (Gerber *et al.*, 1995), and ovarian cancers (Goff *et al.*, 1996) and their application is currently under investigation. In fact the possibilites and developments of PDT in the treatment of cancers are endless. Newer applications of PDT include the treatment of parasitic infections such as trypanosoma (Gottlieb *et al.*, 1995) and viral infections such as HIV and herpes simplex virus (Smetana *et al.*, 1997). However, very little work is being carried out *in vivo* for the treatment of bacterial infections. There have been extensive *in vitro* studies of the lethal photosensitisation of Gram-positive and negative bacteria, mainly using HpD and phthalocyanines (Minnock *et al.* 1996; Merchat *et al.*, 1996;Bertoloni *et al.*, 1992) and there has been some work carried out on the effectiveness of thiazine dyes as photosensitisers (Wilson and Yianni, 1995; Wilson and Pratten, 1994; Soukos, 1996).

1.9 Lasers and their use in dentistry

LASER is an acronym of light amplification by the stimulated emission of radiation. During the 1960's the application of lasers in dentistry was developed. A few years after the discovery of the first Ruby lasers, investigations were undertaken to introduce lasers for 'optical drilling' of teeth (Frentzen and Koort, 1990). Since then, laser technology has developed until today when more than 600 laser media are known that can emit radiation at different wavelengths (Frentzen and Koort, 1990).

The principle on which all lasers work is the generation of monochromatic, coherent and collimated radiation. Laser light that is monochromatic is characterised by radiation in which all waves are of the same frequency (wavelength) and energy. Coherence of laser light is when all waves are in a certain phase relationship to each other, both in space and time. Laser light is collimated radiation, which means that all the waves that are emitted are nearly parallel and the beam divergence is very low (Strang and Moseley, 1988; Frentzen and Koort, 1990). The main difference between laser types is the wavelength, which depends upon laser medium and excitation mode, i.e. whether it is a continuous wave or pulsed. There are three classifications of wavelength: ultraviolet (140-400 nm), visible spectrum (~700 nm) and the infrared (~700-microwave spectrum). The table below (table 1.4) summarises the different lasers used in medicine. The so-called 'hard' lasers used in dentistry include the CO_2 laser, where the laser medium is carbon dioxide gas, Nd:YAG laser whereby the laser medium is yttrium aluminium garnet crystal dotted with solid state neodymium, and the Argon laser where the laser medium is argon gas. Soft lasers such as the HeNe laser (helium neon gas medium)

and diode lasers are mainly applied in biostimulation and are thought to have analgesic effects (Frentzen and Koort, 1990).

Laser	Wavelength (nm)	Mode
CO_2	9000 - 11000	CW, pulsed
Gallium aluminium arsenide	660 - 830	Semi-conductor
Indium gallium arsenide phosphide	670 - 675	Semi-conductor
Nd:YAG	1064	CW, pulsed
Ho:YAG	2100	Pulsed
Er:YAG	2940	Pulsed
Diodes	650 - 950	Pulsed
HeNe	633	CW
Dyes	450 - 1200	CW, pulsed
Argon ion pumped	488	CW
Excimer	190 - 351	Pulsed
Copper vapour pumped dye	< 900	Pulsed
Gold vapour	628	Pulsed

Table 1.4 Table summarising the wavelengths of lasers and mode of excitation. CW = continuous wave. (Table adapted from Frentzen and Koort, 1990).

As mentioned above, the Ruby laser was studied for possible uses in dentistry. However, soon after, it was found that the laser produced significant heat that caused damage to the pulp of the teeth. The only lasers to be cleared for use in

soft tissue dentistry are the CO_2, Nd:YAG, Ho:YAG and Argon lasers (Gillis and Stron, 1983; Das *et al.*, 1997). It has been found that the CO_2 laser when used to make incisions in soft tissue is superior to the scalpel, as the laser incision is slow to heal and hence less scar formation occurs. Furthermore, using pulsed or CW CO_2 lasers could control thermal damage (Widgor *et al.*, 1995). CO_2 lasers were also used in periodontal surgery, and work by Pick *et al.* (1985) who pioneered the surgery, showed that overgrown gingival tissue caused by a number of drugs (Dilantin®, cyclosporine and Procardia) could be removed by using CO_2 lasers. There was minimal damage to the underlying bone, good healing of the gingiva, dry bloodless surgery, instant sterilisation of the surgical site and reduced bacteraemia (Wigdor *et al.*, 1995). The main disadvantage of the technique was that the surrounding epithelium grew faster than the healing connective tissue thereby causing a deep pocket to form, which accumulated bacteria. However, more recently, work carried out by Rossman and Israel (reviewed by Widgor *et al.*, 1995) showed that the laser could be used to remove the epithelium from the connective tissue around the tooth, which also led to inhibition of epithelial growth around this area. Work still needs to be carried out to refine such techniques.

The problems encountered by hard lasers, such as thermal effects, are not an issue with soft lasers as the power of the laser is too low, however photochemical effects cannot be ruled out (Strang and Moseley, 1988). The main uses of low-power lasers are in the treatment of pain by 'laser acupuncture' and in wound healing (Bradley, 1996). HeNe lasers have been used in dentistry in the irradiation of gingival tissue following surgery and it has been claimed that wound healing was better, but this conclusion was drawn

without using any control groups. Much of the work on HeNe and Ga-As lasers has been carried out by Eastern European workers with promising data, but with lack of controls and frequently no mention of energy dose values (Strang and Mosely, 1988).

1.10 AIMS AND OBJECTIVES

The above review has shown that a number of types of anaerobic bacteria are associated with the development of periodontal diseases and that the current treatments, although successful in reducing the bacterial load in the periodontal pocket, cannot reduce the numbers of these pathogenic organisms to such a degree that the disease is eliminated. Disadvantages of using the current treatments include side-effects associated with antimicrobial agents, resistance of bacteria to antibiotics and pain associated with scaling, root-planing and curettage. As the search for new and improved treatments goes on, one possible approach that may be useful in combination with mechanical debridement is PDT. As the above review suggests, PDT, though in its early stages of development, has been successfully used for the treatment of a range of cancers and bacterial infections. The aim of this study was to determine the effects of laser light, used in combination with a photosensitiser, on *Por. gingivalis*, a periodontopathogenic bacterium associated with a number of periodontal diseases.

The aims and objectives can be summarised as follows:

1. To compare the effect of lethal photosensitisation on *Porphyromonas gingivalis* using the positively-charged photosensitser, TBO, in combination with HeNe laser light and the negatively-charged $AlPcS_2$ in combination with

light from a GaAs laser. (TBO was then used for the remaining study).

2. To determine the effect of various dosimetric (increasing TBO concentration, light doses and pre-irradiation times) and physiological conditions (presence of serum, different growth phases and pH values) that may be encountered *in vivo* during the lethal photosensitisation of *Por. gingivalis* using TBO and HeNe laser light. The effect of lethal photosensitisation on the proteolytic activity of *Por. gingivalis* was also determined. As the organism is also present in the oral cavity as a biofilm, the effect of photosensitiser in combination with laser light on its viability was determined.
3. To determine whether a type I and/or type II mechanism(s) is responsible for bacterial killing.
4. To determine whether TBO binds to *Por. gingivalis* cells and is taken up into the cells and whether the uptake plays a role in lethal photosensitisation.
5. To elucidate the possible site of action by determining the site of binding of the photosensitiser. To determine the possible cause(s) of cell death by observing the effects on cellular protein and DNA.
6. The final aim of the study was to determine whether a specific targeting system for *Por. gingivalis* could be developed. The TBO was linked to a *Por. gingivalis*-specific antibody and tested for its ability to selectively kill the organism in the presence of other oral bacteria and human gingival fibroblasts.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Growth of *Porphyromonas gingivalis*

Porphyromonas gingivalis W50 was maintained by twice weekly subculture on Wilkens Chalgren agar (Oxoid Ltd., Basingstoke, U.K.) and incubated at 37°C in an anaerobic cabinet (10 % carbon dioxide, 10 % hydrogen and 80 % nitrogen. Don Whitley Scientific Ltd). For experimental purposes, a few colonies of *Por. gingivalis* were inoculated into liquid medium (BM) which consisted of the following; tryptone soya broth (10 g), proteose peptone (10 g), yeast extract (5 g), glucose (5 g), sodium chloride (5 g) and cysteine-HCl (0.75 g) per litre of distilled water. The pH was adjusted to 7.5, and the broth autoclaved at 121°C for 15 mins. The medium was supplemented with haemin (Sigma Ltd.) and menadione (Sigma Ltd.) prior to use so that the final concentrations were 5 μg/ml and 0.5 μg/ml respectively. The culture was incubated in an anaerobic cabinet.

2.2 Growth of *Streptococcus sanguis*

S. sanguis was subcultured twice-weekly on tryptone soya agar (Oxoid Ltd., Basingstoke, U.K) supplemented with 0.5 % yeast extract (Oxoid Ltd., Basingstoke, U.K.). For experimental purposes a few colonies of *S. sanguis* were inoculated into tryptone soya broth (Oxoid Ltd., Basingstoke, U.K.), supplemented with 0.5 % yeast extract (Oxoid Ltd., Basingstoke, U.K.) and incubated in an anaerobic chamber until it reached stationary phase (approximately 15 h).

2.3 Lasers and Photosensitisers.

2.3.1 Helium/Neon laser in combination with toluidine blue O

The laser used in the studies was a helium/neon (HeNe) gas laser (NEC Corporation, Japan) with a measured output of 7.3 mW which emits light in a collimated beam (diameter of 1.3 mm), with a wavelength of 632.8 nm. The

photosensitiser used in the experiments was toluidine blue O (TBO; Sigma Ltd, Poole, UK).

2.3.2 Gallium aluminium Arsenide laser in combination with aluminium disulphonated pthalocyanine

The laser used in one of the studies was a gallium aluminium arsenide diode laser (GaAs; Omega Universal Technologies Ltd, London, UK) which had a power output of 11 mW and emitted light at a wavelength of 660 nm as a collimated beam. The diameter of the beam was 9 mm. The photosensitiser used in combination with the laser was aluminium disulphonated pthalocyanine ($AlPcS_2$) kindly provided by Prof. D. Phillips (Chemistry department, Imperial College, London).

2.4 Method for lethal photosensitisation studies

An overnight, stationary phase culture of *Por. gingivalis* was centrifuged at 5000 g for 15 minutes and resuspended in 0.85 % (w/v) NaCl so that the suspension gave an absorbance of 1.0 at a wavelength of 560 nm (Ultrospec 2000, Pharmacia, Biotech.). Aliquots (100 μl) of the suspension were added to wells of a 96-well microtitre plate (Sterilin Ltd., Stone UK, see figure 2.1 for layout of microtitre plate). Sterile TBO (100 μl) in saline was added to duplicate wells to give a final concentration of 82 μM (25 μg/ml) unless otherwise stated. The suspensions were continually stirred with microstirring bars during the experiment so that all the bacteria in the suspension were exposed to laser light as it was possible the light did not penetrate down through the whole solution. After 60 s pre-irradiation time, each well was exposed to 0.88 J of light from a HeNe laser (L^+S^+) unless otherwise stated. Control wells were prepared to observe the effects of: i) laser alone, no

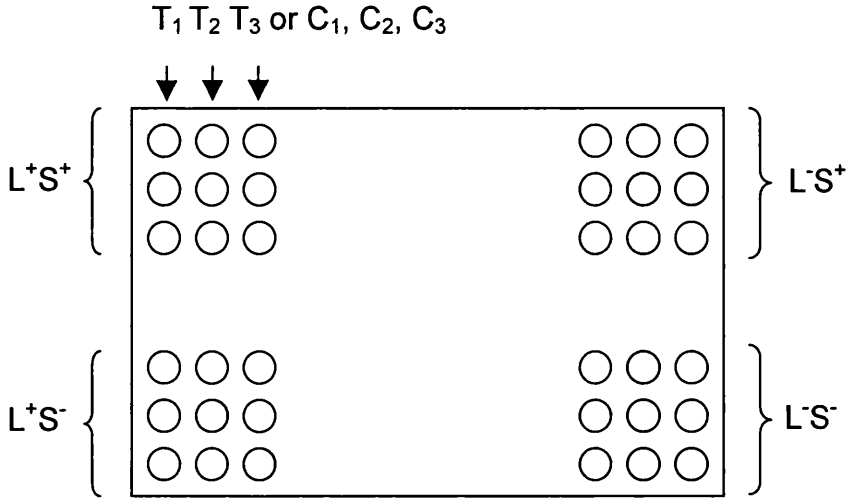
sensitiser being added (L^+S^-) and ii) sensitiser alone, the wells were not exposed to light from the laser (L^-S^+). Further duplicate control wells were prepared to determine the number of viable bacteria present prior to treatment with neither laser nor sensitiser (L^-S^-). Aliquots (50 μl) were plated from each well onto Wilkins Chalgren agar supplemented with 5 % horse blood (Oxoid Ltd). The plates were incubated for 4-6 days in an anaerobic chamber and colonies were enumerated See figure 2.2 for details. Reductions in viable counts were calculated relative to L^-S^-.

The same procedure was carried out using GaAs laser and $AlPcS_2$.

The power of the laser was measured every six months to ensure that it was constant (Laser power monitor, model PD2-A. Optilas, Milton Keynes, UK). It was assumed that the power of the laser and the diameter of the laser beam remained constant throughout the experiment. Hence, as the bacterial suspension was stirred constantly, it was assumed that the light energy was distributed equally between all the bacteria in the suspension.

2.4.1 Calculation of energy dose

Energy dose (mJ) = [length of exposure (seconds)] x [power of laser (mW)]



$T_{1,2,3}$ = Different laser light exposure times (in triplicate).

$C_{1,2,3}$ = Different concentrations of photosensitiser (in triplicate).

Figure 2.1 Layout of microtitre plate for lethal photosensitisation studies

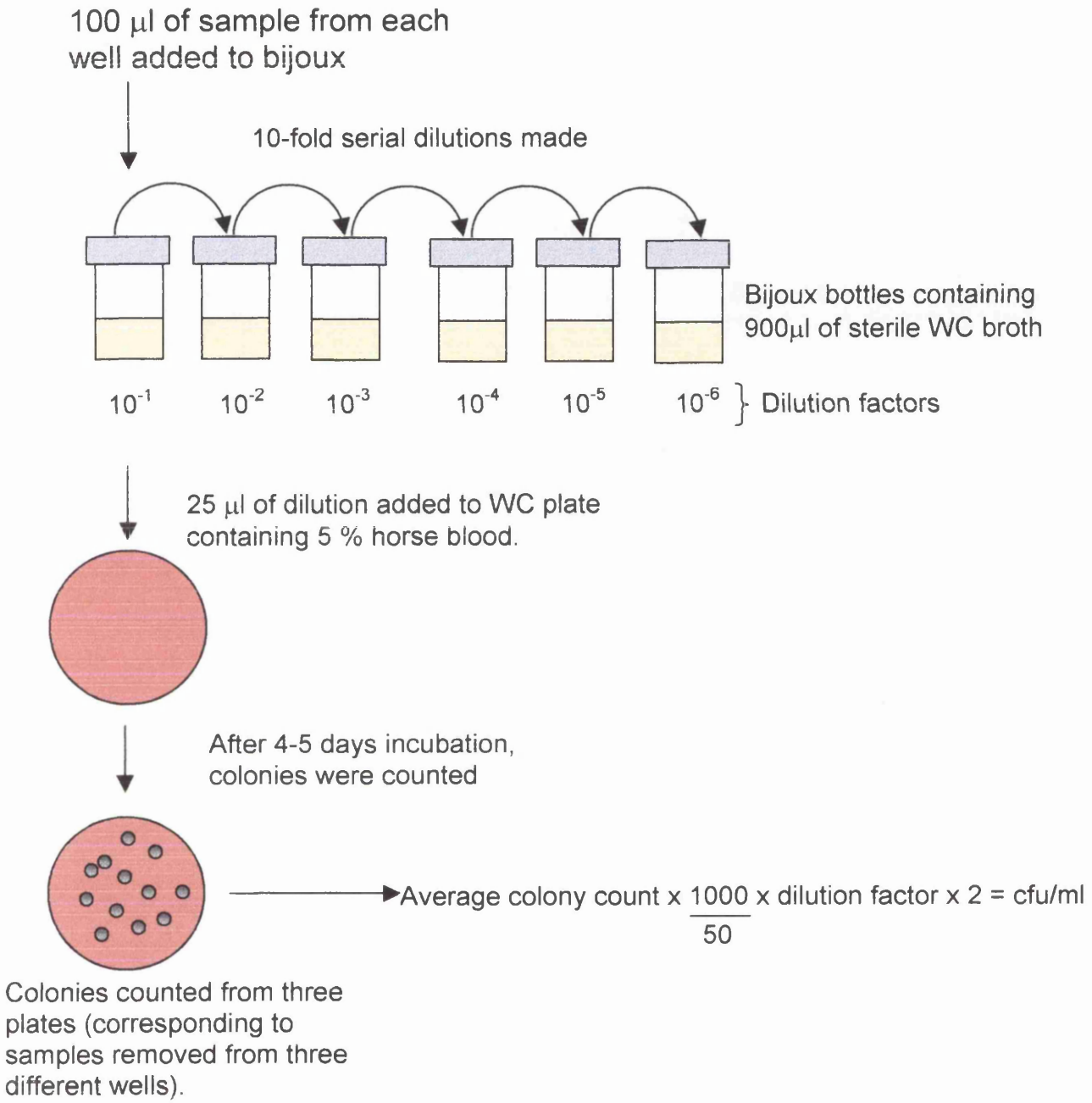


Figure 2.2 Method used to enumerate colonies after lethal photosensitisation studies.

2.4.2 Lethal photosensitisation of *Por. gingivalis* sensitised with increasing concentrations of toluidine blue O and exposed to HeNe laser light

The concentrations of TBO used were 41, 82 and 164 μM (equivalent to 12.5μg/ml, 25 μg/ml and 50 μg/ml, respectively). The laser light dose used was 0.88 J and the pre-irradiation time (PIT) used was 60 s.

2.4.3 Lethal photosensitisation of *Por. gingivalis* using increasing concentrations of aluminium disulphonated phthalocyanine and GaAs laser light

The concentrations of the photosensitiser used were 16, 32 and 64 μM (equivalent to 12.5, 25 and 50 μg/ml, respectively), and the light dose was 1.3 J. The PIT was 60 s.

2.4.4 Lethal photosensitisation of *Por. gingivalis* sensitised with TBO and exposed to increasing laser light doses

TBO was used at a concentration of 82 μM and laser light doses of 0.88, 2.2 and 4.4 J. The PIT was 60 s.

2.4.5 Lethal photosensitisation of *Por. gingivalis* sensitised with aluminium disulphonated phthalocyanine and exposed to increasing light doses

$AlPcS_2$ at a concentration of 32 μM was used and the energy doses used were 1.3 J, 3.3 J and 6.6 J. The PIT was 60 s.

2.4.6 Lethal photosensitisation of *Por. gingivalis* using increasing pre-irradiation times

The effect of increasing PIT was determined by incubating *Por. gingivalis* in 82 μM TBO for 1, 5, 15, 30, 90 and 180 min before exposure to 0.88 J of light.

2.4.7 Lethal photosensitisation of *Por. gingivalis* in the presence of serum

Por. gingivalis cells were resuspended in horse serum (Oxoid Ltd) in place of 0.85 % saline. The PIT was 60 s, and the light dose was 0.88 J. TBO in horse serum was used at a concentration of 82 μM.

2.4.8 Lethal photosensitisation of *Por. gingivalis* at different pH values

Cells from an overnight culture of *Por. gingivalis* were harvested and resuspended in phosphate buffer at a pH of 6.8, 7.3 or 8.0. TBO was dissolved in the appropriate buffers. The pH was measured before and after the addition of *Por. gingivalis* to the buffer in order to ensure the pH did not fluctuate to a great extent. The PIT was 60 s, the concentration of TBO was 82 μM, and the light dose used was 0.88 J. The absorbance spectrum of TBO was measured at each pH in order to determine whether a change in pH had caused a shift in its peak absorbance.

2.4.9 Lethal photosensitisation of *Por. gingivalis* at different growth phases

A growth curve was constructed by inoculating 100 ml of BM broth, supplemented with haemin and menadione, with *Por. gingivalis.* The whole suspension was incubated in an anaerobic cabinet at 37°C. Samples (1 ml) were removed every hour for a total of 55 hours and the absorbance was determined at a wavelength of 560 nm. The susceptibility of *Por. gingivalis* during lag, log and stationary phases of growth was determined. Cells from each growth phase were harvested and

resuspended in saline to give the same cell density ($O.D._{560}$ = 1.0). A final concentration of 82 μM of TBO, a PIT of 60 s, and a light dose of 0.88 J were used.

2.4.10Lethal photosensitisation of different strains of *Por. gingivalis*

The concentration of TBO used was 82 μM and the light dose was 0.88 J. The strains used were P65-3-2, 381, P65-3-1 and P58-2-5 (kindly provided by Mr Milner, Microbiology Department, Eastman Dental Institute).

2.5 SDS-PAGE Gel Electrophoresis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a method of separating proteins within a sample for analysis and molecular weight detemination. The proteins are denatured and rendered monomeric by boiling in the presence of reducing agents (2-merceptoethanol or dithiotheitol) and negatively charged detergent (SDS). Acrylamide is polymerized with a crosslinker, bisacrylamide, to form a matrix through which the protein monomers must pass as they move in an electric field. The size of the monomers being separated determines the percentage of acrylamide used, low percentage to separate monomers of high molecular weight and high percentage to separate out monomers of low molecular weight. The monomer's movement in the electric field is due to the coating of the molecule with the negatively charged SDS. Therefore, the separation of protein monomers in SDS-PAGE is determined solely on size not side chain charge, as is the case in non-denaturing gel electrophoresis.

2.5.1 Method of SDS-PAGE electrophoresis

1.Two glass plates were cleaned throughly by rinsing with water and then with ethanol. The plates were allowed to air dry.

2.The plates were assembled and placed in a pouring holder.

3. Gel components (see below) were mixed together and poured into the sandwiched glass plates.

For a 12 % gel:

7 ml Distilled water

5 ml Tris-HCl (1.5 M, pH 8.8)

200 µl 10 % (w/v) SDS

8.0 ml Bis/Acrylamide (30 % (w/v) acrylamide:0.8 % (w/v) Bisacrylamide; Ultrapure PROTOGEL)

50 µl 10 % (w/v) Ammonium persulphate (Sigma)

10 µl N, N, N', N'-tetramethylethylenediamine (TEMED; Sigma)

4.Water saturated butanol was carefully put on top of the gel to create an even layer on the separating gel.

5. After the separating gel is polymerized, the comb was placed between the glass plates, and the stacking gel (see below) was added between the plates.

Stacking gel

3.05 ml Distilled water

1.25 ml 0.5 M Tris-HCl (pH 6.8)

50 µl 10 % SDS

650 µl Bis/Acrylamide

25 μl 10 % APS

5 μl TEMED

6. Once the stacking gel was polymerized, the comb was removed.

7. The gel assembly was placed into the electrophoresis apparatus and ~ 300 ml of electrophoresis buffer (see below) was added to the apparatus.

Electrophoresis buffer (5X)

1 g SDS

6 g Tris base (Sigma)

28.8 g Glycine (BDH)

8. The samples were boiled for 4 min and loaded into respective wells.

9. The apparatus was then connected to a power supply and run at a constant 30 mA until the dye front was near the bottom of the gel (approximately 60 min).

10. Once the gel electrophoresis was complete, the gel was removed and placed immediately in fix (15 % acetic acid and 30 % methanol). The gel was fixed overnight and then placed in Colloidal blue (Sigma Ltd.) until the bands were visible. Once the bands were visible, the gel was washed with frequent changes of water until there was minimum background.

2.6 Protein Assay

Protein was detected by using Bradford reagent (Sigma Ltd). A standard curve was constructed by preparing samples of bovine serum albumin (BSA, Sigma Ltd.) ranging from 0.01 mg/ml - 0.1 mg/ml and then adding 0.25 ml of the standard to a cuvette (BDH) containing 1.25 ml of Bradford reagent. After 2 min

incubation at room temperature, the absorbance was read at a wavelength of 595 nm. For determination of the protein content of test samples, 0.25 ml of sample was added to 1.25 ml of Bradford reagent and the absorbance then read at a wavelength of 595 nm. The concentration of protein present in the sample was then determined by using the standard curve (see figure 2.3).

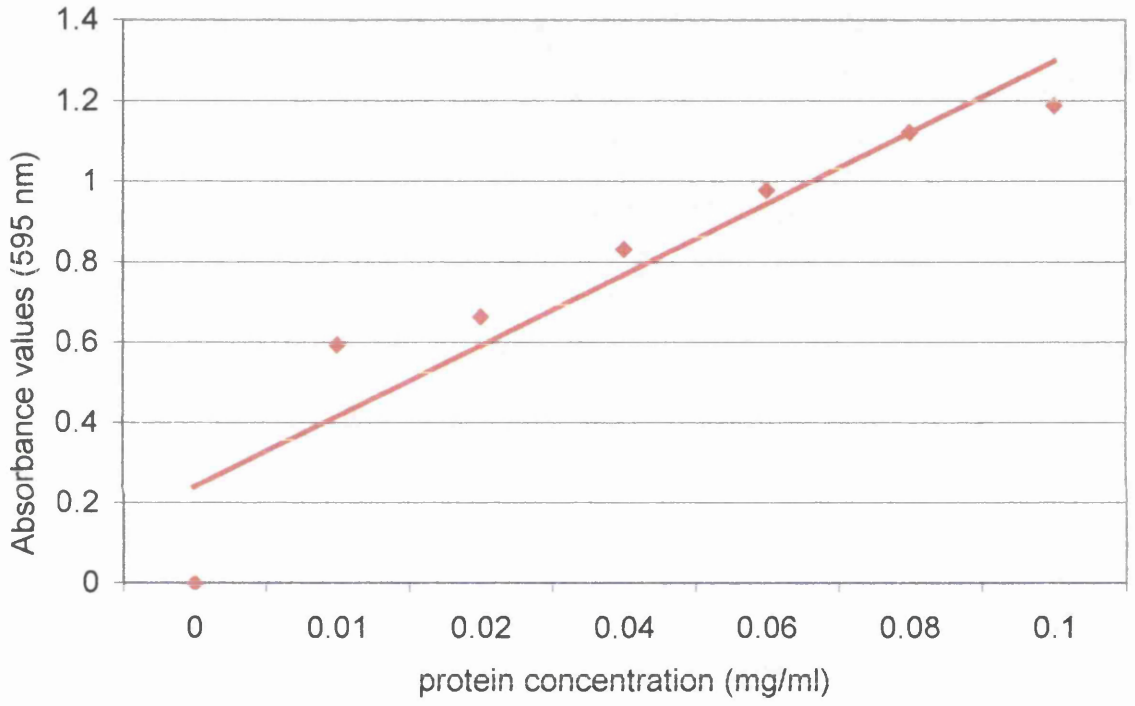


Figure 2.3 Standard curve for protein determination. This represents a typical standard graph for the determination of protein content of samples. A standard was determined for each batch of samples.

2.7 ATP determination

Principles of ATP determination:

$$\text{ATP + Luciferin} \xrightarrow[Mg^{++}]{\text{luciferase}} \text{Adenyl-luciferin +Ppi}^{*} \qquad \text{(A)}$$

$$\text{Adenyl-luciferin + } O_2 \longrightarrow \text{Oxyluciferin +AMP +} CO_2 \text{ + light} \qquad \text{(B)}$$

*Ppi = inorganic phosphates.

Reaction (A) is reversible and the equilibrium lies to the right. Reaction (B) is essentially irreversible. When ATP is the limiting reagent, the light emitted is proportional to the ATP present.

ATP determination was carried out using Bioprobes kit. The cells are placed in a sterile metal container and solution 1 is added which lyses the cells. Solution 2 is then added which contains luciferin and luciferase. A probe is then placed over the metal container so that the latter is completely covered. The probe then measures the light intensity, which is equivalent to the amount of ATP present.

CHAPTER THREE

INFLUENCE OF DOSIMETRIC AND PHYSIOLOGICAL CONDITIONS ON THE LETHAL PHOTOSENSITISATION OF *POR. GINGIVALIS*

3.1 INTRODUCTION

It has been demonstrated by a number of workers (Macmillan *et al*, 1966, Wilson et al, 1992, 1993a, 1993b; Wilson and Mia, 1994; Wilson and Pratten, 1994; Malik et al, 1990, 1991, 1992, 1993, 1996) that bacteria can be killed when irradiated with light in the presence of a suitable photosensitiser. It has been shown that Gram-positive bacteria are more prone to lethal photosensitisation compared to Gram-negative bacteria when negatively charged photosensitisers are used (Valduga *et al*, 1993; Dahl 1988, 1989; Bertoloni *et al* 1990). The first aim of this study was to determine whether *Por. gingivalis* was susceptible to killing using the negatively charged photosensitiser, aluminum disulphonated phthalocyanine ($AlPcS_2$) when used in combination with light from a gallium arsenide (GaAs) and compare it to the kills obtained using TBO in combination with HeNe laser. TBO would then be used to carry out detailed studies to determine whether pure cultures of *Por. gingivalis* could be killed under various dosimetric and physiological conditions which may be encountered in the periodontal pocket.

As the oral cavity harbours various strains of the same bacterial species (Loos *et al*, 1992; Andersen *et al*, 1998), it is possible that the susceptibility of each strain to lethal photosensitisation may vary. The second aim of this study was to determine the susceptibility of different strains of *Por. gingivalis* to lethal photosensitisation using the optimum conditions with the favoured photosensitiser.

Human gingival crevicular fluid contains tissue and serum proteins, free amino acids, immunoglobulins and complement which continually bathe the subgingival bacteria (Loesche, 1994). Antibodies and complement in the crevicular fluid bind to bacteria leading to cytolysis and phagocytosis and hence reductions in the

bacterial load (Newman and Nisengard, 1994). There are a number of reasons why the defence mechanism is not successful in eradicating bacteria from the periodontal pocket such as reductions in neutrophil function (Nisengard and Newman, 1994). Bacteria themselves have mechanisms to disable the host response in the periodontal pocket. Proteases are not only involved in bacterial adherence and colonisation of the oral cavity but are also involved in the degradation of antibodies and cytokines (Fletcher *et al*, 1998). If the protease activity of the bacteria could be reduced or completely extinguished, this would be of great significance *in vivo*. The third aim of the study therefore was to determine whether lethal photosensitisation caused a reduction in the protease activity of *Por. gingivalis.*

Plaque (biofilm) accumulation almost guarantees the development of gingivitis and periodontal disease (Newman and Nisengard, 1994). It has been shown that biofilm-grown bacteria are less susceptible to killing by antimicrobials than planktonic bacteria (Wilson, 1996, Sissons, 1997), therefore, the susceptibility of biofilm-grown bacteria to lethal photosensitisation using TBO in combination with HeNe laser light was determined.

3.2 MATERIALS AND METHODS

3.2.1 Laser and Photosensitiser.

Refer to general materials and methods for details (section 2.2) of HeNe and GaAs laser and photosensitisers and details of experiments (whole of section 2.3).

3.2.2 Determination of protease activity of *Por. gingivalis* during lethal photosensitisation

An overnight culture of *Por. gingivalis* cells was harvested and resuspended in saline. Suspensions were aliquoted (10 ml) and either sensitised with 82 μM TBO, but not exposed to laser light (L^-S^+), sensitised and exposed to laser light (L^+S^+) or neither treated with TBO nor exposed to laser light (L^-S^-). All samples were stirred continually throughout the experiment. Samples (500 μl) were removed after 2, 15, 30, 45 and 60 min corresponding to light doses of 0.88, 6.6, 13.0 19.7 and 26.3 J in order to determine the protease activity. Viable counts were made at each time point following the method described in chapter 2, figure 2.1.

3.2.3 Measurement of protease activity

Cell suspensions (500 μl) were added to 250 μl of 0.6 % (w/v) azocasein (Sigma Ltd) made up in 0.5 M Tris-HCl (pH 7.3) and incubated for 3-4 h. Acetic acid (750 μl of 20 % w/v) was then added to the suspension and centrifuged for 10 min at 4 000 x g, the supernatant was removed and the absorbance was measured at 420 nm. One unit of protease activity is defined as that which caused a change in absorbance of 0.001/h at 420 nm. The results for protease activity were expressed as units of activity.

3.2.4 Lethal photosensitisation of biofilm-grown *Por. gingivalis*

Biofilms of *Por. gingivalis* were prepared by cutting circles from a WCN nitrocellulose membrane (Whatman) using a hole punch to give a diameter of 5.5 mm. The membranes were autoclaved at 121°C for 15 min in a minimum amount of water to reduce curling of the membrane which occurs when autoclaved 'dry'.

After cooling, the membranes were placed onto agar plates and allowed to dry. Each membrane was inoculated with 5 μl of stationary phase *Por. gingivalis* and then incubated at 37°C overnight in an anaerobic cabinet. Biofilms with similar bacterial growth (i.e. the diameter and thickness of the biofilm was similar on each membrane determined by eye) were chosen for the experiments. Duplicate biofilms were sensitised with 82 μM of TBO and after a PIT of 60 s, exposed to 39, 53 and 79 J of light. Biofilms neither exposed to laser light nor sensitised with TBO (L^-S^-) were prepared to determine the number of viable bacteria present prior to any treatment. Biofilms were then placed in 1 ml of WC broth and vortexed thoroughly for 1 min. Serial dilutions were made and plated out on WC agar. The plates were incubated for 4-6 days in an anaerobic chamber and colonies were enumerated.

All statistical analyses were carried out using analysis of variance, single factor (ANOVA). Log reductions were calculated in comparison to L^-S^- samples unless otherwise stated. A p value of <0.05 was considered to be statistically significant.

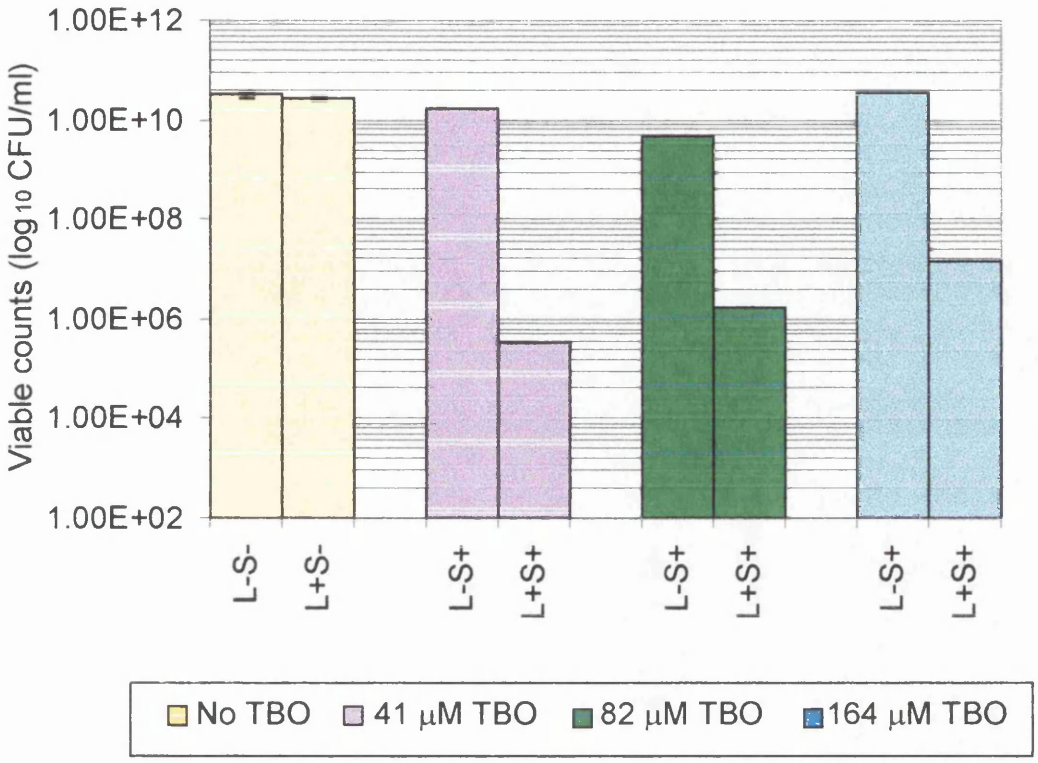


Figure 3.1 Viable counts of *Por. gingivalis* sensitised with increasing concentrations of TBO and exposed to HeNe laser light. The concentrations of TBO used were 41, 82 and 164 μM (equivalent to 12.5μg/ml, 25 μg/ml and 50 μg/ml, respectively). The laser light dose used was 0.88 J and the pre-irradiation time (PIT) used was 60 s.

Figure 3.1 shows the effect on viable counts of increasing TBO concentrations in combination with HeNe laser light. The figure shows that at a light dose of 0.88 J the reduction in viable counts at 12.5, 25 and 50 μg/ml were 5.1 $\log_{10}$, 4.1 $\log_{10}$ and 3.6 $\log_{10}$, respectively. The difference in viable counts between each L^+S^+ was statistically significant.

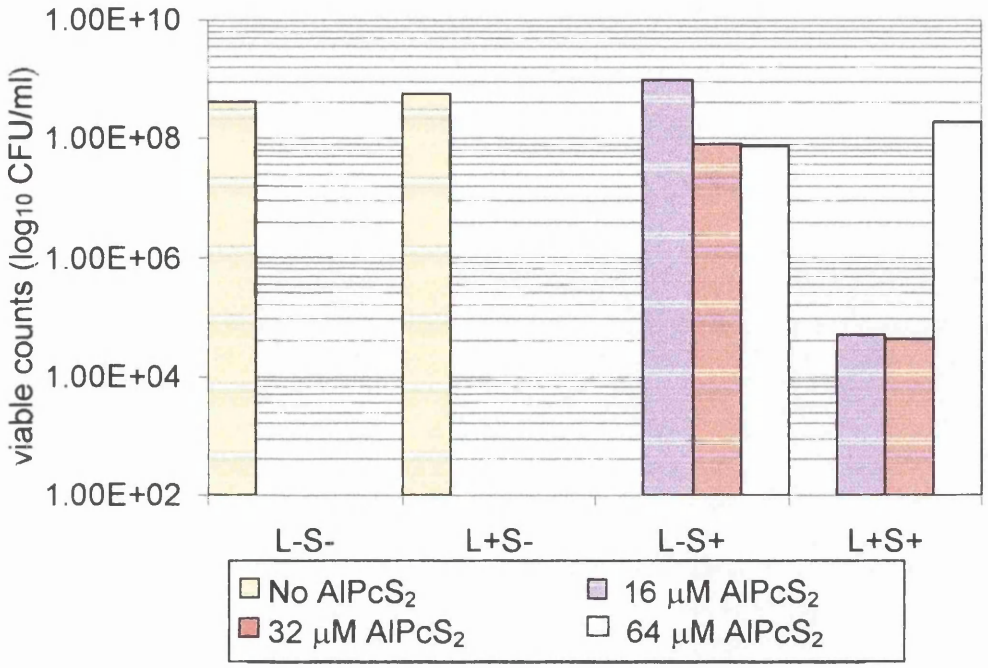


Figure 3.2 Viable counts of *Por. gingivalis* sensitised with increasing concentrations of $AlPcS_2$ and exposed to GaAs laser light. Standard deviations were too low to show error bars. The concentrations of $AlPcS_2$ used were 16, 32 and 64 μM (equivalent to 12.5, 25 and 50 μg/ml, respectively), and the light dose was 1.3 J. The PIT was 60 s.

Figure 3.2 shows the effect on viable counts of increasing $AlPcS_2$ concentrations in combination with GaAs laser light. The results show that at a light dose of 1.3 J, as the concentration of $AlPcS_2$ was increased from 16 to 64 μM, the reduction in viable counts was 4.2 $\log_{10}$, 4.0 $\log_{10}$ and 0.2 $\log_{10}$, respectively. The difference in viable counts between each L^+S^+ was statistically significant.

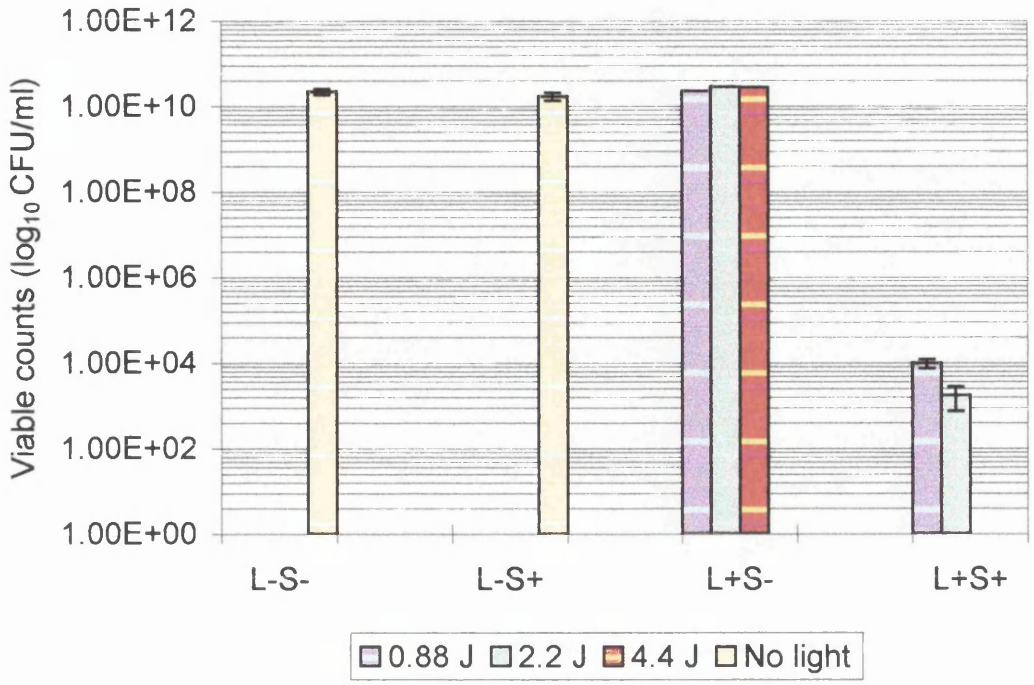


Figure 3.3 Viable counts of *Por. gingivalis* cells sensitised with TBO and exposed to increasing HeNe light doses. Cells were sensitised with TBO at a concentration of 82 μM and exposed to laser light doses of 0.88, 2.2 and 4.4 J. The PIT was 60 s.

The effect of increasing the HeNe laser light dose from 0.88 J to 4.4 J is shown in figure 3.3. There was a 6 log_{10} reduction in viable counts with 0.88 J, 6.5 log_{10} at 2.2 J and 100 % kill was obtained with 4.4 J. The difference in viable counts between each L^+S^+ was statistically significant.

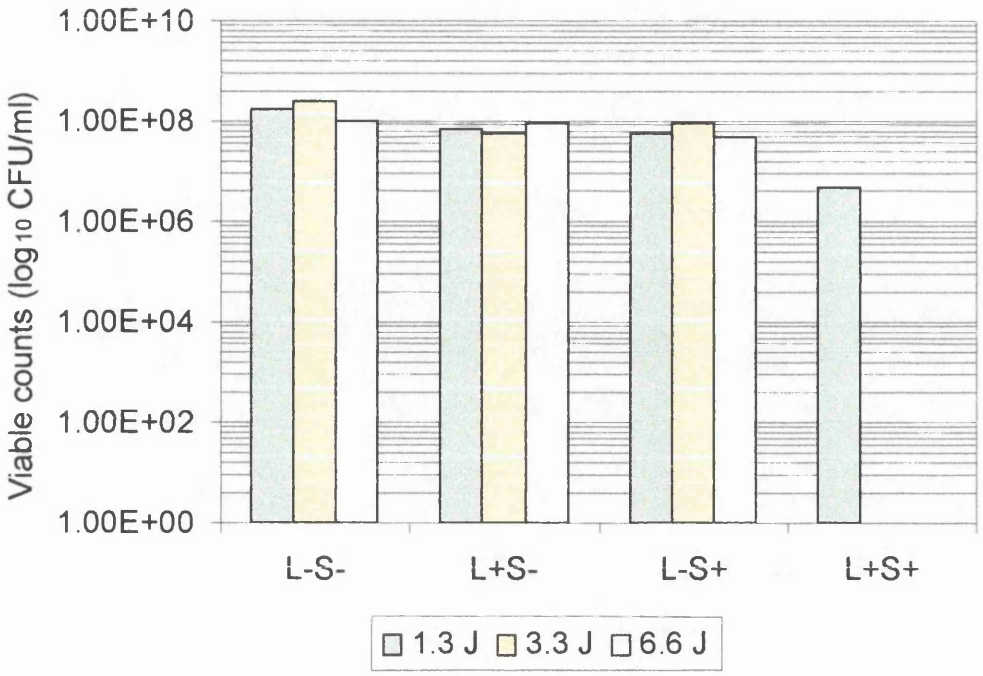


Figure 3.4 Viable counts of *Por. gingivalis* cells sensitised with $AlPcS_2$ and exposed to increasing light doses from GaAs laser. Standard deviations were too low to show error bars. Cells were sensitised with 32 μM of $AlPcS_2$ and the energy doses used were 1.3 J, 3.3 J and 6.6 J. The PIT was 60 s.

Figure 3.4 shows the effect of increasing the GaAs laser light dose on viable counts of *Por. gingivalis* using 32 μM of $AlPcS_2$. The figure shows that as the light dose was increased from 1.3 J to 6.6 J, there was an increase in the numbers of bacteria killed. There was a 1.8 log_{10} reduction in viable counts at 1.3 J and 8 log_{10} reduction (100 % kill) at 3.3 and 6.6 J. The difference in viable counts between each L^+S^+ (that is between 1.3 J and the other two light doses) was statistically significant.

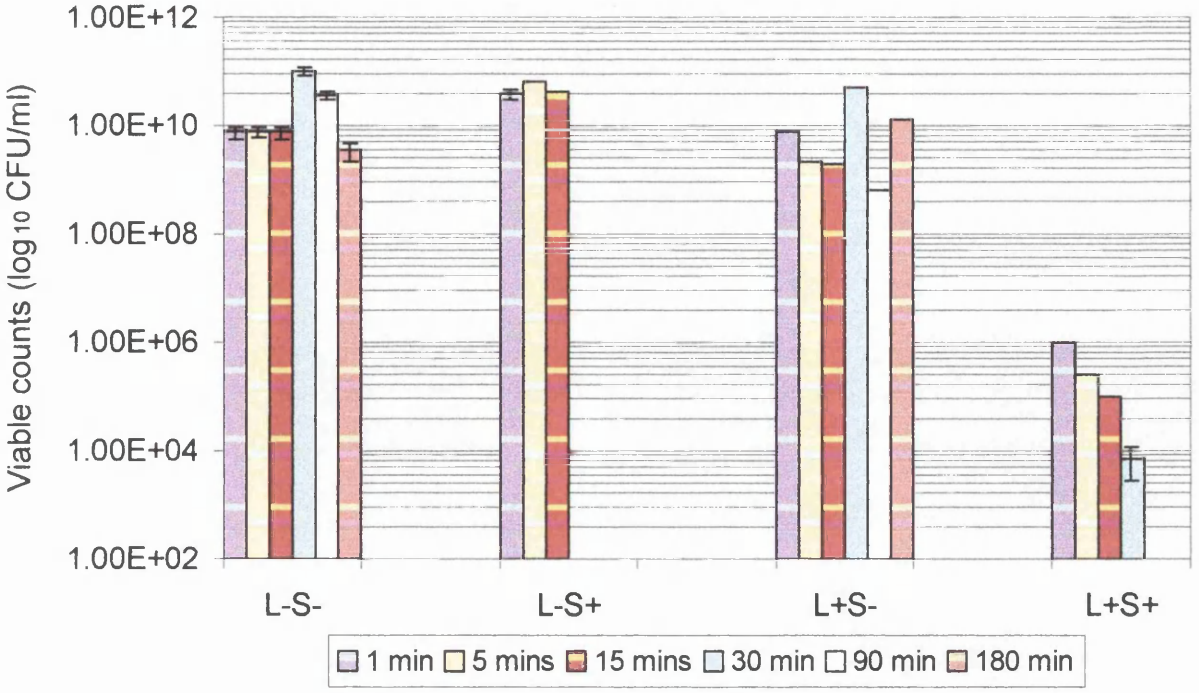


Figure 3.5 Viable counts of *Por. gingivalis* cells incubated in TBO for increasing lengths of time prior to HeNe laser light exposure. Standard deviations were generally too low to show error bars. Cells were incubated in 82 μM TBO for 1, 5, 15, 30, 90 and 180 min before exposure to 0.88 J of light.

Figure 3.5 shows the effect on the kills obtained of varying the time the bacterial suspension was incubated with 82 μM TBO prior to exposure to laser light. As the PIT increased, the proportion of bacteria killed increased. There was a 3.8 $\log_{10}$ reduction at 1 min, 4.5 $\log_{10}$ at 5 min, 5 $\log_{10}$ at 15 min, 7 $\log_{10}$ at 30 min, 8.5 $\log_{10}$ at 90 min and 8.0 $\log_{10}$ reduction at 180 min). The graph also shows that incubating *Por. gingivalis* in TBO alone for 90 and 180 min gave an 8.0 $\log_{10}$ reduction.

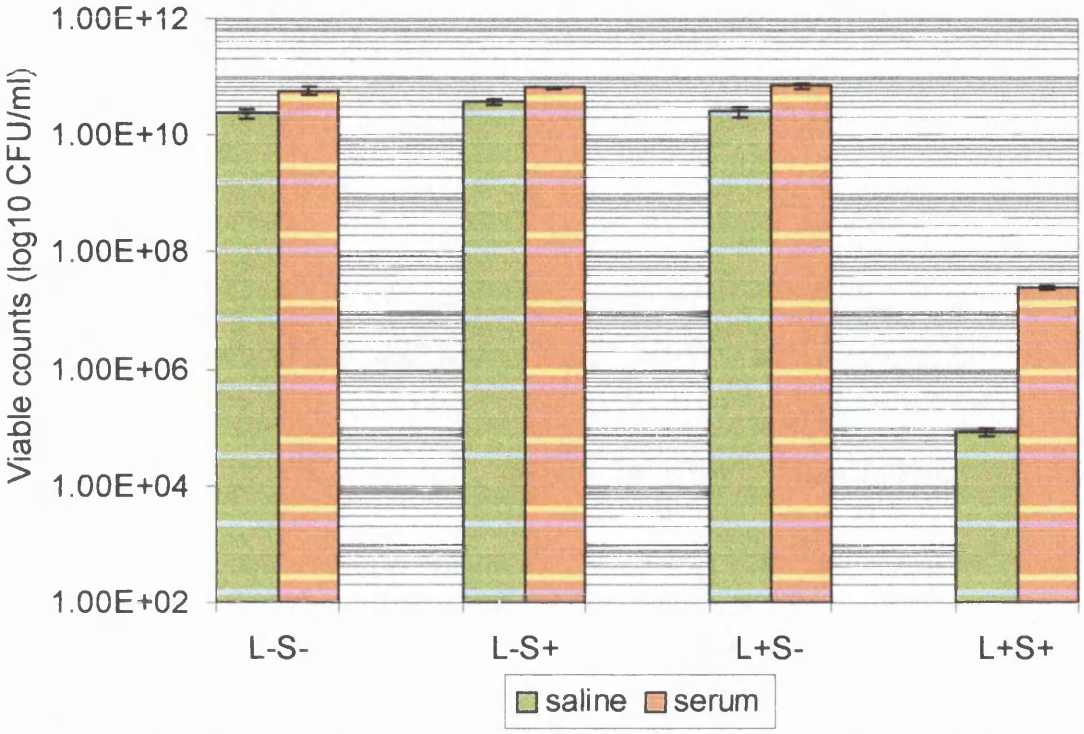


Figure 3.6 Viable counts of *Por. gingivalis* cells sensitised with TBO and exposed to HeNe laser light in the presence of saline and serum. *Por. gingivalis* cells were resuspended in horse serum. Cells were sensitised using 82μM TBO resuspended in horse serum. The PIT was 60 s and the light dose was 0.88 J.

The effect on lethal photosensitisation of suspending *Por. gingivalis* in horse serum is shown in Fig. 3.6. When compared to the controls (L^-S^-), there was a statistically significant reduction in viable counts of *Por. gingivalis* in serum (3 $\log_{10}$) and saline (5.5 $\log_{10}$ reduction).

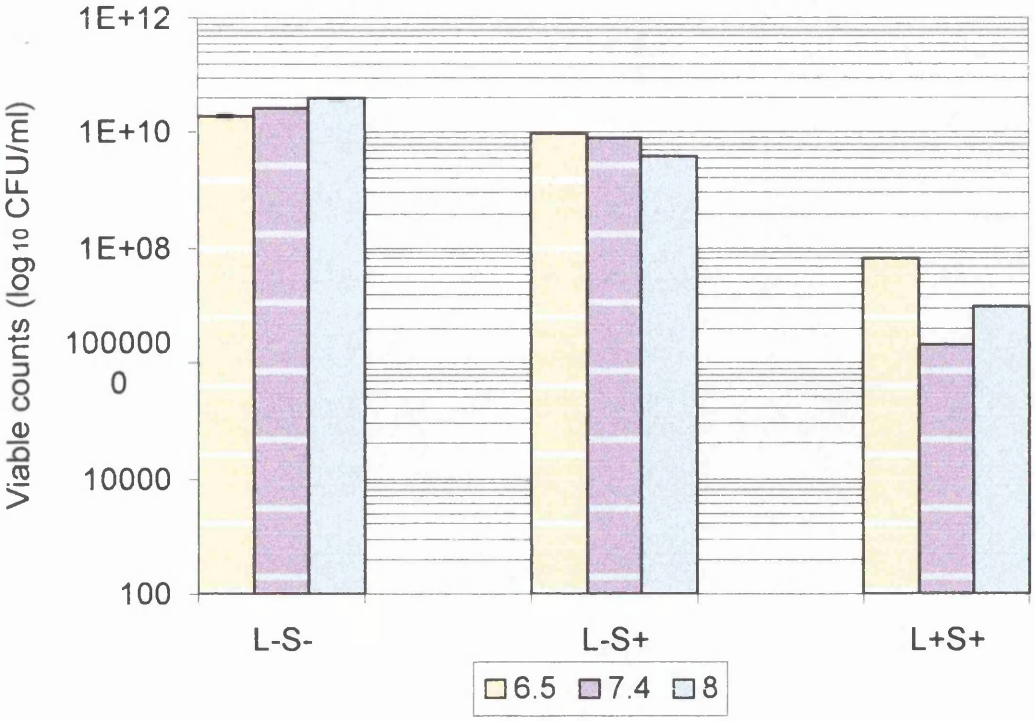


Figure 3.7 Viable counts of *Por. gingivalis* cells sensitised with TBO and exposed to HeNe laser light at various pH values. Standard deviations were generally too low to show error bars. Cells were resuspended in phosphate buffer at a pH of 6.8, 7.3 or 8.0. TBO (at a final concentration of 82μM) was dissolved in the appropriate buffers. The PIT was 60 s and the light dose used was 0.88 J.

The effect of pH on lethal photosensitisation is shown in Figure 3.7. There was a significant reduction in the viable counts at all three pH values. There was a 2.5 $\log_{10}$ reduction in the numbers of viable bacteria at a pH of 6.5, a 4.0 $\log_{10}$ reduction at a pH of 7.4 and a 3.5 $\log_{10}$ reduction at a pH of 8.0. There was no effect on the viable counts following exposure to laser light alone or TBO alone. The peak absorbance of TBO was similar (633 nm) at all three pH values (figure 3.8).

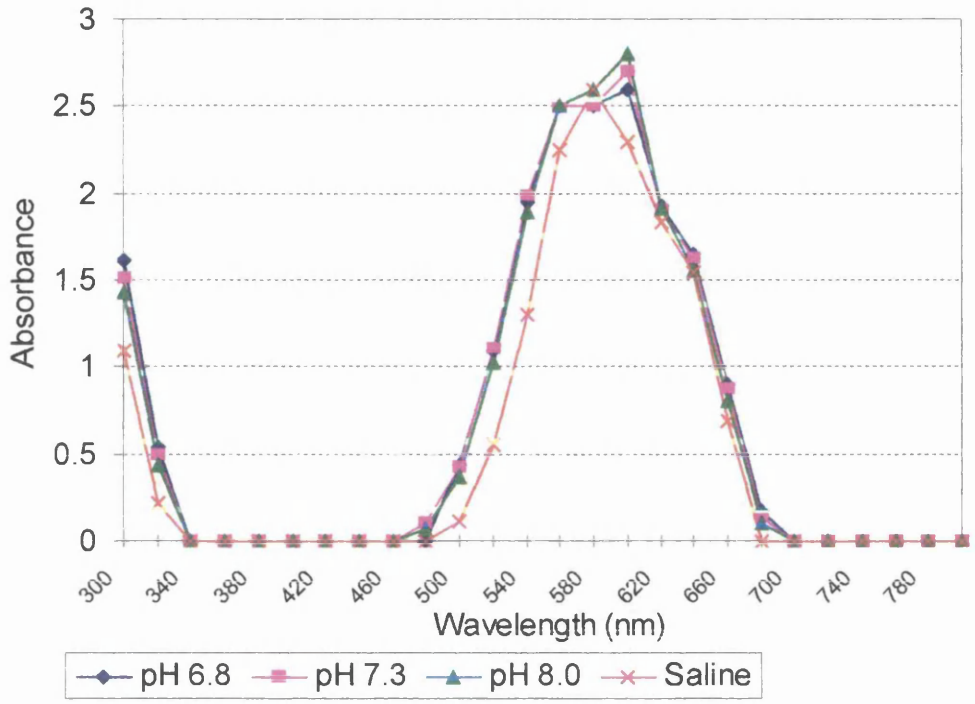


Figure 3.8 Absorbance of TBO at various pH values. TBO was resuspended in phosphate buffer at pH 6.8, 7.3 and 8.0 and the absorbance of the solution was read at increasing wavelengths.

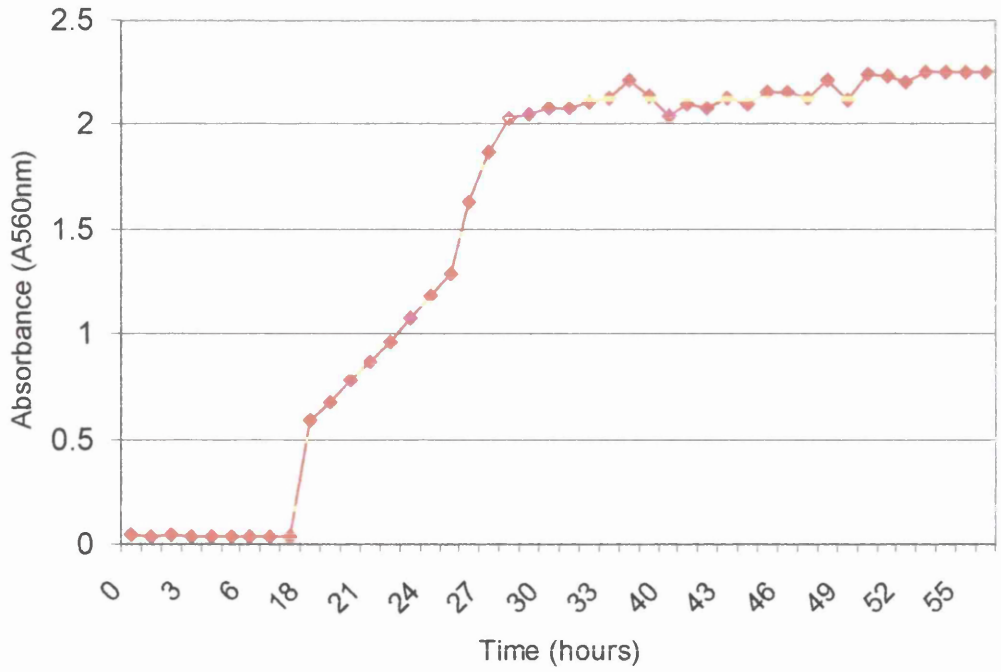


Figure 3.9 *Por. gingivalis* growth curve.

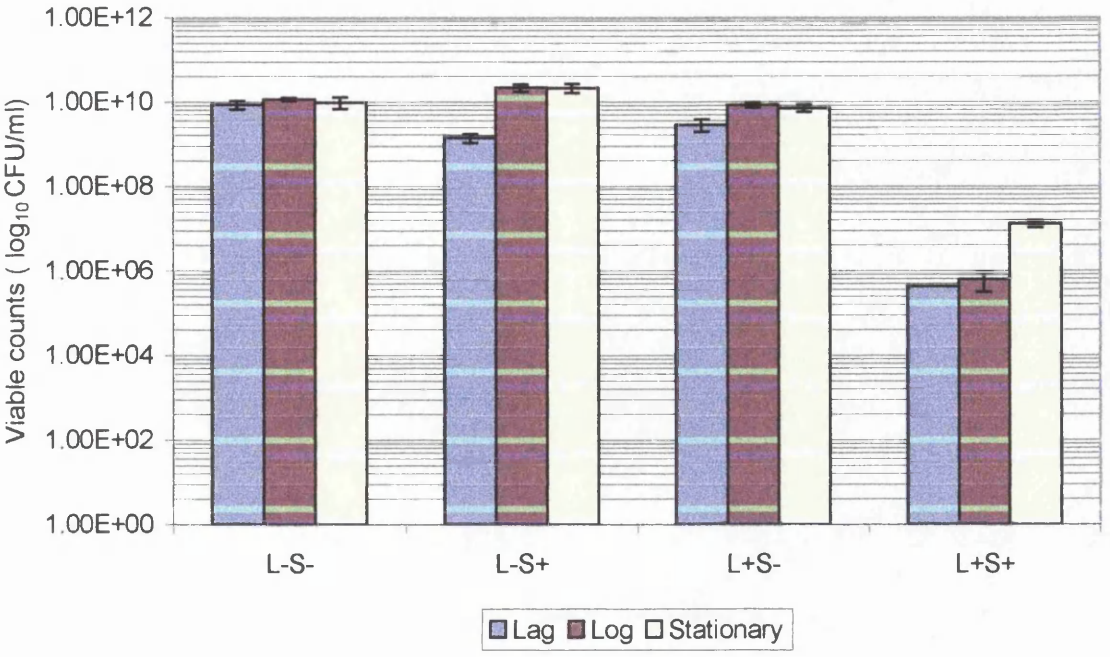


Figure 3.10 Viable counts of *Por. gingivalis* lag, log and stationary phase cells sensitised TBO and exposed to laser light. Cells from each growth phase were harvested and resuspended in saline to give the same cell density ($O.D._{560}$ = 1.0). A PIT of 60 s was used and a final TBO concentration of 82 μM was used. The light dose was 0.88 J.

The growth curve of *Por. gingivalis* is shown in fig. 3.9. The susceptibility of *Por. gingivalis* to lethal photosensitisation when in different growth phases is shown in Figure 3.10. The figure shows that substantial kills were achieved for each growth phase. There was a significant difference between L^-S^- and L^+S^+ lag, log and stationary phase cells. ANOVA showed that lag and log phase cells were equally susceptible to lethal photosensitisation while stationary phase cells were significantly less susceptible compared to lag and log phase cells.

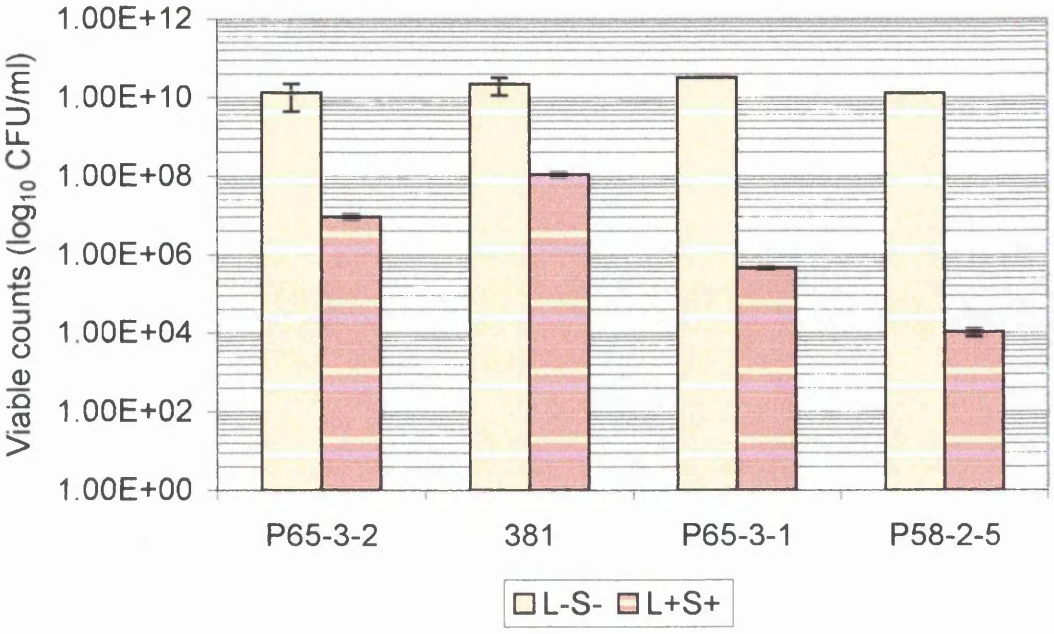


Figure 3.11 Viable counts of different strains of *Por. gingivalis* cells sensitised with TBO and exposed laser light. TBO was used at a concentration of 82 μM and the light dose was 0.88 J. PIT was 60 s.

Figure 3.11 shows the effect of lethal photosensitisation on four different strains of *Por. gingivalis*. The figure shows that the order of susceptibility to lethal photosensitisation, from least to most susceptible was: 381 (2.2 log_{10} reduction), P65-3-2 (2.5 log_{10} reduction), P65-3-1 (4.4 log_{10} reduction) and P58-2-5 (6 log_{10} reduction). Statistical analysis using ANOVA showed that reductions in viability between each of the L^+S^+ were significantly different.

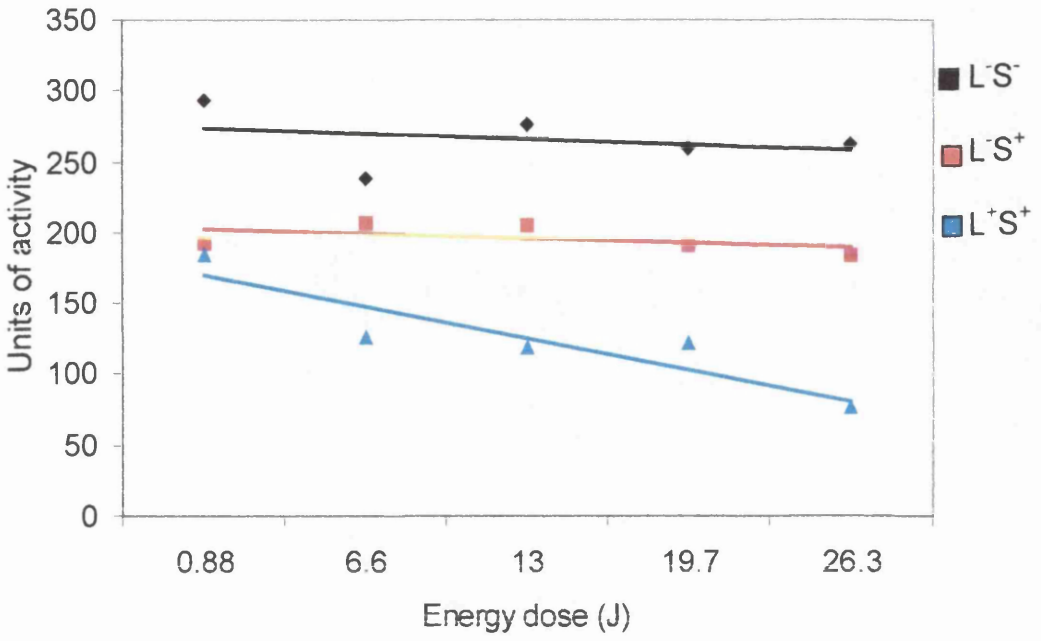


Figure 3.12 Protease activity of *Por. gingivalis* cells exposed to increasing laser light doses. Protease activity of control cells (L^-S^-), cells sensitised with 82μM TBO (L^-S^+) and cells sensitised with 82μM TBO and exposed to laser increasing light doses (L^+S^+). Standard deviations were too low to show error bars (n=3).

Figure 3.12 shows the protease activity of control cells (L^-S^-), TBO sensitised cells (L^-S^+) and cells sensitised with TBO and then exposed to increasing light doses (L^+S^+). The graph shows that compared to L^-S^- cells, there was a statistically significant decrease in protease activity in cells treated with TBO alone (L^-S^+). There was a further statistically significant light dose dependent decrease in the protease activity (184 to 76 units) when sensitised cells were exposed to laser light (L^+S^+). There was a 4.2 $\log_{10}$ reduction in viable counts when sensitised cells were exposed to 0.88 J of light, and there was a 100 % reduction in viable counts thereafter.

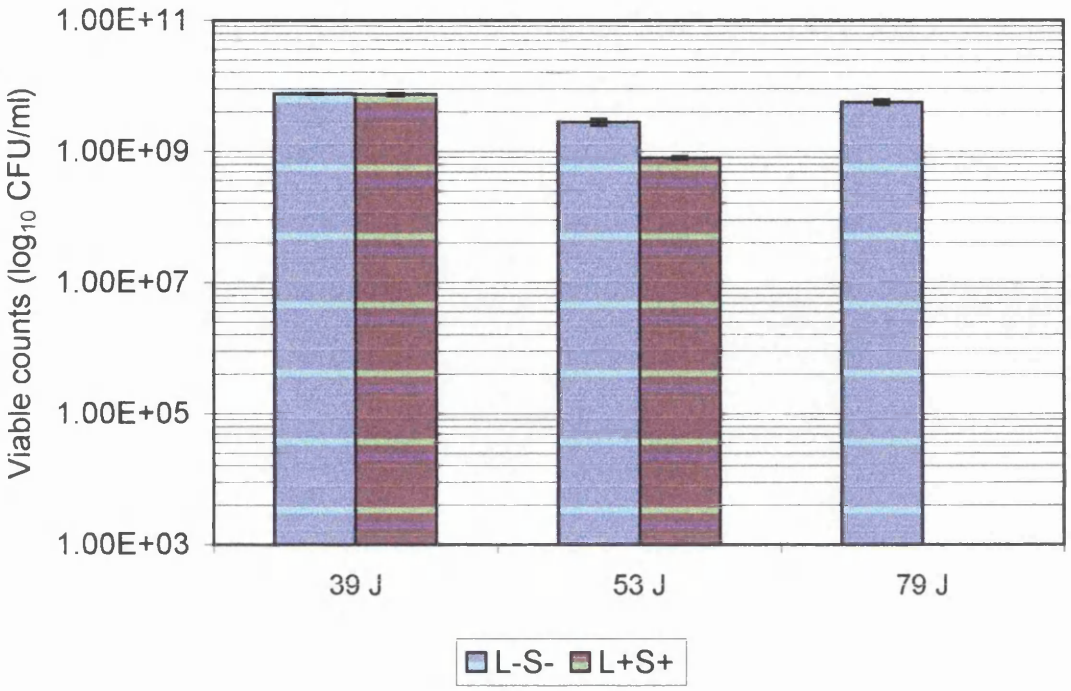


Figure 3.13 Viable counts of biofilm-grown bacteria sensitised with TBO and exposed to increasing laser light doses. Bacterial biofilms were sensitised with 82 μM of TBO and after a PIT of 60 s, exposed to 39, 53 and 79 J of light.

Figure 3.13 shows the viable counts of biofilm-grown bacteria. There was no decrease in viable counts at an energy dose of 39 J, but there was a statistically significant decrease of 0.25 log_{10} at an energy dose of 53 J and a 100 % decrease at an energy dose of 79 J.

3.4 DISCUSSION

It has been well documented that negatively-charged photosensitisers are less efficient at killing Gram-negative bacteria than positively-charged photosensitisers (Macmillan *et al*, 1966; Wilson and Dobson, 1993; Malik *et al*, 1992 and 1993; Millson *et al*, 1996a, 1996b; Minnock *et al*, 1996). The results of this study have

shown that *Por. gingivalis* is sensitive to killing by both TBO and $AlPcS_2$ using laser light at the appropriate wavelength.

In vivo, bacteria in the periodontal pocket would have to be sensitised with TBO before exposure to laser light and, for clinical convenience, this PIT should be as short as possible. It was found that TBO effectively sensitises the bacteria to killing after only 60 s. It was also found that when *Por. gingivalis* cells were incubated in TBO alone for 90 min, the viable counts were reduced by 100 % (figure 3.5). This suggests that TBO itself is toxic to *Por. gingivalis* but only when incubated for 90 min. The toxicity of TBO alone towards *Por. gingivalis* cells may be due to the high redox potential of the photosensitiser (-11 mV, Sprotts *et al.*, 1994). *Por. gingivalis* is expected to survive at a redox potential close to that of the periodontal pocket in disease (-300 mV, Marsh and Martin, 1992) therefore, it is not entirely surprising that a reduction in viable counts was observed when the organism was incubated in TBO. It was also found that in the presence of laser light, as the concentration of TBO was increased from 82 μM to 164 μM, there was a decrease in the numbers of bacteria killed. This may be explained by a decreased penetration of laser light through the suspension due to increasing TBO concentration. A reduced amount of light reaching the sensitised bacteria would reduce the overall efficiency of the system as cytotoxic species would be generated in the supernatant. These cytotoxic species have a short half-life (singlet oxygen has a half-life of ≤ 0.04 μs in water Millson *et al.*, 1996a]), and unless generated close to the bacterial cell, would be unlikely to exert a deleterious effect. Furthermore, TBO at a high concentration (i.e. 164 μM) has a tendency to form aggregates and it has been demonstrated (with $AlPcS_2$ as well as TBO) that when aggregated, photosensitisers have less photobiological efficacy (Phillips, 1997). This may be

because the aggregated photosensitiser quenches cytotoxic species formed before they can have any deleterious effects on the bacterial cells. This may also explain why $AlPcS_2$ at a concentration of 64 μM, showed a greatly reduced deleterious affect on *Por. gingivalis* cells on exposure to GaAs laser light compared to lower concentrations.

The reductions in viable counts in the presence of serum were not as significant when in the presence of saline. A similar effect was observed by Nitzan *et al.* (1983) in lethal photosensitisation studies in the presence of nutrient broth, using *Staphylococcus aureus* and deuteroporphyrin as a photosensitiser. Wilson and Pratten (1995) demonstrated that in the presence of saline, using 12.5 μg/ml $AlPcS_2$ (16 μM) there was a 3 $\log_{10}$ reduction in viable counts of *S. aureus* compared to a 1.5 $\log_{10}$ reduction in the presence of horse serum. Moan *et al.* (1979) found that in the presence of 10 % human serum, the uptake of HP by human cells (NHIK 3025) was reduced by 75 % compared to when in the presence of Hank's solution. Moan *et al* (1979) and Nitzan *et al* (1989) suggested that the difference in uptake might be due to the serum proteins binding the photosensitiser before the latter can bind to the cells. TBO is known to bind to proteins (especially acidic proteins, Brumberg and Pevzner, 1978), and there may be a preference for TBO to bind to serum proteins, resulting in less photosensitiser being available for uptake by *P. gingivalis*, assuming uptake of the photosensitiser by the bacteria is a prerequisite for light-induced killing. It has also been demonstrated that a number of sensitisers, including $AlPcS_2$, TBO (Phillips, 1997) and mTHPBC (5,10,15,20-tetrakis(m-hydroxyphenyl)bacteriochlorin [Grahn *et al*, 1997]) have a tendency to aggregate in the presence of proteins, and aggregation, as mentioned above, reduces the efficacy of the photosensitiser.

This study has also demonstrated that the physiological state of *Por. gingivalis* influences lethal photosensitisation, as cells in the stationary phase were less susceptible to killing than when in the lag or log phase. A review by Kolter (1993) explained the properties of stationary phase cells, which may offer a few explanations as to why these cells are less susceptible to lethal photosensitisation than lag and log phase cells. Firstly, the transition between log and stationary phase involves a change in the relative volume and disposition of the subcellular components, the cytoplasm is condensed and the volume of the periplasm increases. These changes alone may mean that cellular targets of lethal photosensitisation in log phase cells are no longer targets in stationary phase cells. Changes also occur on the surface of the cell, where there is an increase in the expression of hydrophobic molecules. This increases co-adhesion and co-aggregation and makes the cell less permeable, therefore uptake of TBO into the cell and binding of the photosensitiser to the cell surface may be considerably reduced leading to a reduction in kills. The membrane is further made impermeable by a change in the fatty acid composition, which causes a decrease in the membrane fluidity. Another alteration, which may be an important factor, is that stationary phase cells are less prone to oxidative stress, which may make them more "resistant" to cytotoxic species.

Another explanation as to why the stationary phase cells are less prone to lethal photosensitisation compared to cells in the lag and log phase, may be that log phase cells have less surface-associated material (SAM) than cells in the other phases. This may be because the energy of the log phase culture is being channelled into dividing and less into producing proteins and other molecules for incorporation into the cell surface, which is seen more abundantly at early

stationary phase. This may result in a greater uptake of the photosensitiser (if uptake occurs) by the log phase cells giving rise to an increase in kill compared to stationary phase cells. The relative susceptibilities of cells in different phases of growth appear to be species-dependent and also dependent on the photosensitiser used. For example, studies by Venezio *et al* (1985) demonstrated that *S. aureus* sensitised by haematoporphyrin derivative at log and stationary phase was more susceptible to killing than lag phase cells. However Nitzan *et al.* (1983) reported that log phase cells were more susceptible than lag phase cells using haematoporphyrin derivative and white light. While Wilson and Pratten (1994) reported that all phases of *S. aureus* were equally susceptible to lethal photosensitisation using $AlPcS_2$ and light with a wavelength of 660nm.

Another physiological factor that was considered in this chapter was pH. The pH of the periodontal pocket has been reported to be in the region of 7.0-9.0. However, it has been reported most frequently to be around 7.4 (Eggert *et al*, 1991). The results of these experiments have shown that the greatest kills are achieved at a pH of 7.4, and that high kills are also possible at a pH of 6.8 and the more alkaline pH of 8.0. Therefore, lethal photosensitisation would be possible at the pH values most likely to be found in the periodontal pocket. Pottier *et al* (1975) demonstrated, using TBO as a photosensitiser, that as the pH values increase, there is an increase in kills due to an increase in efficiency of singlet oxygen production. However, the results in this chapter show that this is not the case as the kills were less at pH 8.0 compared to pH 7.4. It may be possible that, although there is an increase in the production of singlet oxygen, the uptake of the photosensitiser by the cells has to be taken into account. Work carried out by Wakayama *et al* (1980) using TBO in combination with visible light showed that

the maximum kills of *E. coli* were obtained between pH 7.6-8.0 (the range used was 5.5-8.0). They found that the quantity of dye bound to the bacterial cells was higher at the basic pH values. In the case of *Por. gingivalis*, the uptake and binding of TBO (if it occurs) may be optimal at pH 7.4.

The results of this series of experiments show that *Por. gingivalis* W50 can be killed effectively by using a low energy dose (0.88 J) and low TBO concentration (82 μM). As many strains of *Por. gingivalis* may be encountered in the periodontal pocket, it was essential to determine whether lethal photosensitisation would be as effective on other strains as it was on W50. The results show that all the strains were susceptible to lethal photosensitisation, but strains P65-3-2 and 381 were slightly less susceptible than P65-3-1 and P58-2-5. Furthermore, strains P58-2-5 and W50 were equally susceptible to lethal photosensitisation. The variation in susceptibility could be because of a difference in binding and/or uptake of the photosensitiser (assuming uptake does occur) owing to different cell membrane compositions. In fact, Williams and Holt (1984) showed a difference in membrane protein composition between ten strains of *P. gingivalis* which may contribute to making some strains more prone to lethal photosensitisation than others.

One group of proteins commonly associated with *Por. gingivalis* virulence is the proteases (Shah, 1993). These enzymes are involved in the bacterial colonisation of the host oral cavity (Winkelhoff *et al*, 1993), adherence (Grenier and Mayrand, 1993), and degradation of host antibodies and cytokines (Fletcher *et al.* 1998). The results show that there is a significant decrease in the protease activity as the light energy dose was increased. There was however a decrease in the protease activity with TBO alone. There are two

possible reasons why this may occur. Firstly, the high redox potential of TBO may contribute to a reduction in protease activity, as the optimum working environment of *Por. gingivalis* enzymes is an anaerobic one. Alternatively, TBO may bind to the proteases in such a way as to hinder the enzymatic activity to some degree. This decrease in protease activity in the presence of TBO alone was not however as significant as when sensitised cells were exposed to laser light. This may be because cytotoxic species produced during lethal photosensitisation are required to cause irreversible chemical changes leading to inactivity of enzymes (Tuite and Kelly, 1993). There was a continuing decrease in the activity after 100 % bacterial kill was obtained (after 0.88 J). The fact that the protease activity is present after 100 % reduction in viable count is significant *in vivo,* because even if antimicrobial agents (in current use) manage to kill the pathogenic bacteria in the periodontal pocket, the protease activity can continue to cause adverse effects.

The study has shown that the physiological state of *Por. gingivalis* can influence its susceptibility to lethal photosensitisation. It has been shown that biofilm-grown bacteria are genotypically (Anwar *et al.*, 1991) and phenotypically (Beveridge *et al.*, 1997) different to cells in their planktonic form which may lead biofilm-grown bacteria to be significantly less prone to killing by antimicrobial agents. The data show that biofilm-grown *Por. gingivalis* were less susceptible to lethal photosensitisation compared to the planktonic form. It has been demonstrated by Beveridge *et al.* (1997) using *Pseudomonas aureginosa* that the LPS of the bacterium is involved in the initial attachment to a substrate and that during biofilm formation there are subtle changes to the LPS. LPS is known to play a protective role against cytotoxic species (Bertoloni *et al*, 1990; Malik *et*

al., 1982) and the changes to the LPS may lead to an enhancement of this protective activity when the bacteria are in biofilm form. Another reason may be that the uptake of TBO (if uptake occurs) by biofilm-grown cells may be significantly less than planktonic cells, leading to reduced kills on exposure to laser light. It has been shown by a number of workers that for cell death to occur, penetration of antimicrobials through a bacterial biofilm is an important rate-limiting factor (LeChevallier *et al.*, 1988). It is possible that the PIT used (60 s) was not sufficient to allow permeation of TBO through the whole depth of the biofilm which means that even though the laser light was able to penetrate through the biofilm, only those bacteria sensitised could be killed.

3.5 SUMMARY

1. Sensitising *Por. gingivalis* using $AlPcS_2$ and exposing to increased light doses gave increase in kills. A light dose of 3.3 J gave a 100 % kill. $AlPcS_2$ alone had no effect on the viable counts of the bacteria.
2. Lethal photosensitisation of *Por. gingivalis* using $AlPcS_2$ at concentrations of 16 and 32 μM gave almost 4 $\log_{10}$ reductions in viable counts and a 0.2 $\log_{10}$ reduction at 64 μM.
3. Lethal photosensitisation of *Por. gingivalis* using TBO showed that an increase in the light dose gave an increase in kills. A light dose of 4.4 J and a TBO concentration of 82 μM gave 100 % kills.
4. An increase in the concentration of TBO (41-164 μM) led to a decrease in kills. This may be due to reduced laser light penetration through a concentrated solution of TBO. Another reason may be that there was increased quenching of the cytotoxic species by the TBO in the supernatant.
5. An increase in the PIT gave increase in kills.

6. There was a decrease in the numbers of *Por. gingivalis* killed in the presence of serum compared to saline. This may be due to TBO binding to serum and cytotoxic species produced were quenched before reaching the bacterial cells.
7. Lethal photosensitisation in the presence of increasing pH values (6.8, 7.3 and 8.0) showed that the highest kills were obtained at pH 7.3.
8. All five strains of *Por. gingivalis* tested were susceptible to killing by lethal photosensitisation.
9. The protease activity of *Por. gingivalis* was reduced by TBO alone but to a greater extent by TBO plus laser light.
10. The energy dose required to obtain 100 % kill of biofilm-grown *Por. gingivalis* was 79 J compared to 6.6 J when cells were in the planktonic form.

CHAPTER FOUR

UPTAKE OF TBO BY *POR. GINGIVALIS* UNDER VARIOUS CONDITIONS AND THE CELLULAR DISTRIBUTION OF THE SENSITISER

4.1 INTRODUCTION

Bacterial cells have been shown to take up dyes such as phthalocyanines meso-tetra(hydroxyphenyl)porphine (THPP) and acriflavine. Moan *et al.* (1989a) demonstrated that the uptake is governed by a number of factors such as whether the sensitiser is lipophilic and whether it aggregates. Also, in the case of heterogeneous photosensitisers, the uptake depends on the components of the photosensitiser and whether there is an uptake mechanism for each component, which would also govern its distribution (Moan *et al.* 1989a). Ben-Hur (1989) stated that the uptake also depended on a number of other factors such as the amount of serum in the growth medium, temperature and the structure of the photosensitiser.

Cells have various mechanisms by which they can take up solutes and this, together with the factors mentioned above, suggests that the mechanism of uptake for TBO would be different to that of $AlPcS_2$, which would be different to that of other photosensitisers such as haematoporphyrin. Little is known about photosensitiser uptake by bacteria. However, in the case of other drugs, uptake is known to be dependent on size, charge and hydrophilicity of the molecule (Nikaido, 1993), and also on the number of pores present in the bacterial membrane (Hancock and Bell, 1988).

The differences in uptake and hence distribution of the photosensitiser within the cell would determine what part of the cell would be most prone to the effects of cytotoxic species on exposure to laser light. For example, methylene blue binds to DNA, therefore processes such as cell replication would be disrupted. If a photosensitiser such as congo red binds to the surface of the cell, then it is highly

likely that the structures around the surface such as lipopolysaccharides, outer membrane proteins, and lipids may be affected (Smalley *et al.* 1995).

This study was carried out in order to determine: 1) whether *Por. gingivalis* takes up ^{3}H-TBO. 2) whether the uptake is influenced by the concentration of the ^{3}H-TBO, the presence of serum, growth phase and pH, as it was shown in chapter 3 that lethal photosensitisation was affected by these conditions. 3) if the TBO taken up is responsible for the bactericidal effect, and finally 4) how the photosensitiser is distributed in the cell. The term 'uptake' refers to the ^{3}H-TBO present on the surface of the cell plus the ^{3}H-TBO present intracellularly. All statistical analyses were carried out using ANOVA. The terms 'significance' and 'significantly different' refer to the statistical significance calculated by ANOVA.

4.2 MATERIALS AND METHODS

4.2.1 UPTAKE STUDIES

4.2.1.1 Uptake of ^{3}H-TBO at various time intervals

Por. gingivalis was grown according to the method described in chapter 2. An overnight culture of *Por. gingivalis* was harvested and resuspended in saline to give an absorbance of 1.0 (A_{560}). The bacteria were then incubated in the dark in the presence of ^{3}H-TBO ([^{3}H] Toluidine Blue O, Specific activity 278 Gbq/mmol, 7.5 Ci/mmol, Amersham Life Sciences) for 1-30 min at a final concentration of 82 μM. The suspensions were centrifuged (5 000 x g for 10 min) and the pellet was washed a number of times to remove any unbound TBO. The radioactivity present in the pellet was measured by scintillation spectrometry.

4.2.1.2 Effect of saline and serum on ^{3}H-TBO uptake by lag, log and stationary phase cells

Por. gingivalis cells were harvested and resuspended in either saline or serum. Samples (10 ml) were removed and incubated in the dark for 5 min in the presence of ^{3}H-TBO at final concentrations of 41, 82, 164, 328, and 656 μM. The suspensions were centrifuged (5 000 x g for 10 min) and the pellet was washed a number of times to remove any unbound TBO. The radioactivity present in the pellets and supernatants was measured by scintillation spectrometry.

4.2.1.3 Extraction of surface-associated material from lag, log and stationary phase cells

Cells from the lag, log and stationary phases of growth were harvested and resuspended in 0.85 % NaCl (w/v) to give an absorbance of 1.0 (A_{560}). SAM was removed by following the method of Meghji *et al.* (1992). The cell suspension was continually stirred at 4 °C for 1 h. The supernatant was collected, the pellet was resuspended in saline and the procedure was carried out twice more. The removal of SAM was monitored by electron microscopy. A protein assay was carried out on the SAM extracted from each sample using the Bradford assay (Pierce Ltd, see chapter 2 for details) in order to determine the amount of protein present (referred to as surface associated proteins - SAP) in the SAM.

4.2.1.4 Uptake of ^{3}H-TBO by *Por. gingivalis* cells at various pH values

Por. gingivalis cells were harvested and were resuspended in potassium phosphate buffer at pH values of 6.8, 7.4 and 8.0. Samples (10 ml) were removed and incubated in the dark for 5 min in the presence of ^{3}H-TBO at final

concentrations ranging from 41 to 656 μM. The suspensions were centrifuged and the radioactivity present in the pellet was measured by scintillation spectrometry.

4.2.2 Distribution of ^{3}H-TBO following uptake

4.2.2.1 Binding of ^{3}H-TBO to the Outer Membrane

Stationary phase cells of *Por. gingivalis* were harvested by centrifugation and resuspended in saline. ^{3}H-TBO was added to give a final concentration of 82 μM. After 5 min incubation in the dark, the cells were harvested and washed with saline and resuspended in water containing 10 μM of the protease inhibitor TLCK (Nα-pTosyl-L-Lysinechloro-methyl ketone, Sigma Ltd). The cells were disrupted using a Mickel disintegrator, and incubated with 20 % (w/v) Sarkosyl (L-lauroylsarcosine, Sigma Ltd) for 30 min at room temperature. The outer membrane-peptidoglycan was recovered by centrifugation at 25 000 x g for 80 min, and the pellet was washed with distilled water containing 1 mg/ml of benzamidine. The incorporation of ^{3}H-TBO into the outer membrane (OM) was determined by scintillation spectrometry.

4.2.2.2 Binding of ^{3}H-TBO to Plasma Membrane

Stationary phase cells of *Por. gingivalis* were incubated with ^{3}H-TBO at a final concentration of 82 μM. The cells were washed with distilled water, and isolation of the plasma membrane (PM), was carried out using a method described elsewhere with a few modifications (Sprotts *et al.*, 1994). Spheroplasts of *Por. gingivalis* were produced by treatment of whole cells with 30 mM tris-HCl, 20 % sucrose, 10 mM K-EDTA and 0.5 mg/ml lysozyme. The spheroplasts were disrupted by Mickel disintegration and after incubation with 10 mM K-EDTA and 15 mM $MgSO_4$, the membranes were collected by centrifugation at 15 000 x g for

30 min. Homogenisation of the pellet was carried out in 0.1 M K_2HPO_4 containing 10 mM EDTA at 0°C. The crude membranes were incubated for 30 min at 37°C with 20 mM $MgSO_4$, and 100 μg/ml of DNase and RNase. The membranes were washed a number of times by centrifugation at 1000 x g, with resuspension by homogenisation between steps. The amount of ^{3}H-TBO bound to the plasma membrane was measured by scintillation spectroscopy.

4.2.2.3 Binding of ^{3}H-TBO to Cytoplasmic Constituents of *Por. gingivalis*

Stationary phase cells of *Por. gingivalis* were harvested and resuspended in saline. The cells were incubated in 82 μM (final concentration) of ^{3}H-TBO for 5 min and then washed a number of times in saline. The bacteria were resuspended in 10 ml of distilled water containing 10 μM TLCK and disrupted by Mickel disintegration at 4°C. The cytoplasmic constituents were obtained by a method described elsewhere (Sprotts *et al.*, 1994). Spheroplasts were produced as described above (4.2.2.2) and proteins were precipitated using 20 % trichloroacetic acid (TCA). After centrifugation at 12 000 g for 15 min, the supernatant was decanted. The pellet was resuspended in 10 % TCA and, after centrifugation, the supernatant was pooled with that obtained in the previous step (the pellet was retained for the next step). The incorporation of TBO into the cytoplasmic molecules was measured by scintillation spectrometry. The pellet (from the previous step), was resuspended in 10 ml ice cold 10 % TCA. After aliquoting into test tubes, the suspensions were diluted with an equal amount of water. The samples were boiled for 30 min and cooled for a further 30 min. The resulting protein precipitate was collected on a filter and washed twice with 10 % TCA, once with ice cold 70 % ethanol, twice with 70 % ethanol at 45°C, twice with diethyl ether:ethanol (1:1 at 45°C), and once with diethyl ether. After air drying,

each filter was placed in scintillant and any incorporation of TBO into proteins was measured by scintillation spectrometry.

4.2.2.4 Binding of ^{3}H-TBO to DNA

Stationary phase cells of *Por. gingivalis* were harvested and resuspended in 10 ml of saline. After adding ^{3}H-TBO at a final concentration of 82 μM, the sample was washed and resuspended in 2 ml of saline. Samples (500 μl) were aliquoted into four eppendorf tubes, and DNA was extracted using the following method. 10 μl of 100 mg/ml each of RNase, proteinase K and lysozyme were added to each eppendorf. After placing in a 65°C water bath for 30 min, 100 μl of 10 % SDS was added. This was incubated for a further 15 min at 65°C. Phenol:chloroform (1:1) was added in equal volumes to the eppendorfs and centrifuged at 6 000 x g for 15 min. The upper layer was removed and 2 volumes of absolute ethanol were added. After at least 30 min at -20°C, the samples were centrifuged at 13 000 x g for 10 min. The resulting pellet was dried thoroughly under suction, and was dissolved in a minimum volume of water before incorporation of ^{3}H-TBO was determined.

4.2.2.5 Lethal photosensitisation of *Por. gingivalis* after removing unbound TBO

A stationary phase culture of *Por. gingivalis* was washed with 0.85 % saline (w/v) and aliquoted into four eppendorfs. Two of the aliquots were resuspended in 82 μ M of TBO and the remaining were resuspended in saline. After incubating the cells in TBO or saline for 5 min, the cells were washed a number of times by centrifugation (5 000 x g for 10 min) until the supernatant from the sensitised cells was clear. The washed cells were then resuspended in saline and the suspension was aliquoted (100 μl) into a 96 well microtitre plate. Saline (100μl) was added

to the suspensions and the sensitised cells were either exposed to laser light (L^+S^+) at a dose of 0.88 J or were incubated in the dark (L^-S^+). The unsensitised cells were either exposed to laser light (L^+S^-) at 0.88 J or incubated in the dark (L^-S^-). Serial dilutions of each suspension were made and survivors were enumerated on Wilkins Chalgren (WC) blood agar by incubating at 37° C for 4-6 days in an anaerobic chamber.

4.3 RESULTS

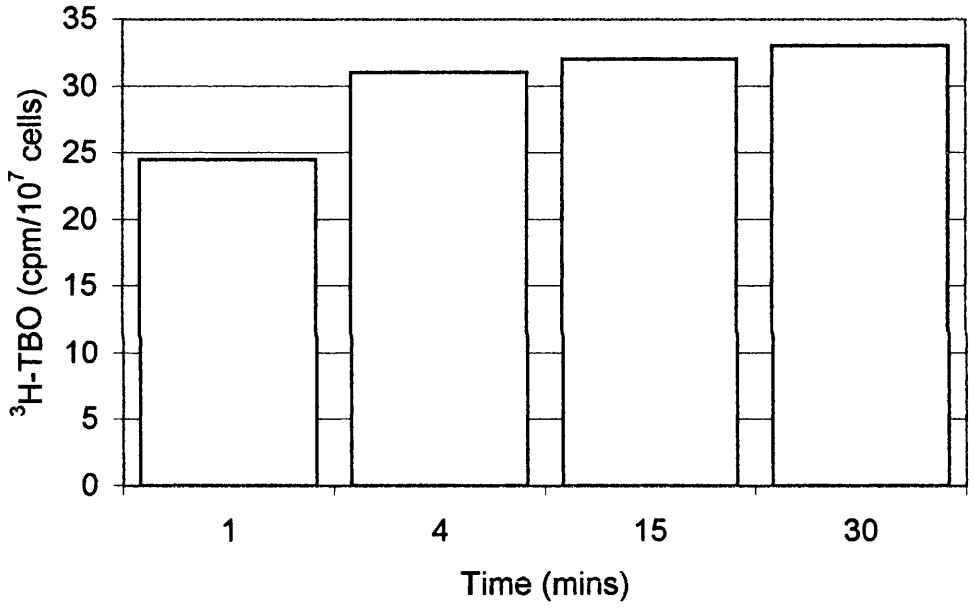


Figure 4.1 Uptake of ^{3}H-TBO at various time intervals. Standard deviation was too low to show error bars. *Por. gingivalis* was incubated in the dark in the presence of 82 μM ^{3}H-TBO for 1-30 min. The pellet was washed a number of times to remove any unbound TBO. The radioactivity present in the pellet was measured by scintillation spectrometry.

Figure 4.1 shows the effect on ^{3}H-TBO uptake of increasing the time *Por. gingivalis* was incubated in photosensitiser. The graph shows that at 4, 15 and 30

min, the amount of photosensitiser taken up was very similar (31,32 and 33 cpm/10^7 cells, respectively). However statistical analysis showed that the difference between each value was significant. The graph shows that of the total amount taken up after 30 min (33 cpm/10^7 cells), 75 % of this was taken up within the first 60 s of incubation.

Concentration of ^{3}H-TBO (μM)	Supernatant (CPM x1000)	Pellet (CPM x 1000)	Total (supernatant + pellet) CPM x1000	Percentage taken up by 10^{10} cells	Uptake by 10^{10} cells (μM)
41	20	5	25	20	8.2
82	40	5	45	11	9.0
164	50	10	60	17	27.9
328	75	10	85	12	39.4
656	85	20	105	19	124.6

Table 4.1a Uptake of ^{3}H-TBO by Lag phase cells in the presence of saline

Concentration of ^{3}H-TBO (μg/ml)	Supernatant (CPM x1000)	Pellet (CPM x 1000)	Total (supernatant + pellet) CPM x 1000	Percentage taken up by 10^{10} cells	Uptake by 10^{10} cells (μM)
41	25.5	2.5	28	9	3.7
82	55.5	5	60.5	8	6.6
164	85.5	5.1	90.6	6	9.8
328	105	5.8	110.8	5	16.4
656	130	5.8	135.8	4	26.2

Table 4.1b Uptake of ^{3}H-TBO by Lag phase cells in the presence of serum

Concentration of ^{3}H-TBO (μg/ml)	Supernatant (CPM x1000)	Pellet (CPM x 1000)	Total (supernatant + pellet) CPM x 1000	Percentage taken up by 10^{10} cells	Uptake by 10^{10} cells (μM)
41	20	19	39	48	19.7
82	45	40	85	47	38.5
164	60	40	100	40	65.6
328	80	40	120	33	108.2
656	110	70	180	39	255.8

Table 4.2a Uptake of ^{3}H-TBO by Log phase cells in the presence of saline

Concentration of ^{3}H-TBO (μg/ml)	Supernatant (CPM x1000)	Pellet (CPM x 1000)	Total (supernatant + pellet) CPM x 1000	Percentage taken up by 10^{10} cells	Uptake by 10^{10} cells (μM)
41	35	5	40	13	5.3
82	85	8	93	6.6	5.4
164	110	2.5	112.5	5.2	8.5
328	145	10	155	6.5	21.3
656	180	12	192	6.25	269

Table 4.2b Uptake of ^{3}H-TBO by Log phase cells in the presence of serum

Concentration of ^{3}H-TBO (μg/ml)	Supernatant (CPM x1000)	Pellet (CPM x 1000)	Total (supernatant + pellet) CPM x 1000	Percentage taken up by 10^{10} cells	Uptake by 10^{10} cells (μM)
41	22.5	5	27.5	18	7.4
82	35	7	42	17	13.9
164	65	10	75	13	21.3
328	93	10	103	10	32.8
656	110	22.5	132.5	17	111.5

Table 4.3a Uptake of ^{3}H-TBO by Stationary phase cells in the presence of saline

Concentration of ^{3}H-TBO (μM)	Supernatant (CPM x1000)	Pellet (CPM x 1000)	Total (supernatant + pellet) CPM x 1000	Percentage taken up by 10^{10} cells	Uptake by 10^{10} cells (μM)
41	30	2.5	32.5	8	3.3
82	45.5	2.8	48.3	6	4.9
164	82	5	87	6	9.8
328	107	7	114	6	19.7
656	135	10	145	7	45.9

Table 4.3b Uptake of ^{3}H-TBO by Stationary phase cells in the presence of serum

Table 4.1a shows the uptake of ^{3}H-TBO by lag phase cells in the presence of saline. The table shows that as the concentration of the photosensitiser was increased, the amount of TBO in μM taken up by 10^{10} cells was significantly increased. The increase in uptake at each concentration in the presence of serum (table 4.1b) was also statistically significant. There was a 3-fold decrease in the

uptake of the photosensitiser by lag phase cells in the presence of serum compared to saline over the concentration range 41 -656 μM.

Table 4.2a shows the uptake of ^{3}H-TBO by *Por. gingivalis* log phase cells. There was a significant increase in the uptake of the photosensitiser as the concentration was increased in the presence of saline. There was an increase in the uptake of the photosensitiser in the presence of serum (table 4.2b) but the difference in increase was only significant after 82 μM. The uptake in the presence of saline was almost 6 times greater compared to that which occurred in the presence of serum.

Table 4.3a shows the uptake of ^{3}H-TBO by stationary phase cells in the presence of saline. As the concentration of the photosensitiser was increased, there was a statistically significant increase in its uptake. The same was true in the presence of serum (table 4.3b). Furthermore, there was a 2-fold decrease in uptake of ^{3}H-TBO in the presence of serum compared to saline.

Comparing the average uptake between lag and log phase cells in the presence of saline, over 41 - 656 μM ^{3}H-TBO, shows the uptake by log phase cells was 2.3-fold higher than lag phase cells, and there was a 2.6-fold higher uptake by log than stationary phase cells. A comparison in the average uptake in the presence of serum between lag and log phase cells over 41 - 164 μM showed that there was no significant difference in the uptake. However, at 328 and 656 μM the difference was statistically significant. The same was observed between log and stationary phase cells.

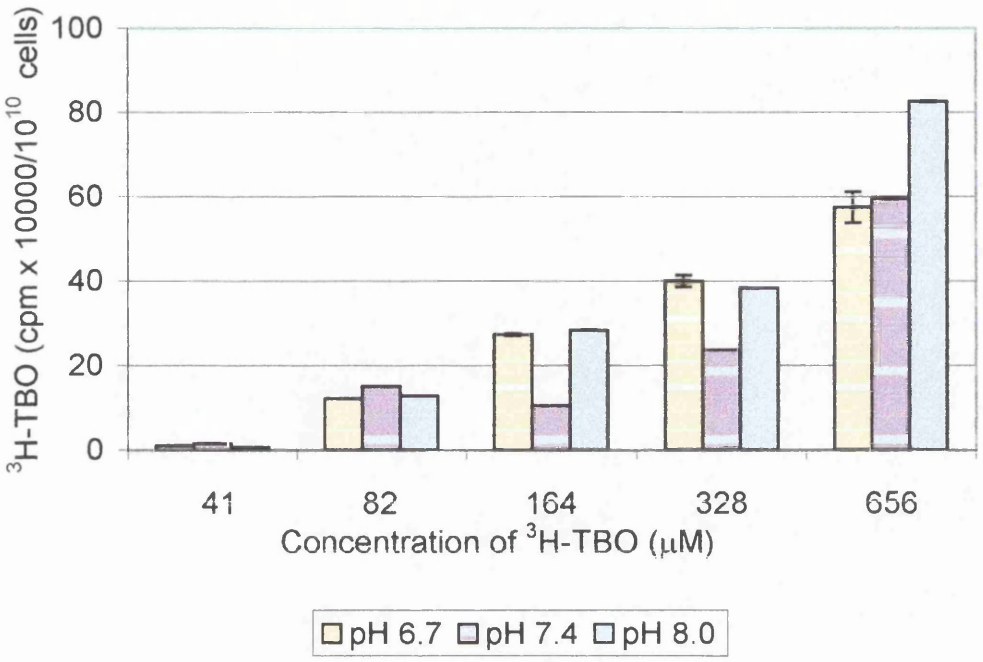


Figure 4.4 Incorporation of ^{3}H-TBO into *Por. gingivalis* cells at various pH values. *Por. gingivalis* cells were resuspended in potassium phosphate buffer at pH values of 6.8, 7.4 and 8.0. Samples (10 ml) were removed and incubated in the dark for 5 min in the presence of ^{3}H-TBO at final concentrations ranging from 41 to 656 μM. The suspensions were washed and the radioactivity present in the pellet was measured by scintillation spectrometry.

The uptake of ^{3}H-TBO at different pH values is shown in figure 4.4. The graph shows that there was no easily discernible pattern to the amount taken up as the pH values increased. The relative amount taken up at pH 7.4 was greatest at 41 and 82 μM but least at 164 and 328 μM. At the highest concentration of 656 μM, the greatest amount taken up was at a pH value of 8.0.

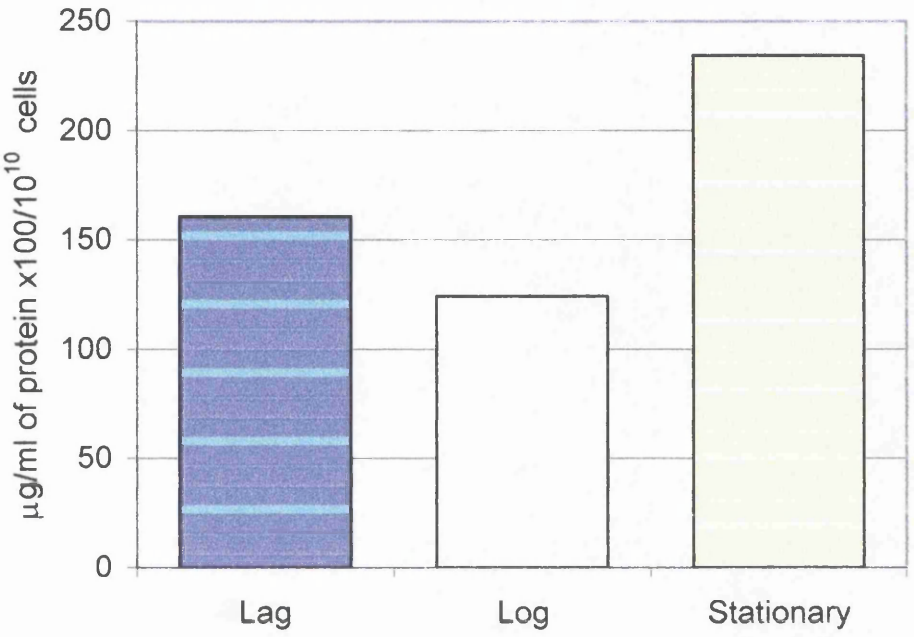


Figure 4.5 Amount of protein present in the SAM from lag, log and stationary phase cells. Cells from the lag, log and stationary phases were harvested and resuspended in 0.85 % NaCl (w/v) to give an absorbance of 1.0 (A_{560}). SAM was removed by continually stirring the cell suspension at 4 °C for 1 h. This was carried out a total of three times. A protein assay was carried out on the SAM extracted from each sample using the Bradford assay in order to determine the amount of protein present (referred to as surface associated proteins - SAP) in the SAM.

Figure 4.5 shows the amount of surface associated proteins (SAP) on *Por. gingivalis* cells at lag, log and stationary phases of growth. Stationary phase cells had the most SAP (2.5 times greater than that of the log phase and 1.5 times more than the lag phase) and the least amount was found on the log phase cells.

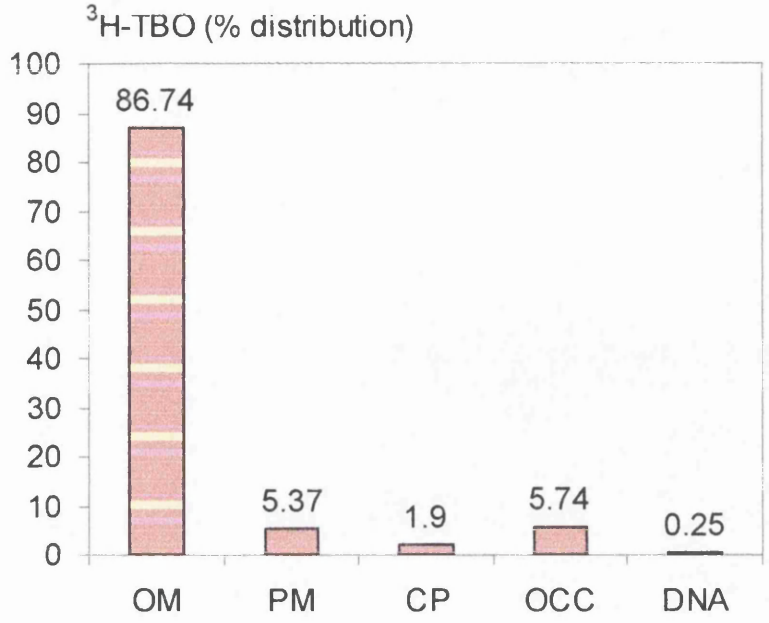


Figure 4.6 Distribution of ^{3}H-TBO within *Por. gingivalis* cells. Whole cells were incubated in 82 μM ^{3}H-TBO and outer membrane (OM), plasma membrane (PM), cytoplasmic proteins (CP), other cytoplasmic constituents (OCC) and DNA were extracted and the incorporation of the photosensitiser was measured by scintillation spectroscopy.

The distribution of ^{3}H-TBO within *Por. gingivalis* cells is shown in figure 4.6. Most of the ^{3}H-TBO (86.7 %) was found to bind to the outer membrane, 5.4 % to the plasma membrane, 1.9 % to the cytoplasmic proteins, 5.7 % to other cytoplasmic constituents and 0.25 % to DNA.

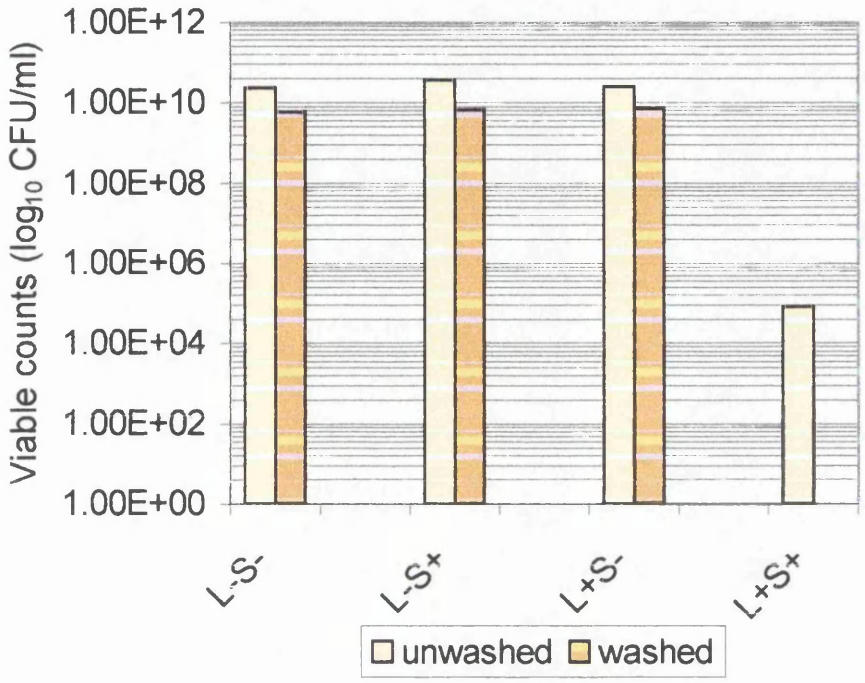


Figure 4.7 Viable counts of *Por. gingivalis* cells exposed to laser light in the presence and absence of excess TBO. Cells were resuspended in 82 μM of TBO and the remaining were resuspended in saline. After incubating the cells in TBO or saline for 5 min, the cells were washed a number of times until the supernatant from the sensitised cells was clear. Aliquots were either exposed to laser light (L^+S^+) at a dose of 0.88 J or were incubated in the dark (L^-S^+). The unsensitised cells were either exposed to laser light (L^+S^-) at 0.88 J or incubated in the dark (L^-S^-).

Figure 4.7 shows the effect of eliminating TBO from the supernatant before exposing to laser light. Greater kills were obtained (100 %, i.e. 10 $\log_{10}$ reduction) when only cellular-bound TBO was present compared to when TBO was also present in the supernatant (5 $\log_{10}$ reduction).

The Table below shows the collated results of the uptake studies and those obtained from the photosensitisation studies (chapter 3).

Parameters tested	Photosensitisation studies	Uptake studies
Concentration of TBO	TBO at 41-164 μM gave substantial kills at all concentrations, though kills decreased slightly at 164 μM	Uptake was increased as concentration of ^{3}H-TBO was increased from 41-656 μM
PIT	Similar kills were obtained as the PIT increased (1-15 min)	Of the total amount of ^{3}H-TBO taken up, 75 % was taken up within the first 60 s of incubation
PH	Kills greatest at pH 7.4 compared to 6.8 and 8.0	Uptake was greatest at pH 7.4 at 82 μM TBO
Serum	Kills were significantly decreased	Uptake was 2.5 fold lower in the presence of serum
Growth phase	Stationary phase cells were less susceptible than lag and log phase cells	Uptake by log phase cells was greater than the other two phases

Table 4.4 Summary of results

4.4 DISCUSSION

The aim of this part of the study was to determine whether *Por. gingivalis* takes up TBO and what part this cellular-bound TBO plays in lethal photosensitisation. The amount of ^{3}H-TBO (tables 4.1a - 4.3b) taken up by the bacteria increased as

the concentration of the photosensitiser was increased both in the presence of saline and serum. The uptake by all growth phases in the presence of serum was significantly reduced compared to saline suggesting that serum greatly affects the uptake of TBO and the results from chapter 3 show that it greatly influences lethal photosensitisation.

There may be a number of reasons why serum may reduce photosensitiser uptake and, as a result, influence lethal photosensitisation. One reason may be that more of the TBO binds to the proteinaceous components of serum. As mentioned in chapter 3 (discussion) photosensitisers have a tendency to aggregate when in the presence of proteins. Therefore, if there was aggregation of TBO, it may have been difficult for the cells to take up the photosensitiser. However, at high TBO concentrations in the presence of serum, there was an increase in the amount of TBO taken up compared to the lower concentrations. TBO also has a tendency to aggregate when present in high concentrations. Therefore, the presence of serum at high TBO concentrations may have altered the properties of the photosensitiser such that the binding capacity may have increased. The SAP from each growth phase has a distinct composition, and it may be that SAP on stationary phase cells (where most uptake was seen) has a higher affinity for the TBO when aggregated under these conditions.

The difference in the composition and amount of SAP from each growth phase may offer an explanation as to why the uptake in the presence of saline was different for each growth phase. The difference in the uptake may be due to the varying amount of SAP (figure 4.5) at each growth phase. An increase in the amount of protein on a cell would give rise to more TBO binding to the cell. Log

phase cells have less SAP than cells in the other phases because the energy of the log phase culture is being channelled into dividing and less into producing proteins for incorporation into the cell surface which is seen more abundantly at early stationary phase. The results in chapter 3 showed that the cells in early stationary phase were significantly less susceptible to killing than cells in the other two phases when 82 μM of TBO was used. This difference in killing by lethal photosensitisation does not seem to correlate directly with the amount taken up by each phase. In early stationary phase cells, which were shown to have abundant SAP (figure 4.5), TBO may have been bound at a considerable distance from the target site so that cytotoxic species may not have reached their most effective site of action (assuming the SAP is not the main site of action). Although there was more SAP on the lag phase cells than the log phase, the reason why both were equally susceptible to lethal photosensitisation may be because the site of action of the cytotoxic species is different at each phase (Tuite and Kelly, 1993). As the cells reach different phases of growth, protein expression and lipid content vary in order to adapt to the different environmental conditions the cells encounter which may lead to a difference in the binding and/or uptake of solutes.

Figure 4.1 shows that nearly 75 % of the total amount of TBO taken up after 30 min (33 cpm/10^7 cells), was taken up within the first 60 s of incubation. Uptake of photosensitiser continued for the remaining 29 min, though the difference in uptake between each subsequent time point was less significant. Chapter 3 showed that similar kills were obtained as the PIT was increased suggesting that a PIT of 60 s is sufficient in order to obtain substantial kills which would be clinically advantageous.

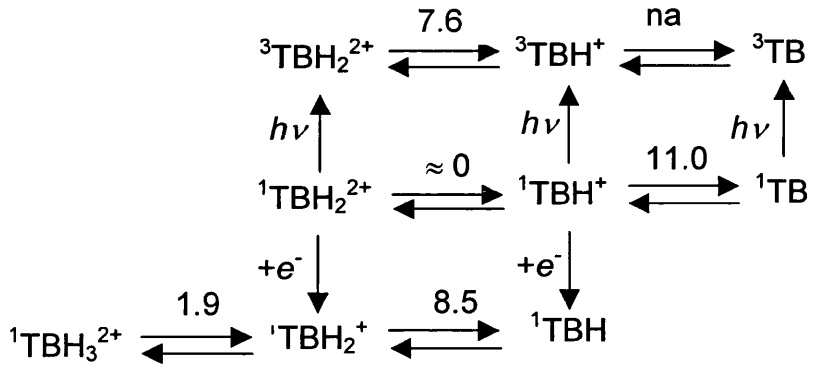
If uptake is a pre-requisite to cell death, then it is possible that the TBO not taken up by the cell, and therefore the cytotoxic species produced by the TBO in the surrounding medium, do not play an essential part in lethal photosensitisation. To test whether this was the case, cells were incubated in TBO for 60 s, and then the TBO from the surrounding medium, and loosely bound TBO from the cells, were removed by repeated washings in saline. The washed cells were then exposed to laser light. It was found that there was a 100 % (10 $\log_{10}$) decrease in viable counts on removal of the surrounding photosensitiser, and there was a 99.99 % (4.8 $\log_{10}$) reduction in the presence of excess TBO. Therefore, it seems that the cytotoxic species produced in the surrounding medium during lethal photosensitisation do not seem to play an important role in the killing of *Por. gingivalis* cells.

The data also show that the state of the photosensitiser influences the lethal photosensitisation of *Por. gingivalis*. The pH can influence the state of TBO as shown below in the diagram (after Tuite and Kelly, 1993). The pK_a values of TBO are shown in red. The amount of photosensitiser taken up at low concentrations (41 and 82 μM) was greatest at pH 7.4. It was suggested by Bongard *et al.* (1995) that TBO in its reduced form (protonated) is more lipophilic and can therefore be taken up more easily by endothelial cells, which may also apply to bacterial cells. Berg and Moan (1997) stated that the neutral form of a photosensitiser could easily penetrate the plasma membrane. This may also explain why there was a greater kill at pH 7.4 (chapter 3: TBO at a concentration of 82 μM).

At higher concentrations of TBO (164 and 328 μM), the relative amount taken

up at pH 7.4 was decreased. At pH values close to neutral, the dye can exist in the protonated and non-protonated form and both have different lifetimes, the non-protonated form has a longer lifetime than the protonated form (Tuite and Kelly, 1993). It may be that at higher concentration of TBO the chemical properties of the photosensitiser change, as there may be added complications such as aggregation of photosensitiser molecules. This in turn may affect the amount taken up by the cells.

It has been shown that the quantum yield of $^1O_2$ by TBO falls over the wider pH range of 9.0 to 5.0 (Phillips, 1997). Therefore, when exposed to laser light, TBO at a higher pH should give more kills. It was actually found that the kills were slightly higher at 7.4 than 8.0 (chapter 3). However, this may be an implication that a difference in the kills over such a small pH range may not be related to the $^1O_2$ quantum yields.



Acid-base properties of TBO. Values are pKa's (after Tuite and Kelly, 1993)
na = not applicable.

A value below 1 (in diagram above) is regarded as denoting a strong acid and that above 4 is mildly (Vollhardt, 1987). The pK_a of triplet state TBO is 7.6, making it weakly basic. Tuite and Kelly (1993) stated that the lifetime of the

triplet state is increased at higher pH values due to the greater basicity of the excited state compared to its ground state. However, as mentioned above, these measurements are made over a wide range of pH values, and the experiments carried out in this present chapter and chapter 3 were done so over a narrow pH range of 6.8 - 8.0 as *Por. gingivalis* cannot survive outside this range. It was demonstrated by Oldham and Phillips (1999) using TBO as a photosensitiser that the yield of singlet oxygen falls by 60 % from a pH of 7.0 to 4.5. They concluded that as TBO can kill *C. albicans* (which can survive at acidic pHs) at very low pH values, a type II mechanism alone cannot be involved in killing. In fact they suggested both type I and type II mechanisms were involved in killing of *C. albicans* and *Por. gingicalis*. Therefore, it is possible that even if the periodontal pocket reaches acidic pHs, *Por. gingivalis* could still be killed using TBO and HeNe laser light.

The uptake studies alone do not, of course, indicate where the sensitiser is located in the cell. An investigation into the distribution of the photosensitiser showed that of the TBO present in the cell, 87 % was present in the outer membrane. Very little was actually bound to the rest of the cellular components (fig. 4.6). However, this distribution may change when cells are exposed to laser light, as there may be damage to the membrane, which in turn may allow additional photosensitiser to enter the cell. However the present distribution study shows that as most of the TBO is bound to the outer membrane the initial damage on exposure to laser light is most likely to be incurred by the outer membrane.

From studies of a wide range of compounds, it is known that the likelihood of

any molecule being taken up by an organism depends upon its charge (Moan *et al.* 1987; Berg *et al.*, 1990). In chapter 3 it was shown that $AlPcS_2$ was not as effective as TBO in killing *Por. gingivalis.* Minnock *et al.* (1996) showed that Gram-negative bacteria *(E. coli* and *Pseudomonas aeruginosa*) were photosensitised by cationic PPC (zinc pyridinium phthalocyanine), but not with the neutral TDPEC (tetra-diethylonamine phthalocyanine) or negatively charged TSPC (tetra-sulphonated phthalocyanine). Uptake studies showed that the lack of activity of TSPC was due to its low affinity for the bacteria, TBO was taken up rapidly by *Por. gingivalis*, which indicates its high affinity for the dye as it was possible to achieve 99.99 % reductions in viable counts at low concentrations and low light doses (chapter 3). However, Minnock *et al.* (1996) found that uptake alone is not responsible for differences in kill as TDPEC and PPC are both taken up by the bacteria but only PPC shows activity (assuming 'activity' refers to the amount of cytotoxic species produced). They found that the localisation and subcellular distribution of the dyes might be a crucial factor in determining whether kills were obtained. They also found that the tightly bound fraction of PPC (like TBO) was responsible for the killing of bacterial cells on exposure to laser light.

The mechanism of uptake of charged molecules in *Por. gingivalis* is poorly understood. However, diffusion of molecules across the outer membrane of *E. coli* has been found to be via various types of porins. For example, it was found that the OmpF and OmpC (outer membrane protein F and C) favoured the diffusion of cations over anions but with PhoE, the opposite was true (Samartzidou and Delcour, 1999). TBO is a cationic molecule and it was taken up rapidly by *Por. gingivalis*, so it may be possible that something equivalent to OmpF and OmpC

may be present in *Por. gingivalis.* It is difficult to say what the mechanism(s) of uptake by *Por. gingivalis* cells is, however the studies herein demonstrate that of the total amount of photosensitiser in the cell, 87 % binds to the outer membrane and it seems most likely that it is the membrane bound fraction which is responsible for killing of *Por. gingivalis* on exposure to laser light. However, the intracellular bound TBO may play an equally important role.

4.5 SUMMARY

1. Of the amount of photosensitiser taken up by *Por. gingivalis* cells, 75 % was taken up within the first 60 s of incubation.
2. Serum reduced the amount of photosensitiser taken up by lag, log and stationary phase cells.
3. pH also influenced the uptake of TBO. The highest uptake at low TBO concentrations (41 and 82 μM) was at pH 7.4.
4. The fraction that is tightly bound to the surface of *Por. gingivalis* cells may be responsible for most of the reduction in viable counts on exposure to laser light.
5. Of the TBO distributed within the cell, most was bound to the outer membrane (86.7 %) and the rest was distributed between the plasma membrane (5.4 %), cytoplasmic proteins (1.9 %), other cytoplasmic constituents (5.7 %) and DNA (0.25 %).

CHAPTER FIVE

A STUDY INTO THE POSSIBLE MECHANISM OF LETHAL PHOTOSENSITISATION OF *PORPHYROMONAS GINGIVALIS*

5.1 INTRODUCTION

The mechanism by which TBO induces cell death has not yet been completely elucidated, though it is thought to involve both type I and type II mechanisms (Phillips, 1997). A type I mechanism involves a direct transfer of electrons between sensitiser and molecular oxygen to produce the superoxide radical anion which, at low pH values, can be protonated to give the reactive hydroxyl radical. A type II mechanism involves energy transfer from the excited dye molecule to an oxygen molecule resulting in the formation of singlet oxygen (Phillips, 1997). The aim of this study was to elucidate whether a type I and/or type II mechanism is involved in the killing of *Por. gingivalis* using TBO in combination with HeNe laser light. In chapter 4 it was shown that TBO binds to the outer and plasma membranes of *Por. gingivalis*. This study therefore included an investigation into whether there was any structural damage to the membrane by measuring lipid peroxidation and internalisation of radiolabelled photosensitiser after exposing sensitised cells to laser light.

5.2 MATERIALS AND METHODS

5.2.1 Lethal photosensitisation of *Por. gingivalis* in the presence of deuterium oxide

A stationary phase culture of *Por. gingivalis* was centrifuged at 5 000 x g for 15 min and resuspended in saline (0.85 % NaCl w/v) so that the suspension gave an absorbance of 1.0 (A_{560}). The suspension was aliquoted equally into four tubes and centrifuged. The pellets were resuspended in a sterile solution of NaCl in D_2O or H_2O (referred to only as D_2O and H_2O in the subsequent text). Aliquots (100 µl) of each suspension were added to wells of a 96-well microtitre plate (Sterilin Ltd., Stone UK). Filter-sterilised TBO in H_2O or D_2O (100 µl) was added to duplicate

wells to give final concentrations of 82 μM. The suspensions were continually stirred with microstirring bars during the experiment. After 60 s PIT, each well was exposed to 0.7 J or 4.4 J of light from the HeNe laser (L^+S^+). Controls (L^-S^-) were incubated in the dark. The bacteria were not incubated in the D_2O to allow for the exchange of intracelluar H_2O for D_2O as the bacteria did not survive the incubation time (3 hours). Therefore, the experiment determined the effect of prolonging the lifetime of singlet oxygen produced extracelluarly.

5.2.2 Lethal photosensitisation of *Por. gingivalis* in the presence of L-tryptophan

The same procedure as above was used, however increasing concentrations of L-tryptophan were added to the saline suspension of bacteria ($5x10^{-8}$-$5x10^{-3}$ M).

5.2.3 Lethal photosensitisation of *Por. gingivalis* in the presence of DABCO, ascorbic acid, sodium azide , methionine and propyl gallate

The same method as above was used with the radical and singlet oxygen scavengers, DABCO (0.1 - $5x10^{-7}$ M), ascorbic acid (0.1 - $5x10^{-7}$ M), sodium azide ($5x10^{-4}$ - $5x10^{-7}$ M), methionine (0.1 - $5x10^{-7}$ M) and propyl gallate (0.05 - $5x10^{-7}$ M).

5.2.4 Measurement of ATP

Por. gingivalis cells suspended in saline were sensitised with 82 μM of TBO and after a PIT of 60 s, exposed to 0.88, 4.4 or 13 J of light. Samples (100 μl) were removed and placed in a sterile sample holder, the procedure for detection of ATP was carried out as described in chapter 2. The amount of ATP present in cells sensitised with TBO (L^-S^+) and control cells (L^-S^-) was also determined.

5.2.5 Lipid peroxidation

The method used for measuring lipid peroxidation was that described by Placer *et al.* (1996) with a few modifications. Samples (1 ml) of irradiated and unirradiated cells were taken every 10 min (for a total of 60 min corresponding to a light dose of 26 J), added to 1 ml of 2-thiobarbituric acid [4,6-dihydroxypyrimidine-2-thiol] (TBA reagent, Sigma Ltd.) and boiled for 15 min. After cooling, 1ml of ethanol:butanol (1:1) and 1 M NaOH was added. After centrifugation at 4 000 x g the absorbance of the supernatant was determined at a wavelength of 534 nm (absorption maximum of malonyldialdehyde, MDA). Blanks were treated in exactly the same way as described, except either saline or 82 μM of TBO was added in place of the bacterial sample. The concentration of MDA from each sample was determined from a standard curve prepared with MDA (Sigma Ltd).

5.2.6 Lethal photosensitisation of *Por. gingivalis* in the presence of sepharose bead - bound TBO

5.2.6.1 Method of binding TBO to sepharose beads

TBO (164 μM) was prepared in water and added to 15 ml of ice-cold coupling buffer (0.1 M sodium borate, pH 8.0). The solution was filter-sterilised and cooled to 4°C. Cyanogen bromide-activated sepharose beads (1 g, Sigma) was added to 35 ml of 1 mM HCl and incubated at room temperature for 15 min. After this time, the bead suspension was placed in a funnel lined with filter paper (Whatman) and washed with 200 ml of 1 mM HCl under suction. The suspension was washed a further 3 times with coupling buffer (total of 30 ml). The bead suspension was then added to the TBO solution and incubated in the dark at 4°C for 20 hours with continuous rolling. After this time, the mixture (TBO-bead) was allowed to settle for 30 min. The supernatant was removed and the absorbance was measured at

633 nm in order to determine the amount of TBO that had bound to the beads. The TBO-bead suspension was washed under suction with 50 ml of coupling buffer, and incubated in ethanolamine (30 ml) for 2 hr at room temperature with continual rolling after which time the excess ethanolamine was removed by gentle suction. The TBO-bead suspension was washed with alternating high pH (pH 8.0) and low pH (pH 4.0) buffer, incubating the TBO-beads in each buffer for 5 min. The TBO-beads were then resuspended in PBS and stored at 4°C.

5.2.6.2 Lethal photosensitisation of *Por. gingivalis* using TBO-beads

An overnight culture of *Por. gingivalis* was prepared as described in chapter 2. *Por. gingivalis* (100μl) was added to duplicate wells. Bacterial suspensions were then sensitised with either 100μl of i) TBO-beads ii) unbound TBO in the presence of beads (TBO+beads) iii) free TBO. After a PIT of 60 s the suspensions were exposed to 4.4 J of laser light. Controls (L^-S^-) were included.

5.2.7 Lethal photosensitisation using TBO bound to *Por. gingivalis* in the presence of D_2O.

A stationary phase culture of *Por. gingivalis* was washed with 0.85 % saline (w/v) and aliquoted into four eppendorfs. Two of the aliquots were resuspended in 82 μM of TBO and the remaining were resuspended in saline. After incubating the cells in TBO or saline for 5 min, the cells were washed a number of times by centrifugation (5 000 x g for 10 min) until the supernatant from the sensitised cells was clear. The washed cells were then either resuspended in saline or D_2O and then centrifuged once more. The pellets were either resuspended in saline or D_2O and aliquoted (100 μl) into a 96 well microtitre plate. Cells were either exposed to laser light (L^+S^+) at a dose of 4.4 J or incubated in the dark (L^-S^-). Serial dilutions of each

suspension were made and survivors were enumerated on Wilkins Chalgren (WC) blood agar by incubating at 37° C for 4-6 days in an anaerobic chamber.

5.2.8 Lethal photosensitisation of *Por. gingivalis* in the presence of ^{3}H-TBO

An overnight culture of *Por. gingivalis* cells was harvested and resuspended in saline. The cells were then treated as shown in figure 5.1, below.

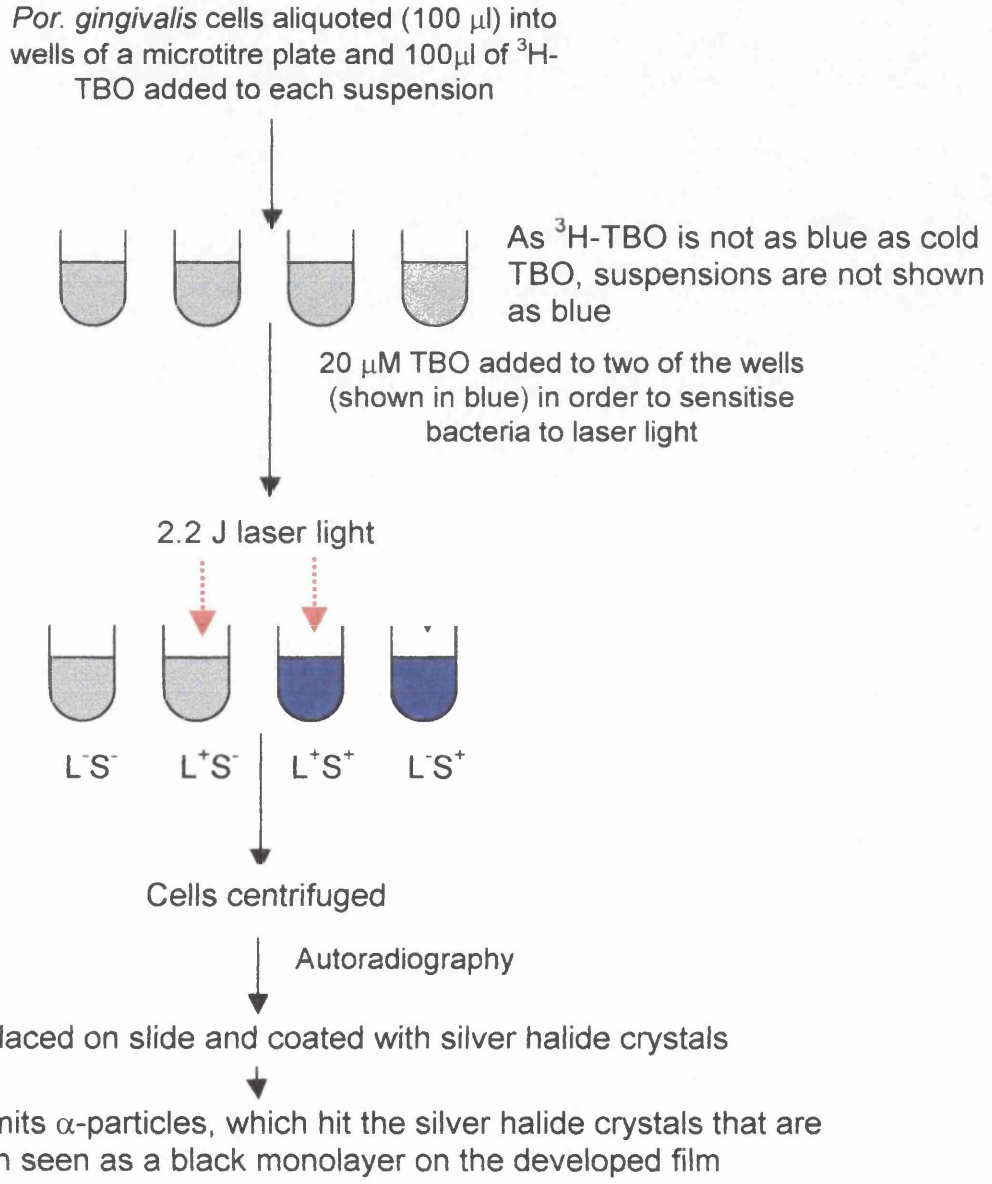


Figure 5.1 Method describing lethal photosensitisation of *Por. gingivalis* cells in the presence of ^{3}H-TBO.

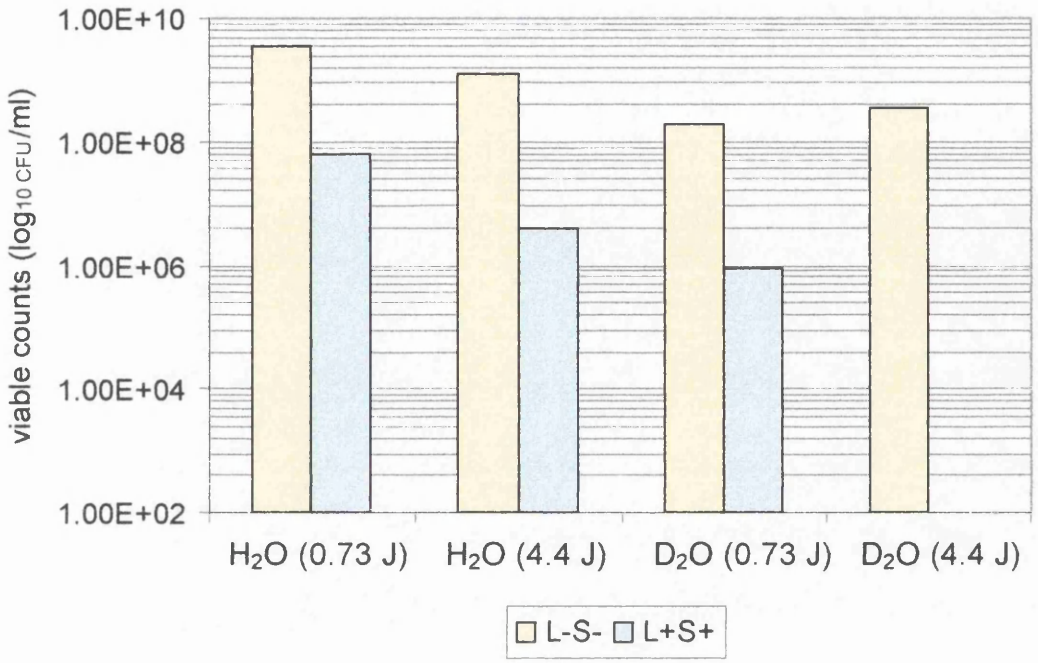


Figure 5.2 *Por. gingivalis* viable counts when in the presence of NaCl in H_2O or D_2O. Standard deviations were too low to show error bars. Cells were resuspended in a sterile solution of NaCl in D_2O or H_2O (referred to only as D_2O and H_2O). Aliquots (100 μl) of each suspension were continually stirred with microstirring bars during the experiment. TBO (82 μM) was added to appropriate well and after 60 s PIT, each well was exposed to 0.7 J or 4.4 J of light from the HeNe laser (L^+S^+). Controls (L^-S^-) were incubated in the dark.

The effect of replacing water with D_2O on the effectiveness of lethal photosensitisation is shown in fig. 5.2. In the presence of water, there was a 1.5 and 2.3 $\log_{10}$ reduction in the viable counts after exposure to 0.73 and 4.4 J of light respectively, compared to a 2.1 and 8.2 $\log_{10}$ (100 %) reduction respectively, in the presence of D_2O.

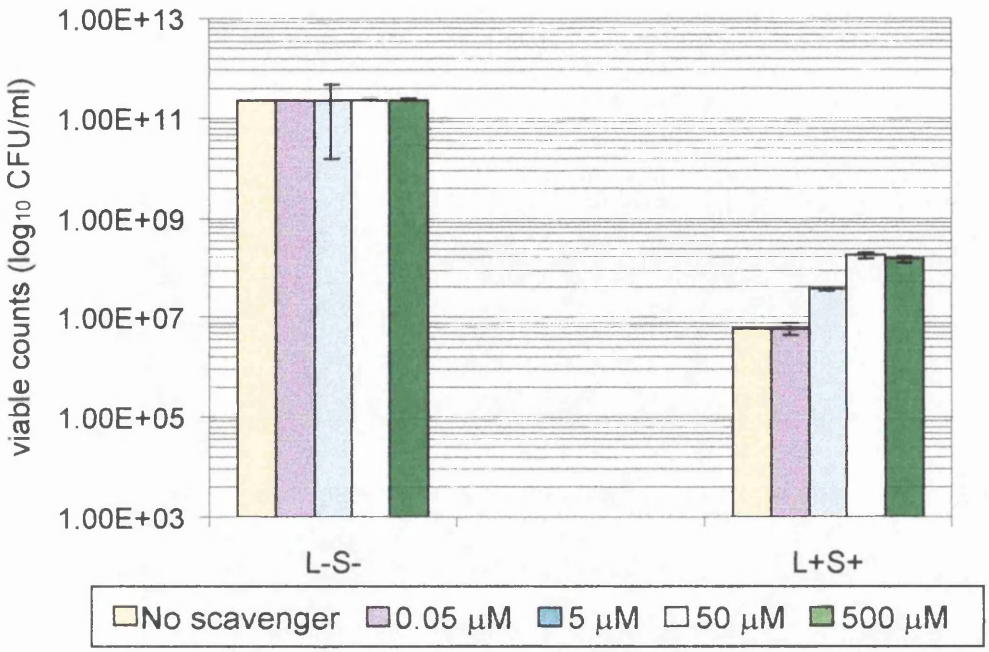


Figure 5.3 Viable counts of *Por. gingivalis* control cells (L^-S^-) and cells sensitised with 82 μM of TBO and exposed to 0.88 J of laser light (L^+S^+) in the presence of increasing concentrations of L-tryptophan.

Fig. 5.3 shows that in the presence of the hydroxy radical and singlet oxygen scavenger L-tryptophan, as the concentration of the scavenger was increased from 0.05 to 50 μM, there was a statistically significant decrease in the numbers of bacteria killed. The difference in kills between 50 and 500 μM however was not significant.

When the experiment was carried out in the presence of DABCO, ascorbic acid, sodium azide, methionine and propyl gallate, the cells were killed in the presence of the scavenger alone at the higher concentrations (0.1-0.0005 M). At concentrations where the cells were not killed ($5x10^{-5}$ - $5x10^{-7}$ M) there was no protection afforded by the scavenger during lethal photosensitisation.

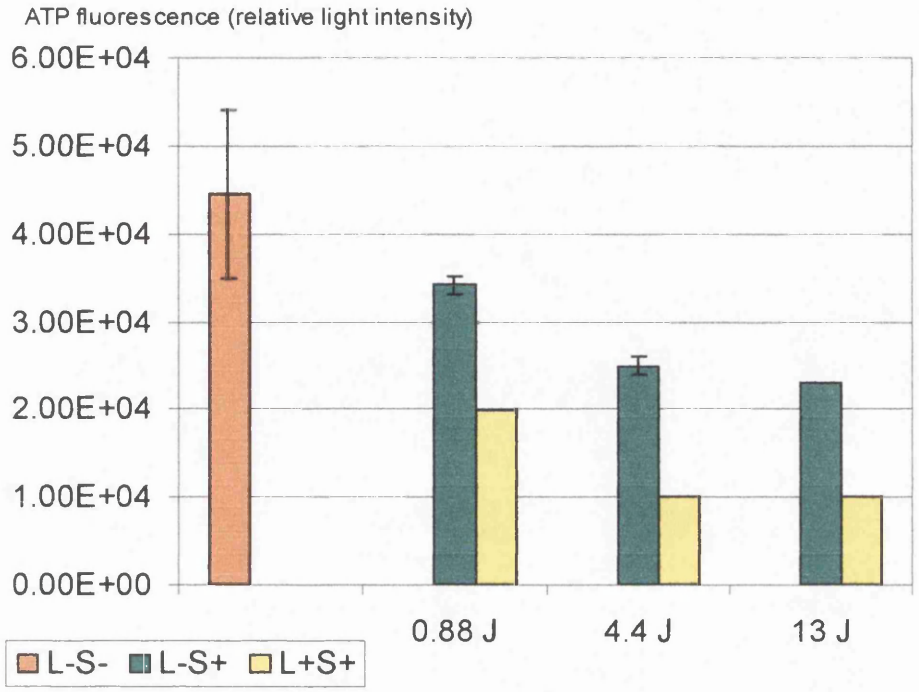


Figure 5.4 A comparison of ATP fluoresence of control and sensitised cells exposed to laser light. The y-axis shows the ATP fluorescence of whole *Por. gingivalis* control cells (L^-S^-), cells sensitised with 82 μM TBO (L^-S^+) and cells sensitised and exposed to increasing doses of laser light (L^+S^+). Standard deviations were generally too low to show error bars.

Figure 5.4 shows the effect of lethal photosensitisation on levels of ATP. The decrease in levels of ATP between 0.88 and 4.4 J was found to be statistically significant. There was no further decrease in ATP after exposure to 4.4 J of light. The amount of ATP was reduced by 55 %, 78 % and 78 % compared to the control cells (L^-S^-) after exposure to 0.88, 4.4 and 13 J of light, respectively. The reductions in ATP levels when cells were exposed to TBO alone for 2, 15 and 30 min were 23 %, 44 % and 48 %, respectively.

There was no evidence of lipid peroxidation. Suspensions containing sensitised bacteria were washed in saline to eliminate TBO from the supernatant and reduce the possible interference of the photosensitiser with the assay. The energy dose was increased from 23 J to 100 J as it was thought that perhaps lipid peroxidation would only be detected if cells were exposed to high energy doses. The volume of bacteria was reduced from 20 ml to 5 ml and the energy dose was unchanged so that viable counts were reduced by 100 %. After each of these conditions was used, no lipid peroxidation was detected.

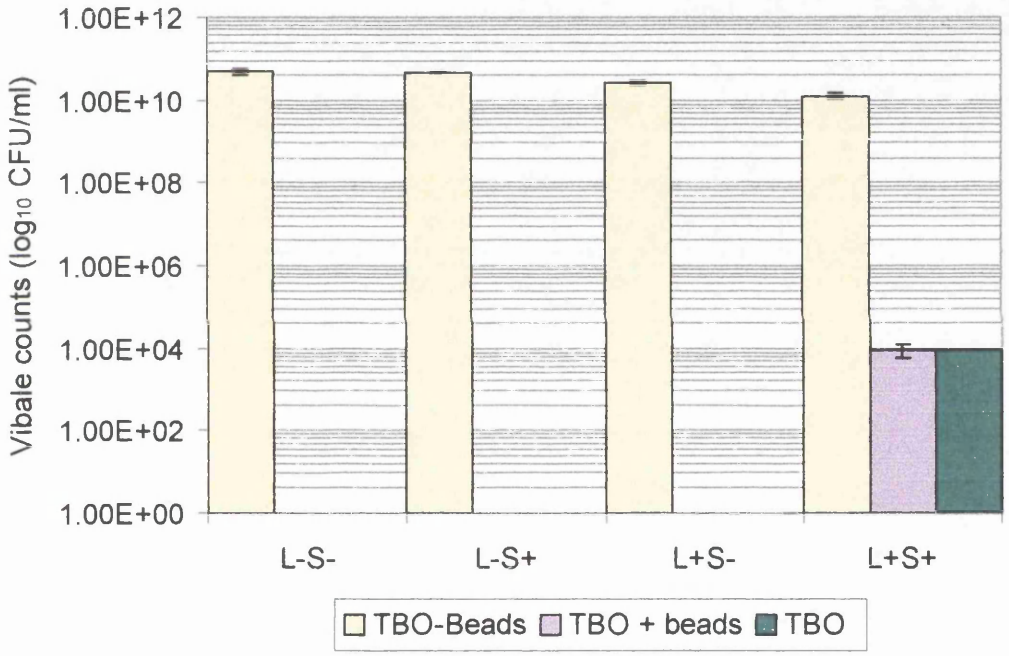


Figure 5.5 Viable counts of cells sensitised with free TBO or TBO-bead and exposed to laser light. The y-axis shows the viable counts of *Por. gingivalis* control cells (L^-S^-, not sensitised with TBO-beads), cells sensitised with TBO-beads (L^-S^+), cells not sensitised, but exposed to laser light (L^+S^-). The graph also shows the viable counts of cells exposed to laser light after sensitisation with TBO-beads, TBO in the presence of free beads (TBO+beads) or free TBO.

Figure 5.5 shows the effect on the lethal photosensitisation of *Por. gingivalis* cells when sensitised with TBO bound to sepharose beads. The figure shows that compared to L^-S^-, there was a small but statistically significant 0.2 log_{10} reduction in viable counts when bacteria were sensitised with TBO-beads and exposed to laser light. In contrast, there was a 6.2 log_{10} reduction in viable counts in the presence of free TBO and TBO+beads. It was calculated from a standard concentration curve of TBO that 36 μg/ml of TBO was bound to the beads.

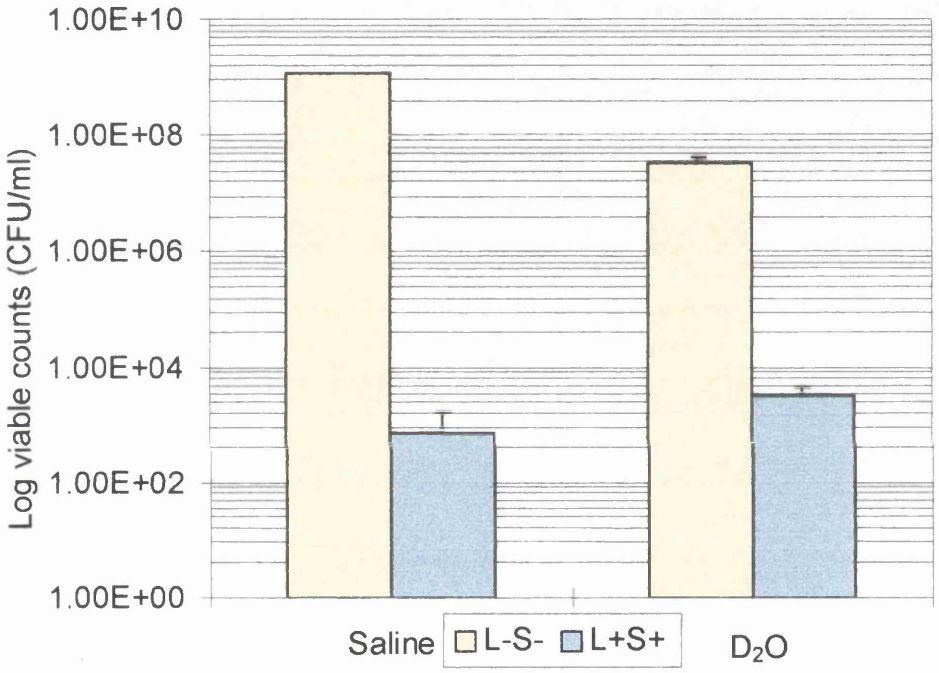


Figure 5.6 Viable counts of *Por. gingivalis* cells suspended in either saline or D_2O and exposed to laser light in the absence of excess TBO. Aliquots were either resuspended in 82 μM of TBO or saline. After incubating the cells in TBO or saline for 5 min, the cells were washed a number of times until the supernatant from the sensitised cells was clear. The washed cells were then either resuspended in saline or D_2O and then centrifuged once more. The pellets were either resuspended in saline or D_2O and aliquoted (100 μl) into a 96 well microtitre plate. Cells were either exposed to laser light (L^+S^+) at a dose of 4.4 J or incubated in the dark (L^-S^-).

Figure 5.6 shows that when excess TBO was removed from the bacterial cells, and the cells were exposed to laser light in the presence of saline, there was a 6.1 $\log_{10}$ reduction in viable counts. When the cells were exposed to laser light in the presence of D_2O, there was a 4 $\log_{10}$ reduction in viable counts.

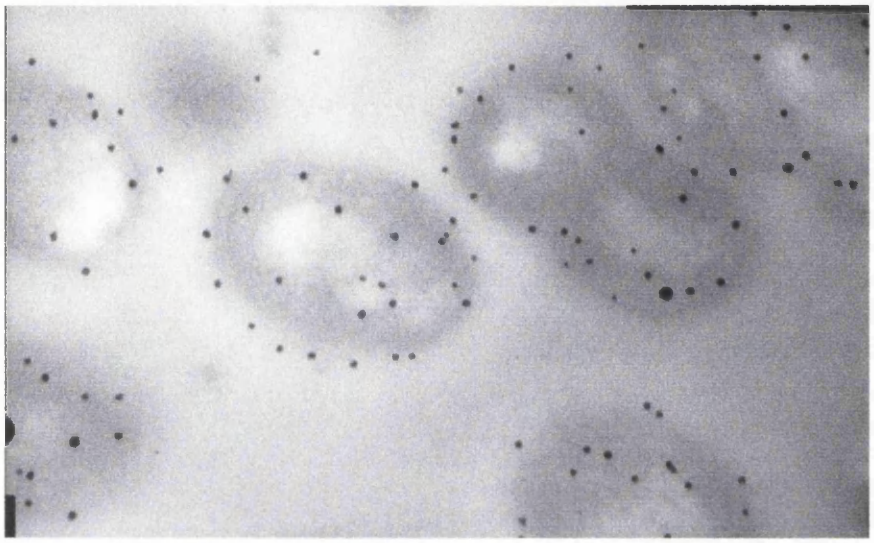


Figure 5.7a TEM *of Por. gingivalis* in the presence of ^{3}H-TBO (L^-S^-). Magnification x23 000

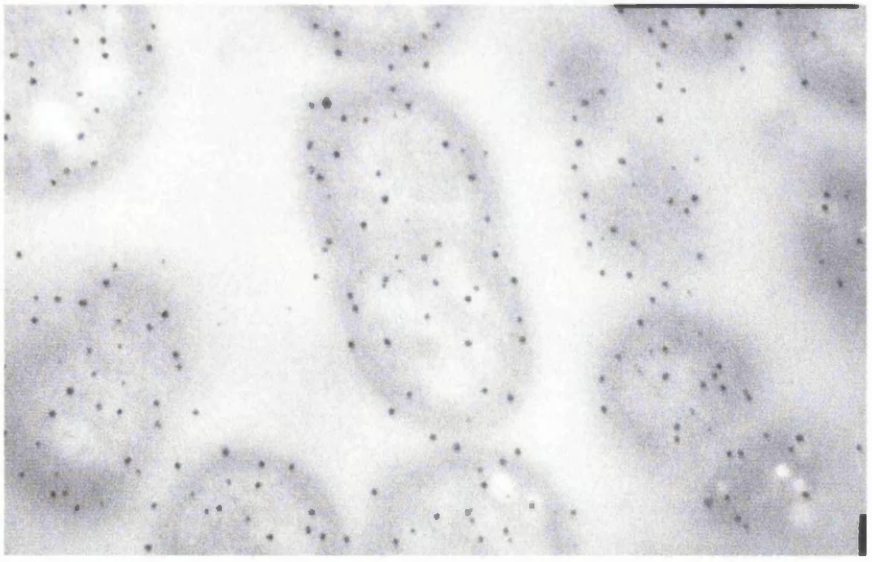


Figure 5.7b TEM of *Por. gingivalis* in the presence of ^{3}H-TBO and exposed to laser light (L^+S^+). Magnification x23 000

Figure 5.7a shows whole *Por. gingivalis* cells in the presence of ^{3}H-TBO. The ^{3}H-TBO shows up as black dots when in the presence of silver halide crystals. Figure 5.7b shows *Por. gingivalis* cells in the presence of ^{3}H-TBO sensitised with 20 μM of

TBO and exposed to 2.2 J laser light. In figure 5.7a the ^{3}H-TBO is mainly on the surface of the cell and in figure 5.7b the ^{3}H-TBO is also present intracellularly.

5.4 DISCUSSION

Many investigators have suggested that the main agent responsible for cellular damage during lethal photosensitisation is singlet oxygen (Michaeli and Feitelson, 1995; Kochevar *et al.*, 1996; Rosenthal and Ben-Hur, 1995). Indeed, Oldham (1997) demonstrated that *Por. gingivalis* whole cells generated singlet oxygen when sensitised with TBO in the presence of D_2O, suggesting the involvement of a type II reaction during lethal photosensitisation. The results of the present study show that prolonging the lifetime of singlet oxygen leads to a significant decrease in the viable counts of *Por. gingivalis* compared to lethal photosensitisation in the presence of H_2O (lifetime of singlet oxygen is ~3 μs in H_2O and ~53 μs in D_2O, [Rossi *et al.*, 1981]). In fact, in the presence of D_2O, a 100 % (6.5 $\log_{10}$) kill was obtained at a light dose as low as 0.73 J, and a 2.5 $\log_{10}$ reduction in viable counts was obtained in the presence of H_2O at the same light dose. As the cells were not incubated in D_2O for a long period of time prior to the experiment, and hence there was probably no exchange of D_2O for intracellular H_2O, the kills seen are probably due to the increase in the lifetime of extracelluar singlet oxygen.

The involvement of a type I reaction cannot, however, be ruled out. The involvement of free radicals was investigated using *Por. gingivalis* cells incubated in propyl gallate (radical scavenger, Nitzan *et al.*, 1989). However, the scavengers themselves had a deleterious effect on *Por. gingivalis* at what should have been the effective concentrations (ranging from 0.1 M - 5 x 10^{-7}

M derived from work previously carried out by Burns *et al.*, (1996, 1997) on a range of bacteria). Lower concentrations of the scavenger were also used (less than 10^{-7} M) but no protection was afforded, as the reduction in viable counts was the same as that obtained when no scavenger was present.

For a type I reaction to occur, the photosensitiser has to be either bound to the bacteria or in very close proximity to it (less than δ, figure 5.7). Binding TBO to beads (TBO-beads) eliminates the type I reaction as the TBO needs to be in close proximity to the bacterial cell, and binding the photosensitiser to the beads eliminates this (Philips, 1997). Lethal photosensitisation of *Por. gingivalis* was carried out using TBO-beads and compared to lethal photosensitisation in the presence of free TBO. In order for a type I reaction to occur in the presence of TBO-beads the photosensitiser has to be bound to the surface of the beads and be in direct contact with the bacterial cells. It was found by Philips, that only 0.15 % of the TBO bound to the beads will meet this criterion and concluded that a type I reaction cannot be responsible for cell death. If this is the case, then any kills obtained with TBO-beads would be due to a type II reaction. Figure 5.7 below shows that the bacteria (green dots) have to be within the diffusion length of singlet oxygen (δ) before cell death can occur. It may be possible that singlet oxygen is quenched by the beads, however, Philips concluded that this is not the case as the beads (which are made up of 4 % agar) have no constituents which would quench singlet oxygen with significant efficiency. The results showed that binding TBO to beads led to a significant decrease in the numbers of bacteria killed, however, the decrease was not as significant as that obtained when cells were sensitised with free TBO. This may be due to some TBO being bound inside the bead, which would render it unavailable to the bacteria.

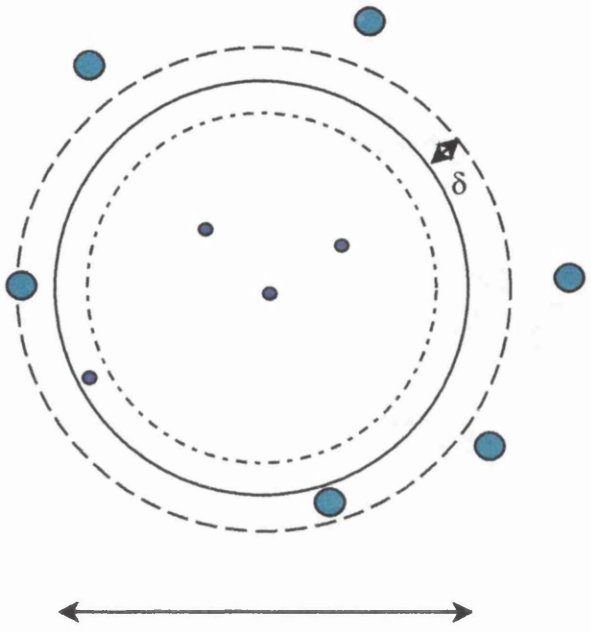


Figure 5.8 Cross-section of a sepharose bead. δ indicates the diffusion length of singlet oxygen. The diffusion length is not necessarily the same inside the bead (-----) as it is into the surrounding solution (–––). The blue dots represent TBO and the green dots represent bacteria (not drawn to scale).

It was shown in chapter 4 that when cells were exposed to laser light after removing unbound TBO, 100 % kill was achieved; this may suggest that a type I reaction is involved in cell death as the photosensitiser has to be in close proximity to the cell for a type I reaction to occur. However, this does not suggest that a type II reaction was not involved. In order to demonstrate whether a type II reaction occurred when unbound TBO was eliminated from the system, excess TBO was removed from the cells and the sensitised cells were then exposed to laser light in the presence of D_2O. It was found that 99.99 % kills were obtained in the presence of either H_2O or D_2O. If singlet oxygen was involved in cell death when the excess TBO was washed from the cells then

increasing the lifetime of singlet oxygen should increase the kills (to 100 %). However, as the results suggest that the kills in the presence of D_2O were very similar to those in the presence of H_2O, it is possible that only a type I reaction may be involved in cell killing when excess TBO is removed from the system.

As mentioned above (and chapter 4), membrane-bound TBO is involved in inducing cell death which suggests that membrane damage would occur as a result of lethal photosensitisation. Figure 5.6a shows that most of the sensitiser (^{3}H-TBO) was bound to the outer surface of the cell and figure 5.6b shows that after exposing to laser light, the sensitiser is located intracellularly. The fact that the ^{3}H-TBO was less blue than the cold TBO (method section; figure 5.1) suggests that the photosensitiser, when tritiated may have degraded. This would mean that binding of the ^{3}H-TBO to the cell, on the whole, may have been reduced when compared to cold TBO. The difference this makes to the experiment carried out in chapter 4 and above (figure 5.7a and b) is in the amount of photosensitiser present, not the actual distribution or the location of the photosensitiser before and lasing. The result shown in figure 5.7a and b would strongly suggest that outer membrane and plasma membrane damage occurs as a result of lethal photosensitisation. Any disruption of the membrane would adversely affect the energy-generating system associated with the plasma membrane, which produces ATP. Compared to control cells, the reduction in the amount of ATP present in cells incubated in photosensitiser alone (L^-S^+) for 2, 15 and 30 min was 23 %, 44 % and 48 %, respectively. On exposure of sensitised cells to increasing doses of laser light, the actual decrease in ATP, once the decrease attributable to TBO is taken into consideration, was 33 %. It is possible that the decrease in ATP was due to the

cell using its stores of ATP as an energy source for the repair of structures such as the membrane which had been damaged. Furthermore, if the membrane is adversely affected, this may have an adverse effect on the *production* of ATP, which the cell cannot replenish causing the cell to eventually die. It can be argued however, that the 33 % reduction in the amount of ATP was actually due to its degradation as the cells were non-viable. It is difficult to assess from these data alone whether the reduction in ATP is due to the action of cytotoxic species or simply due to cell death. The data from this chapter and chapter 4 strongly indicate that membrane damage occurs as a result of lethal photosensitisation. Therefore it is very likely that the damaged cell membrane leads to an impairment of the energy production which, depletes the ATP content of the cell, leading to its death.

Membrane damage as a result of the action of cytotoxic species usually involves lipid peroxidation (Moan *et al.*, 1979; Girotti, 1990; Chen and Yu, 1994). Lipid peroxidation is one of the most widely used indicators of membrane damage (Placer *et al.*, 1966; Girotti, 1985 and 1990) and the main product is the chemically stable malondialdehyde (MDA) which is detected using a colorimetric assay (Placer *et al.*, 1966). MDA could not be detected during the lethal photosensitisation of *Por. gingivalis,* but this does not necessarily mean that it did not occur. A possible reason why none was detected may be the products of lipid peroxidation were rapidly reduced, as the micro-environment in the bacterial cell is highly anaerobic (-300 mV, Marsh and Martin, 1992). It is highly likely that lipid peroxidation does occur as a result of lethal photosensitisation, as there is evidence of membrane damage, and cytotoxic species are known to cause degradation of membrane lipids (Girotti, 1985; Pooler, 1989).

5.5 SUMMARY

1. At a light dose of 4.4 J, greater kills were obtained when using TBO in the presence of D_2O compared to when TBO was used in the presence of H_2O. This indicates that a type II reaction is most likely to be involved in the lethal photosensitisation of *Por. gingivalis* when sensitised with TBO and exposed to HeNe laser light.

2. There was a 33 % decrease in the amount of ATP present in cells sensitised with TBO and exposed to laser light compared to control cells. This decrease is most likely due to disruption of energy-generating mechanisms by cytotoxic species rather than merely reflecting cell death.

3. No lipid peroxidation as a result of lethal photosensitisation was detected. However, there was evidence of membrane damage as ^{3}H-TBO was internalised when cells sensitised with TBO were exposed to laser light.

4. There was a 0.2 log reduction when cells were exposed to laser light after sensitising with TBO-beads compared to 6.2 log reduction when sensitised with free TBO.

5. There was a 99.99 % reduction in viable counts when excess TBO was eliminated and cells were exposed to laser light in the presence of either H_2O or D_2O. This may suggest that a type I reaction could be involved in cell death when excess TBO is removed from the system.

Overall, the data suggests that both types I and II are involved in cell death of *Por. gingivalis* using TBO and HeNe laser light.

CHAPTER SIX

EFFECT OF LETHAL PHOTOSENSITISATION ON MACROMOLECULES AND CELL ULTRASTRUCTURE

6.1 INTRODUCTION

Thiazine dyes bind predominantly to the surface of bacterial and mammalian cells (Ito, 1978) and therefore, it is thought that any damage incurred to the sensitised cell on exposure to laser light would be to the membrane. TBO is also known to bind to proteins, therefore it is likely that damage would occur to membrane proteins. However, in chapter 4 it was shown that a small proportion of TBO penetrate whole *Por. gingivalis* cells, and results from chapter 5 suggested that there may be an increase in the amount of photosensitiser penetrating the cell during lethal photosensitisation as a result of membrane damage, possibly leading to damage to cytoplasmic constituents. The aim of this part of the study therefore, was to ascertain whether there was any alteration in the molecular masses of membrane proteins, and alterations in DNA structure, as a result of lethal photosensitisation. Changes in the ultrastructure of *Por. gingivalis* cells as a result of lethal photosensitisation were also ascertained by transmission and scanning electron microscopy.

6.2 MATERIALS AND METHODS

6.2.1 Measurement of tryptophan fluorescence.

For maintenance of *Por. gingivalis*, refer to chapter 2.

Before attempting to elucidate whether proteins from various components of the cell were adversely affected by cytotoxic species, damage to proteins in the whole cell was assessed by using fluorescence detection to monitor photo-oxidation of the tryptophyl residue. Cells from a stationary phase culture of *Por. gingivalis* were harvested by centrifugation at 5 000 x g for 10 min and resuspended in saline so that the suspension gave an absorbance of 0.1 at a wavelength of 560 nm.

TBO was added to half the suspension to give a final concentration of 82 μM (once the TBO was added all the subsequent steps were carried out in near darkness as the fluorescence of trp (tryptophan) residue decreases in the presence of any light). After 60 s incubation, the cells were washed a number of times until the supernatant was clear. Samples (1 ml) of TBO-sensitised and unsensitised cells were added to quartz cuvettes (standard fluorimeter cells, 10 mm pathlength, Starna). One of the sensitised samples was exposed to light from the HeNe laser, which had an output of 8.0 mW, and the fluorescence was measured at increasing light doses of 0.48 J, 0.96 J, 1.92 J and 4.32 J using a fluorimeter (Perkin-Elmer spectrofluorimeter.). The fluorescence (peak detected at 330 nm and excited at 290 nm, 10 nm slits) of control samples (L^-S^+ and L^-S^-) was measured at the same time points.

6.2.2 Effects of lethal photosensitisation on proteins from various components of *Por. gingivalis.*

A stationary phase culture of *Por. gingivalis* cells (1.5 l), was harvested by centrifugation at 5 000 x g for 15 min. The cells were resuspended in saline and aliquoted into three equal volumes. TBO at a final concentration of 82 μM was added to two of the aliquots, and after a PIT of 5 min, the cells were centrifuged and washed with saline. The samples were then resuspended in saline containing 10 μM of the protease inhibitor TLCK (Nα-pTosyl-L-Lysinechloro-methyl ketone, Sigma Ltd) and one TBO-treated sample was exposed to light from a 7.3 mW HeNe laser with continuous stirring, at a dose of 105 J. TLCK was used as it has been found to be most effective in inhibiting *Por. gingivalis* protease activity (Curtis, personal communication). The samples were then centrifuged at 5 000 x g for 15 min and resuspended in saline (this method unless stated, was used prior to all

the following isolations) The outer membranes and plasma membranes were obtained using the methods described in chapter 4.

6.2.2.1 Isolation of endotoxin.

Endotoxin was obtained from L^-S^- and L^+S^+ cells by following the method described by Meghji *et al.* (1995). L^-S^- and L^+S^+ cells were harvested and the pellets were resuspended in 1:1 mixture of saline/butan-1-ol. The mixture was centrifuged at 5 000 x g for 15 min, the aqueous phase was retained and the organic phase was extracted once again with saline. The aqueous phases (endotoxin) were pooled, and dialysed against dionised water for 3 days, with frequent changes. The volume of the endotoxin solution was reduced to ~30 ml by placing the dialysis tubing in polyethylene glycol, wrapping in foil and placing at 4°C until volume was reduced. The resulting solutions were freeze-dried and reconstituted with sterile water before washing twice at 45 000 x g for 1 hour.

6.2.2.2 Isolation of periplasmic proteins.

Periplasmic proteins were isolated as described by Ferro-Luzzi Ames *et al.* (1984). An overnight culture of *Por. gingivalis* was harvested and after resuspending in saline, aliquoted (100μl) into a 96 well microtitre plate. TBO (100 μl) or saline (100 μl) was added to duplicate wells. Suspensions were either exposed to laser light at a dose of 0.88 J (L^+S^+) or were incubated in the dark (L^-S^-). L^-S^- and L^+S^+ cells were harvested and the supernatants were decanted. The pellets were resuspended by brief vortexing in the residual supernatant. Chloroform (20 μl) was added and, after brief vortexing, the tubes were maintained at room temperature for 15 min before adding 200 μl of 0.01 M Tris-HCl (pH 8.0). The suspension was centrifuged for 20 min at 6 000 x g and the supernatant containing the

periplasmic proteins was retained.

The energy doses were chosen as they all gave 99-100 % reductions in viable counts.

All of the samples obtained were boiled in SDS buffer and the constituent proteins separated by SDS-PAGE using 12 % polyacrylamide gels (see chapter 2 for details). Protein bands were visualised by staining gels in colloidal blue (Sigma Ltd).

6.2.2.3 Effect of lethal photosensitisation on DNA.

DNA from *Por. gingivalis* was isolated from irradiated and unirradiated cells as described in chapter 5. The DNA was run on a 0.4 % agarose gel containing 0.0025 % ethidium bromide. The bands were visualised under UV light.

6.2.3 Transmission electron microscopy (TEM).

All samples for TEM and SEM, were processed immediately after lethal photosensitisation. Irradiated (L^+S^- and L^+S^+) and unirradiated (L^-S^- and L^-S^+) cell suspensions were harvested at 5 000 x g for 15 min and fixed by resuspending in 2.5 % (v/v) glutaraldehyde. The suspension was incubated at 4°C for 4 h and the cells harvested at 5 000 x g for 10 min. The supernatants were discarded, and the pellets were dehydrated by incubating for 10 min in increasing concentrations of ethanol (50, 70, and 90 %). The final dehydration step involved incubating the pellets for 15 min (3 times) in 100 % ethanol. LR white resin (The Resin Company) was then introduced into the cells by the following method. A small sample of each pellet was transferred to a gelatin capsule and the samples were incubated at 4°C for 30 min in 1:1 absolute ethanol:resin, 1:2 absolute ethanol:resin, 100 % resin,

and finally overnight in 100 % resin at 4 °C. The 100 % resin was replaced once more and after 30 min at 4°C replaced with resin containing accelerator (The Resin Company). This was then incubated at 4°C for 24 hrs. The samples were trimmed and then cut using a microtome into 0.5 μm sections, placed on an electron microscope grid and then stained with uranyl acetate (5 min) and Reynolds lead citrate (5 min). The samples were then viewed using an electron microscope (JEOL, version 1CX, U.K.)

6.2.4 Scanning electron microscopy (SEM).

Irradiated and unirradiated cell suspensions were harvested at 5 000 x g for 15 min, resuspended in 3 % glutaraldehyde in 0.1 M sodium cacodylate buffer, and incubated overnight at 4°C. Cells were then post-fixed in 1 % osmium tetroxide at 4°C for 2 h. Dehydration of the samples was then carried out by incubating for 15 min in increasing amounts of ethanol: 20 %, 50 %, 70 %, 90 % and finally in three changes of 100 % ethanol, 10 min each time. The samples were then incubated in three changes of 100 % acetone for 10 min each time. The samples were then transferred to a Critical Point Dryer (Balzers CPD020). The samples were then viewed using a scanning electron microscope (Cambridge 90B, Cambridge, U. K.)

6.3 RESULTS

The effect of laser light on tryptophan fluorescence from sensitised whole cells is shown in figure 6.1. As the laser light dose was increased to 4.3 J, the tryptophan fluorescence intensity (INT) decreased from 135 to 100 units.

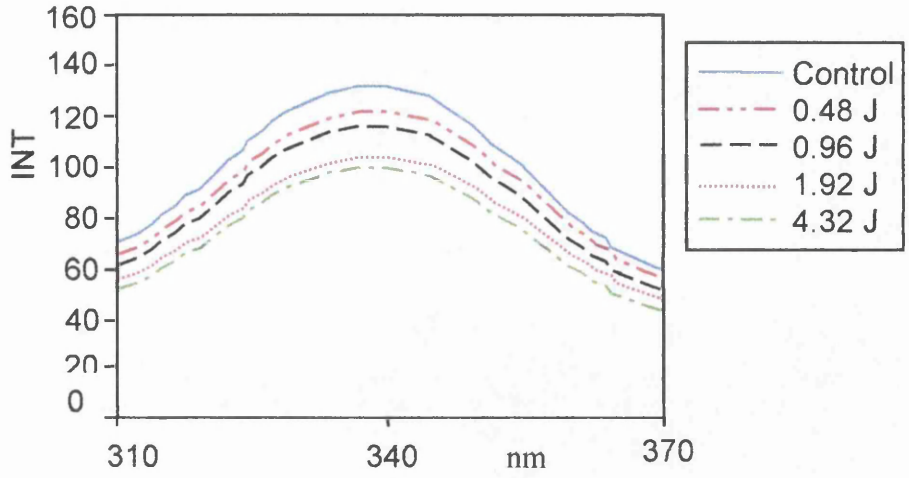


Figure 6.1 The figure shows the tryptophan fluorescence of whole *Por. gingivalis* cells sensitised with 82 μM TBO and exposed to increasing laser light doses. The y-axis shows the intensity of the fluorescence (INT) and the x-axis shows the absorbance wavelength.

The effect of lethal photosensitisation on proteins from the outer membrane (OMp), and PM (PMp) of *Por. gingivalis* is shown in figure 6.2. Lane 1 shows the wide-range molecular weight markers (Sigma). Lanes 2 and 3 show OMp (L^-S^-) and OMp (L^+S^+), respectively. The results show that high molecular mass (HMM) proteins (116-66 kDa), are not present in the treated (L^+S^+, lane 3) outer membrane samples compared to control samples (L^-S^-, lane 2). Lanes 4 and 5 show PMp (L^-S^-) and PMp (L^+S^+), respectively. Both HMM and low molecular mass (LMM) proteins (116-20 kDa) were affected (L^+S^+, lane 5). Furthermore, a sharp band was seen at 14 kDa, which may represent the breakdown products of the HMM proteins.

There were no detectable alterations in the molecular masses of endotoxin or periplasmic space proteins.

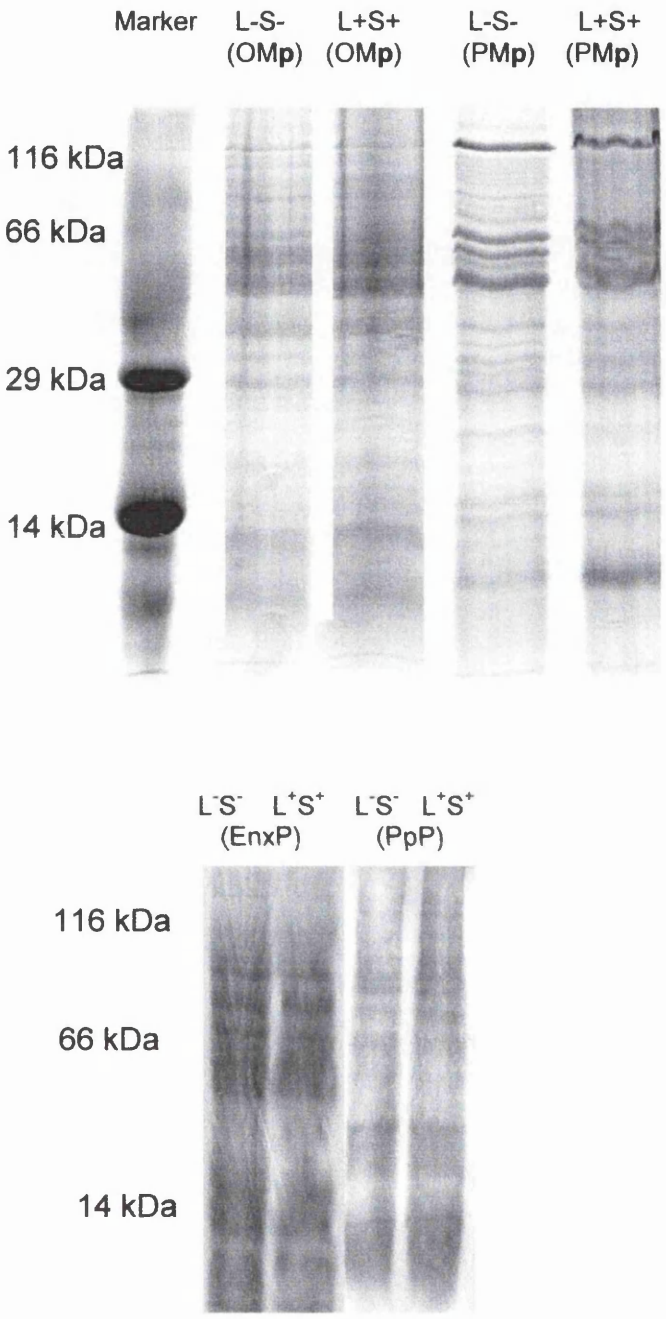


Figure 6.2 SDS-PAGE of various components of *Por. gingivalis* L^-S^- and L^+S^+ cells.

Figure 6.2 Lane 1 shows the molecular weight markers ranging from 116-14 kDa. Lane 2 shows (L^-S^-) outer membrane proteins (OMps) isolated from cells neither

sensitised nor exposed to laser light. Lane 3 shows (L^+S^+) OMps isolated from sensitised cells exposed to laser light. Lane 4 shows (L^-S^-) plasma membrane proteins (PMps) and lane 5 shows (L^+S^+) PMps. Lane 6 shows (L^-S^-) periplasmic proteins (Pps) and lane 7 shows (L^+S^+) Pps. Lane 8 shows (L^-S^-) endotoxin (EnXp) and lane 9 shows (L^+S^+) EnXp

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Figure 6.3 shows that DNA from control cells (L^-S^-) has a mass of approximately 12 000 kbp and that exposing whole *Por. gingivalis* cells sensitised with TBO to laser light causes degradation of the DNA (lane 2).

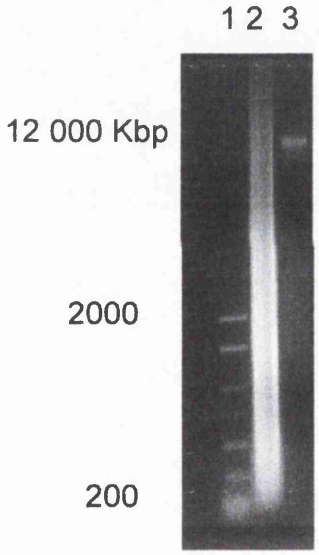


Figure 6.3 Figure 6.3 Lane 1 shows the DNA ladder ranging from 2000 to 100 kilobase pairs (Kbp). Lane 2 shows the DNA from *Por. gingivalis* cells sensitised with 82 μM of TBO and exposed to laser light (L^+S^+). Lane 3 shows the DNA isolated from control cells (L^-S^-).

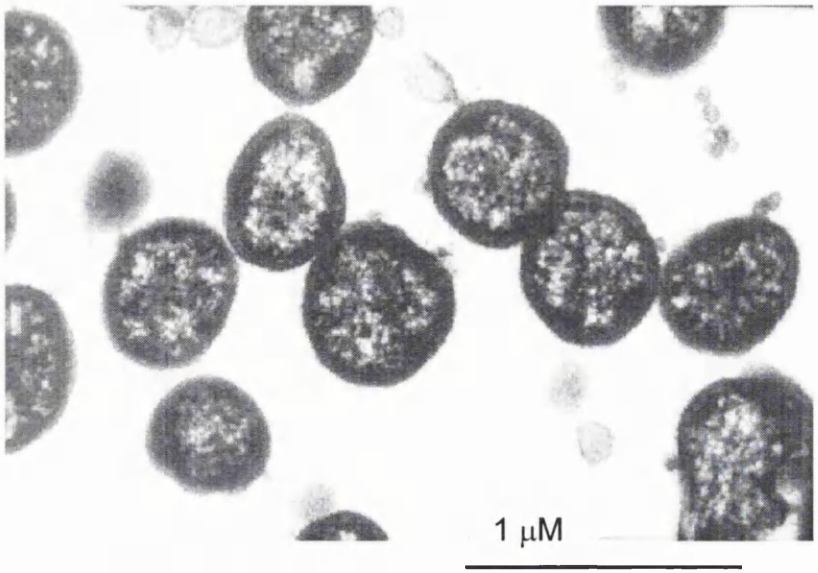


Figure 6.4a TEM of *Por. gingivalis* L^-S^- cells

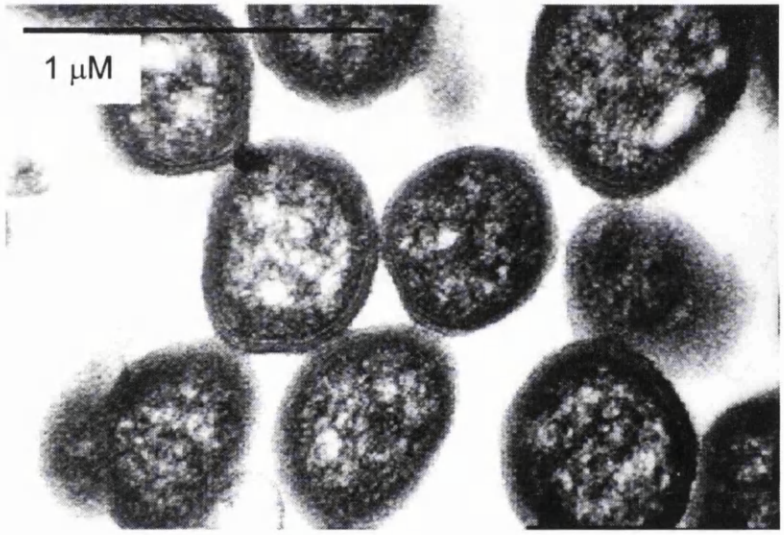


Figure 6.4b TEM of *Por. gingivalis* L^+S^- cells

Figures 6.4a and 6.4b show the ultrastructure of *Por. gingivalis* control cells (L^-S^-) and cells exposed to laser alone (L^+S^-). (Magnification = x23 000).

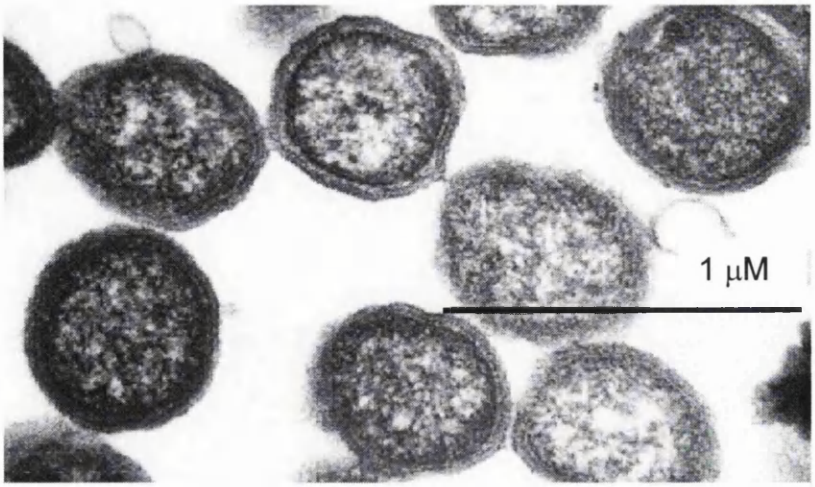


Figure 6.4c TEM of *Por. gingivalis* cells exposed to TBO alone (L^-S^+)

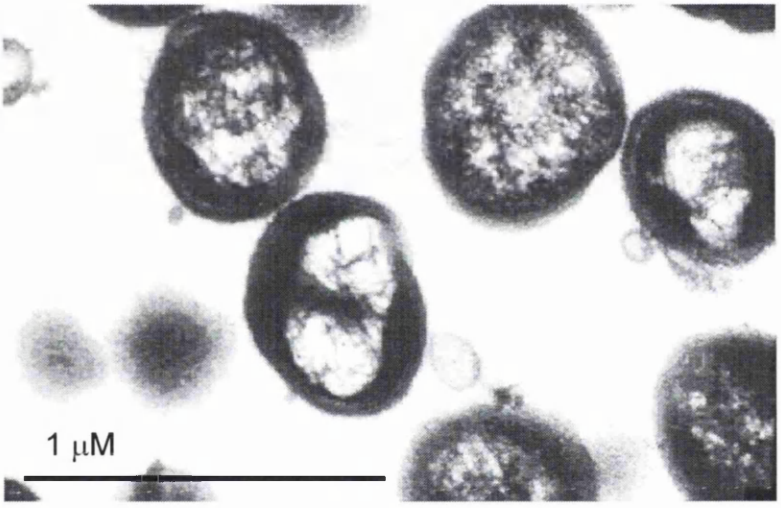


Figure 6.4d TEM of *Por. gingivalis* cells sensitised with TBO and exposed to laser light (L^+S^+) (Magnification = x23 000)

Figures 6.4b-c show that the cells look very similar in comparison to L^-S^- cells. The outer membrane and periplasmic space cannot be seen as distinctly in figure 6.4d, compared to the cells in figures 6.4a-c. The membranes appear more condensed, and the cytoplasm is much more vacuolated in figure 6.4d compared to the cells in figures 6.4a-c.

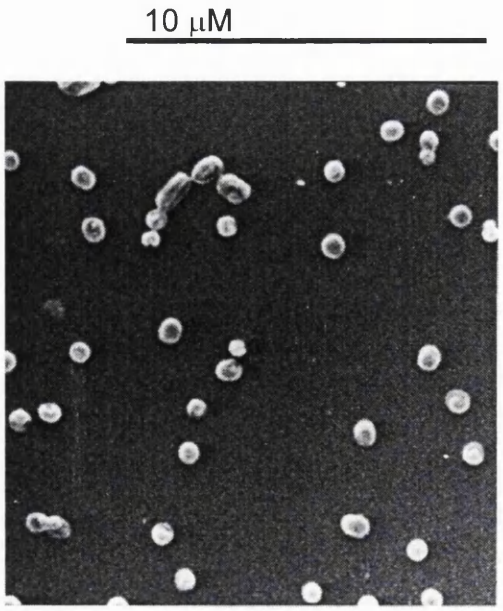


Figure 6.5a SEM of *Por. gingivalis* whole cells (L^-S^-)

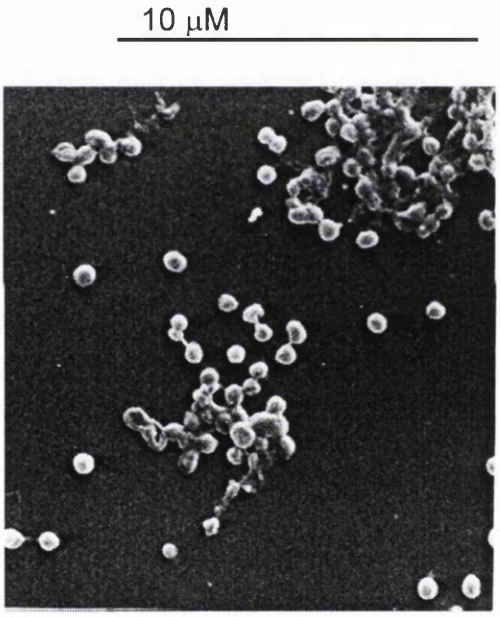


Figure 6.5b SEM of *Por. gingivalis* whole cells (L^+S^+)
(Magnification = x5 000)

Figure 6.5a shows an SEM of *Por. gingivalis* control cells (L^-S^-) and figure 6.5b shows treated cells (L^+S^+). *Por. gingivalis* cells in figure 6.5b are more clumped and have a flattened appearance compared to those in figure 6.5a. Furthermore, there are many strand-like structures linking 2-3 cells together, which are not seen in the control cells.

6.4 DISCUSSION

The results of this part of the study show that the fluorescence due to tryptophyl residues in whole *Por. gingivalis* cells decreased as the light dose was increased. Tryptophan (trp), together with phenylalanine and tyrosine, has an aromatic side chain, but unlike the other two amino acids, the trp side chain contains an indole ring joined to a methylene group. This side chain is responsible for the fluorescence emitted during excitation of the amino acid at a wavelength of 340 nm. It was found that when whole *Por. gingivalis* cells were sensitised with TBO and exposed to laser light, the fluorescence decreased. This indicates that the trp side chain may have either been cleaved from the amino acid, or 'folded' into the structure so that the fluorescence could no longer be detected. These initial results suggested that lethal photosensitisation affects cellular proteins and that singlet oxygen is probably responsible for the reduction in the tryptophan fluorescence (Wakayama *et al.*, 1980). Further analysis by SDS-PAGE showed that the molecular masses of the outer membrane and plasma membrane proteins altered after lethal photosensitisation. An alteration in the outer membrane proteins was expected, as most of the cellular bound TBO is localised in the outer membrane (chapter 4). Damage to plasma membrane proteins was also expected as many investigators have suggested that the main target of lethal photosensitisation is the plasma membrane (Shen *et al.*, 1996; Dahl *et al.*, 1989; Ehrenberg *et al.*, 1993; Kochevar *et al.*, 1995; Malik *et al.*, 1993). Damage to the outer membrane alone may not be the main cause of the significant reductions in viable counts (99.9 - 100 %, chapter 3) as the results in chapter 5 showed that binding TBO to beads (stopping the internalisation of the photosensitiser) gave a 0.2 $\log_{10}$ reduction in viable counts. This reduction was not as great as that obtained in the presence of free TBO (7 $\log_{10}$ reduction). The reduction in viable counts using TBO-beads can be

assumed to be due solely to damage to the outer membrane as the thickness of *Por. gingivalis* outer membrane is 0.1 μm (calculated from TEM) and singlet oxygen can diffuse only 11.5 nm (Nir *et al.*, 1991) during its lifetime and the diffusion of hydroxyl radicals is even less.

It has been suggested that during lethal photosensitisation, singlet oxygen and perhaps hydroxyl radicals (Shen *et al.*, 1996) interact with photo-oxidizable amino acid residues such as His, Cys, Trp and Tyr in one protein molecule to produce reactive species, which may in turn interact with residues or free amino groups in another protein to form a cross-link. Disulphide cross-linking, and lipid-protein cross-linking, protein strand scission, lipid-lipid cross-linking, fatty acid oxidation and release of MDA from oxidised fatty acids (Feher *et al.*, 1995) can also occur as a result of lethal photosensitisation. If cross-linking did occur during lethal photosensitisation of *Por. gingivalis* then it is possible that cross-links were formed between MDA and membrane lipids which would mean that MDA could not be released into the supernatant and hence could not be detected when assaying for lipid peroxidation (chapter 5).

The data show that there was an adverse affect on the molecular masses of a number of outer membrane proteins. Of the proteins detected, three high molecular mass (HMM) bands from the treated OMp samples were absent compared to the controls. These bands may correspond to haemagglutination factors or proteases found in the 45-115 kDa region such as arginine-specific cysteine proteases, lysine-specific cysteine proteases, thiol proteases, and cystine proteases from the streptopain family (Pike *et al.*, 1996, Pavloff *et al.* 1995 and Nakagawa *et al.*, 1995). Proteases such as Lys-gingipain have been reported to be involved in haemin

uptake (Pike *et al.* 1996). However, cell death cannot be attributed to the lack of uptake of haemin alone as *Por. gingivalis* has been shown to grow successfully for a number of successive subcultures on blood-free plates. Any deleterious effect on proteases would be significant *in vivo*, however, as proteases are important in the nutrition and adherence of *Por. gingivalis* as they have been implicated in the degradation of host proteins colonisation of the oral cavity by interaction with Gram-positive early colonisers of tooth surfaces (Christersson *et al.*, 1991), and destruction of host connective tissue (Genco, 1996). Extraction of the outer membrane by sarkosyl does not allow the detection of very low molecular weight proteins (less than 14 kDa) by SDS-PAGE (Deslauriers *et al.*, 1990). Therefore, it is possible that proteins with a mass lower than 14 kDa were affected in addition to those observed.

As already mentioned, haemagglutination factors, which are involved in haemin uptake and the colonisation and adherence of *Por. gingivalis*, might be affected by lethal photosensitisation. The SEM results indicate that there is indeed increased co-adherence of *Por. gingivalis* bacteria. However, this increase may be due to a number of factors. Vesicles are thought to be involved in adherence (Madden *et al.*, 1992), although on the basis of TEM there was no indication of a change in the production of vesicles. As vesicles are thought to be formed by extrusion of the outer membrane, there may be a difference in the structure and/or composition of the vesicles as a result of lethal photosensitisation leading to increased coadherence. Hydrophobicity is also thought to be involved in the adherence of *Por. gingivalis* cells (Okuda, 1993), so any change in the hydrophobic nature of the bacterial membrane due to alterations in its lipid and/or protein content, may lead to an increase in adherence. One or all of these factors may be involved in the

increased co-adherence of *Por. gingivalis* cells. Whether this is detrimental to the cell in itself cannot be ascertained from these data alone. SEM also revealed what appear to be filamentous structures emanating from one cell and linking it to another. A possible reason for this may be that the damaged membrane of one cell is "repaired" to the damaged membrane of another because the cells are in such close proximity to each other. Alternatively, one cell may be forming protein-protein and/or protein-lipid cross-links with the other cell. This would be unlikely where the filaments between two cells are quite long, in which case cell division and / or separation may have been affected. In fact, Malik *et al.* (1990) showed that on irradiation of Gram-positive and Gram-negative cells sensitised with porphyrin for 1 hr, the cells could not complete cell wall synthesis or cell division. There was an increase in the numbers of bacteria that were connected to the parent cell by unfinished septa.

Many investigators have suggested that cell death as a result of lethal photosensitisation may be due to damage to the plasma membrane, with great emphasis being placed on damage incurred by the action of singlet oxygen (Dahl *et al.*, 1989). Ehrenberg *et al.* (1993) and others (Kochevar *et al.*, 1995; Malik *et al.*, 1996), found that on exposure of sensitised cells to light there was leakage of ions such as K^+ ions and this was attributed to cytoplasmic membrane damage. It was shown by Paardekooper *et al.* (1993, 1995a and 1995b) that photosensitisation of yeast cells by TBO caused a drastic change in plasma membrane permeability, and Ehrenberg *et al.* (1993) deduced from polarisation experiments that photosensitised damage to protein sites in the cell, probably in the membrane, may be the cause of cell death induced by light-activated photodynamic processes. The results in this chapter have shown that a range of PMp (116-20 kDa) was affected by lethal

photosensitisation. It seems highly likely that the effect on PMp may be one of the factors responsible for cell death as without a functional plasma membrane the cell would not survive as it is the site of energy generation, anabolic and catabolic metabolism and active transport. In fact it was found that the production of ATP was significantly reduced when cells were irradiated after sensitisation, although the reduction was not conclusively shown to be due to the action of cytotoxic species (chapter 5). However, it seems highly likely from these data that the reduction in ATP was due to adverse effects on the plasma membrane.

The results also show that there was damage to DNA. The damage that occurred during lethal photosensitisation could have been due to breakage of the main chain, degradation of bases (it is thought that the main targets of lethal photosensitisation are thymine and guanine [Tuite and Kelly, 1993]), or cleavage of hydrogen bonds. Damage may become permanent or it may be repaired. If left unrepaired it may result in mutations, or chain breakage (Feher, 1995). The smearing pattern of the DNA from L^+S^+ cells was characteristic of the smearing observed by Nir *et al.* (1991). They found that incubating *S. aureus* cells in haemin in the dark lead to smearing of the plasmidial and possibly chromosomal DNA as observed when run on an agarose gel. They concluded this smearing was due to single strand breaks. Gantchev *et al.* (1994) showed that photo-irradiation of a solution of DNA in the presence of $AlpcS_4$ and $ZnPcS_4$ lead to strand breaks which they concluded was due to singlet oxygen and liberation of nucleobases, which was due to hydroxyl radicals. The role of singlet oxygen in DNA strand breakage has been shown by Lafleur *et al.* (1987). They found that singlet oxygen-induced inactivation of DNA is not due to DNA backbone breakage nor to interstrand crosslinking, but rather to some form of damage to the base or sugar moiety. Di

Mascio *et al.* (1989) used Rose Bengal that had been physically separated from a solution of plasmid or bacteriophage DNA by immobilising the photosensitiser on a glass plate so that only singlet oxygen could reach the plasmid or DNA solution. After exposing to 400-600 nm laser light they found that there was DNA strand breakage. From these data it seems highly likely that singlet oxygen plays an important role in DNA damage during lethal photosensitisation. Interestingly, work carried out by OhUigin *et al.* (1987) using methylene blue (MB) under aerated and de-arated conditions showed there was direct cleavage of phosphodiester bonds and other bonds were rendered labile to alkali. It was shown that MB binds to DNA in at least two ways, firstly, there is intercalative binding whereby the photosensitiser induces helical unwinding (extent of unwinding was 24° per every MB^{+}). The second is non-intercalative binding where there is little unwinding. Intercalation is not required for photolysis and strand breakage is oxygen-enhanced. It was also found that cleavage was guanine-specific. These findings may be relevant to TBO as the two photosensitisers are chemically related. If intercalation is not a requirement for photolysis, and strand breakage is enhanced by oxygen, then it is very likely that on exposure to laser light, the singlet oxygen derived from TBO that is bound or unbound to cytoplasmic constituents, is responsible for guanine-specific damage to *Por. gingivalis* DNA.

Work carried out by Villanueva *et al.* (1993) showed, using a range of photosensitisers, that mesotetra (4N-methylpyridyl) porphine, MB and TBO were the best photosensitisers with regard to inducing tryptophan degradation and DNA-protein cross-linking on exposure to laser light. They concluded that these effects were due to singlet oxygen. Therefore, it is very likely that not only is there degradation of DNA, but also crosslinking of DNA and cytoplasmic proteins,

which suggests extensive damage to the *Por. gingivalis* cytoplasm as a result of lethal photosensitisation.

It has been shown by Valduga *et al.* (1993) that damage incurred by singlet oxygen to Gram-positive and Gram-negative cells altered the cytoplasm and genetic material when viewed by TEM. A study of the ultrastructure of *Por. gingivalis* showed that the cytoplasm of L^+S^+ cells was contracted, and there was condensing or compacting of the membrane in comparison to controls. This alteration in the cytoplasmic structure may have been due to direct damage to cytoplasmic constituents by cytotoxic species. Indirect damage may have occurred via the membrane. If the membrane were altered in some way, the fixative used during the preparation of the sample may not have been able to penetrate the cells properly. If this occurs, the cytoplasm would not have been fixed and during the dehydration step the cytoplasm would have contracted. The reason why the fixative may not have been able to penetrate the cell properly could be because of membrane condensation, perhaps as a result of cross-linking. It has been suggested that contraction of the membrane may be due to damage to hopanes, which are equivalent to cholesterol found in mammalian cells. It has been found by a few investigators (Girotti 1985) that cholesterol (and hopanes) resembles other unsaturated lipids in being susceptible to peroxidative degradation when exposed to a sensitizing agent, exciting light of suitable wavelength and molecular oxygen. Again indicating that lipid peroxidation probably occurred during lethal photosensitisation of *Por. gingivalis.* Bertoloni *et al.* (1992) showed that sensitising *C. albicans* cells with Hp lead to a limited alteration in the structure of the plasma membrane which allowed further penetration of the dye into the cell causing cytoplasmic damage. A similar observation was made

when *Por. gingivalis* cells sensitised with ^{3}H-TBO and TBO were exposed to laser light (chapter 5). To further demonstrate whether internalisation of ^{3}H-TBO occurs as a result of membrane damage, cells sensitised with radioactive and cold TBO could be exposed to increasing light doses and progress of the ^{3}H-TBO followed at each time point.

The results have also demonstrated that there was no alteration in the molecular masses of endotoxin and periplasmic proteins. However, there may have been an alteration in the tertiary and possibly quaternary structures rather than changes in primary structure of the proteins, changes in tertiary and quaternary structures would not have been observed by SDS-PAGE. Another possibility is that there was no binding of the TBO to endotoxin or periplasmic proteins and because the lifetime of the cytotoxic species formed is so short, those produced by outer and plasma membrane-bound TBO on exposure to laser light may have dispersed before reaching the endotoxin or periplasmic proteins.

6.5 SUMMARY

1. High molecular mass proteins from the outer membrane and high and low molecular mass proteins from the plasma membrane were affected by lethal photosensitisation.
2. DNA from treated samples was damaged, possibly by single strand breaks.
3. The ultrastructure of *Por. gingivalis* was altered by lethal photosensitisation. There was contraction of the cytoplasm, and condensing of the membrane. SEM studies showed there was co-adherence of the cells and strand-like structures linking two cells together which may be evidence that cellular division is adversely affected by lethal photosensitisation.

CHAPTER SEVEN

DETERMINATION OF THE EFFECT OF LETHAL PHOTOSENSITISATION ON THE MEMBRANE FLUIDITY OF *POR. GINGIVALIS*

7.1 INTRODUCTION

The physical properties of biological membranes are determined by the composition of the membrane lipids and fatty acids (Keweloh and Heipieper, 1996). Some environmental factors, such as temperature and organic solvents, have a strong influence on the membrane fluidity and therefore the physiological properties of the membrane. Micro-organisms can adapt to environmental changes by modifying their membrane to maintain a constant degree of fluidity, known as homeoviscous adaptation (Herman *et al.*, 1994). Whilst eukaryotic cells regulate their membrane fluidity by changing the cholesterol content, bacteria vary the degree of saturation of the fatty acids. Figure 7.1 shows how a transition between cis and trans fatty acids can cause a decrease in membrane fluidity. This increase in rigidity may affect uptake of many solutes. One of the aims of this study was to determine whether there was a change in the membrane fluidity of *Por. gingivalis* cells at pH values of 6.8, 7.4 and 8.0 which may help explain the differences in uptake of the photosensitiser (chapter 4) and differences in kills obtained during lethal photosensitisation at these pH values (chapter 3).

It has been demonstrated that a decrease in membrane fluidity of bacterial and mammalian cells occurs as a result of damage to the membrane lipids by cytotoxic species (Fukuzawa, 1997). However, lipid peroxidation was not detected during lethal photosensitisation of *Por. gingivalis* cells (chapter 5). It was suggested in chapter 5 that this may be due to limitations of the assay used to determine lipid peroxidation. The second aim of this study therefore, was to determine whether a decrease in membrane fluidity occured as a result of lethal photosensitisation, which would provide evidence for the involvement of lipid

peroxidation in the light-induced killing of *Por. gingivalis*.

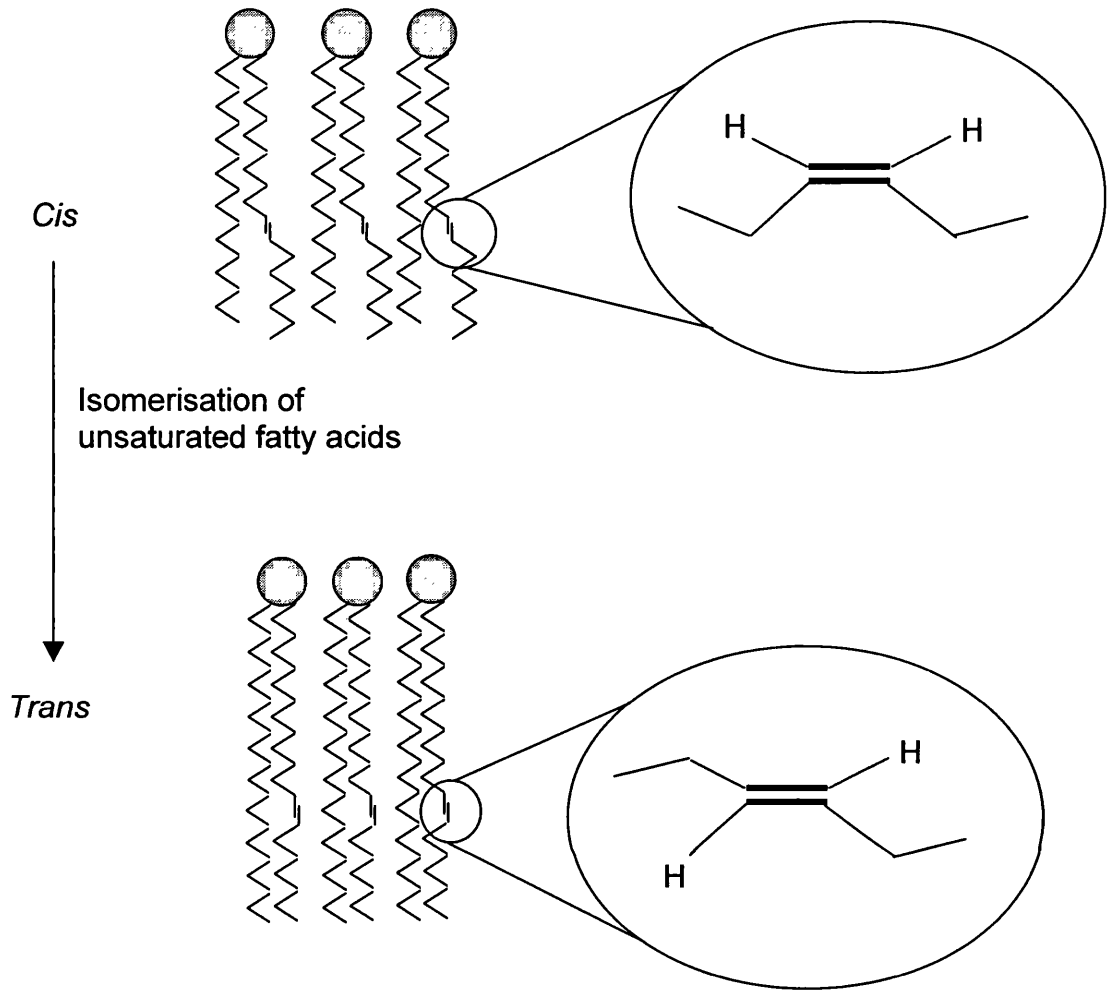


Figure 7.1 A transition from a *cis* to *trans* fatty acid configuration leads to a decrease in membrane fluidity as the phospholipid packing is increased. (Adapted from Keweloh and Heipieper, 1996).

7.1.1 Principles of measuring membrane fluidity

Figure 7.2 shows the basic principles of membrane fluidity determination. 'H' indicates the horizontal component of light and 'V' indicates the vertical component. Four readings are taken by changing the angle of both polarisers relative to the sample. The sample is excited at 340 nm and the light intensity (light emitted by the sample) is measured at a wavelength of 425 nm.

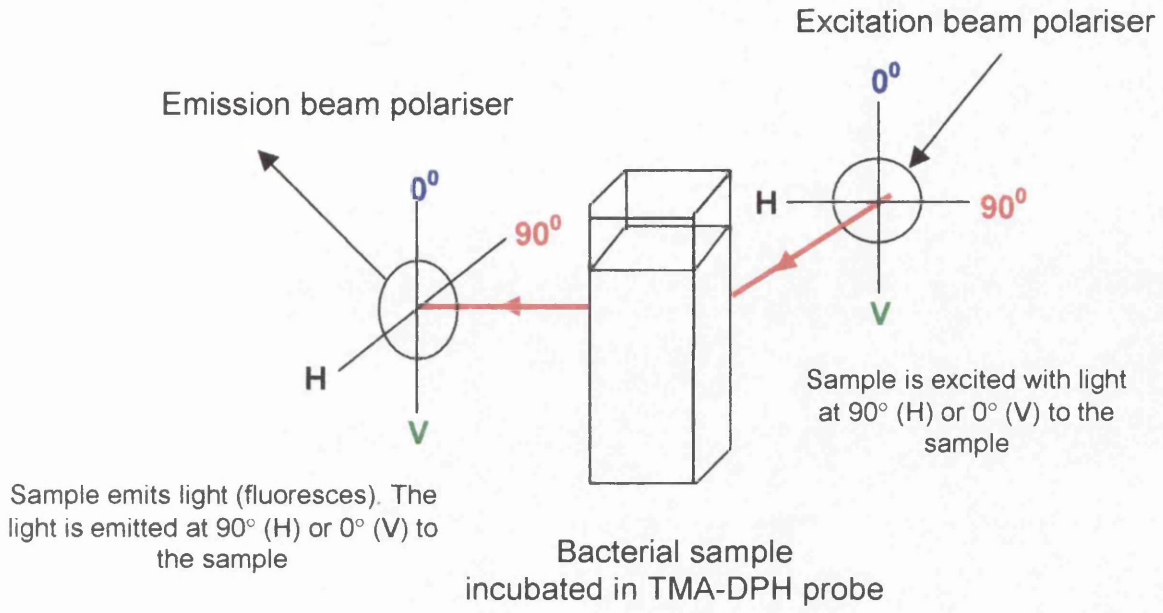


Figure 7.2 Representation of planes of incident and fluorescent light. When the sample is excited by light delivered 90° to the sample, or when light is emitted at 90^0 to the sample, this is denoted by 'H'. When the sample is excited by light delivered 0° to the sample or when light is emitted 0^0 to the sample, this is denoted by 'V'. The values that are obtained when the sample is excited with light at either 90° or 0° to the sample are then used to determine the polarisation (*P*), or fluidity of the membrane using the following equation:

$$P = \frac{V_V - L_V (V_H/L_H)}{V_V + L_V (V_H/L_H)}$$

V_V = excitation and emission polarisers are at 0° to the sample.

L_V = excitiation polariser is at 0° and emission polariser is at 90°

V_H = excitiation polariser is at 90° and emission polariser is at 0°

L_H = excitiation and emission polarisers are at 90°.

7.2 MATERIALS AND METHODS

7.2.1 Measurement of membrane fluidity

7.2.2 Trimethylammonium diphenyl hexatriene (TMA-DPH)

TMA-DPH (Bioprobes Ltd) is a linear polyene that contains a cationic trimethylammonium substituent which acts as a surface anchor and readily incorporates the molecule into the plasma membrane. It binds in proportion to the available membrane surface and because it is non-fluorescent in water, excess need not be removed.

7.2.3 Effect of lethal photosensitisation on membrane fluidity of *Por. gingivalis* cells

Stationary phase *Por. gingivalis* cells were harvested and resuspended in 0.85 % NaCl in D_2O. A thick bacterial suspension is required when measuring membrane fluidity, which means that a high energy dose is required to attain substantial kills (99 %). The experiment was carried out in the presence of D_2O as it prolongs the lifetime of singlet oxygen and substantial kills could be obtained at low energy doses so that the bacteria were kept in an aerobic environment for no longer than necessary. The method for the lethal photosensitisation of *Por. gingivalis* is described in chapter 2. Cells were sensitised with 82 μM TBO (PIT was 60 s) and exposed to light at a dose of 0.88 J. Cells were harvested and washed twice in sterile water. The pellets were resuspended in 3 ml of sterile water and transferred to an UV cuvette (standard fluorimeter cells, 10mm pathlength, Starna). The polarisation anisotropy (measurement of membrane fluidity, designated as *P*) was recorded using a fluorimeter (Perkin-Elmer) to obtain background values. The cells were incubated in TMA-DPH (dissolved in tetrahydrofuran) at a final concentration of 3×10^{-5} M for 3 min at room temperature, after which time the anisotropy was

measured as described above (introduction).

7.2.4 Effect of pH on membrane fluidity

Stationary phase cells were harvested, washed and resuspended in potassium phosphate buffer at pH 6.8, 7.4 or 8.0. The suspensions were neither exposed to laser light nor incubated in TBO. Membrane fluidity measurements were carried out as described above.

7.3 RESULTS

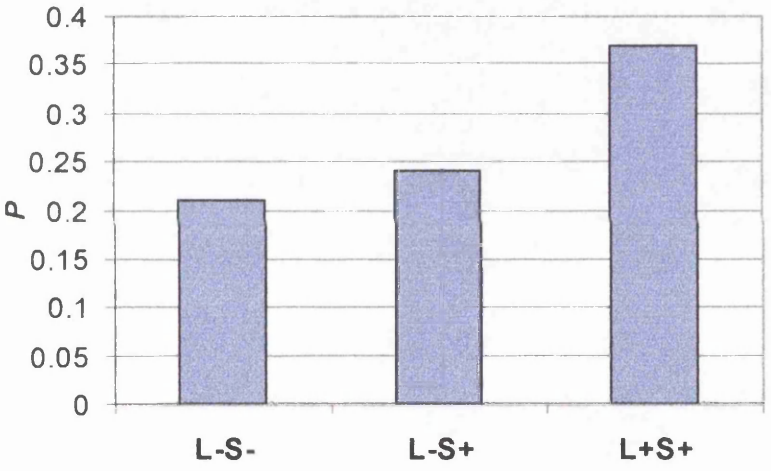


Figure 7.3 The anisotropy (*P*) of control cells, cells sensitised with 82 μM TBO and cells sensitised and exposed to laser light in the presence of D_2O. Standard deviation was too low to show error bars (n=3).

Fig 7.3 shows the anisotropy (*P*) of *Por. gingivalis* cells in the presence of D_2O (pH = 5.0). The anisotropy of control (L^-S^-) cells was 0.21. The anisotropy increased significantly to 0.24 when cells were sensitised with TBO (L^-S^+). There was a further statistically significant increase to 0.37 when sensitised

cells were exposed to 0.88 J of laser light (L^+S^+). There was no decrease in the viable counts in the presence of TBO, however when sensitised cells were exposed to laser light there was a 7 $\log_{10}$ reduction in viable counts.

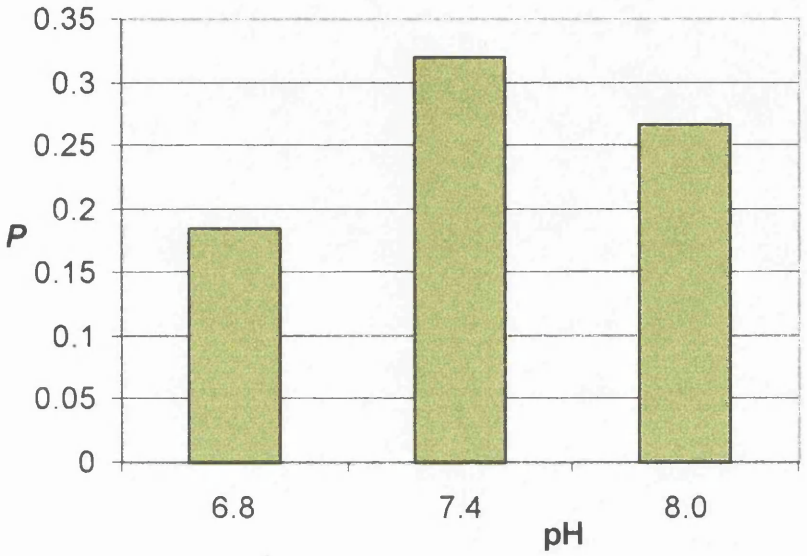


Figure 7.4 Anisotropy of cells at increasing pH values. Standard deviation was too low to show error bars (n=3). Stationary phase cells were harvested, washed and resuspended in potassium phosphate buffer at pH 6.8, 7.4 or 8.0. The suspensions were neither exposed to laser light nor incubated in TBO.

Figure 7.4 shows the anisotropy of cells (L^-S^-) in suspensions of phosphate buffer at pH of 6.8, 7.4 and 8.0. The anisotropy of whole cells was 0.19, 0.32 and 0.27, respectively. The anisotropy was greatest at the physiological pH of 7.4.

7.4 DISCUSSION

The results suggest that the rigidity of the *Por. gingivalis* membrane was significantly affected by lethal photosensitisation (a decrease in the value of *P*

corresponds to an increase in membrane fluidity). Many investigators have shown in mammalian cells that a decrease in membrane fluidity is most likely due to lipid peroxidation (Kaplan *et al.*, 1995; Girotti, 1990; Chen and Yu, 1994;). Lipid peroxidation can occur when cytotoxic species attack phospholipids containing unsaturated fatty acyl moieties. The effects of lipid peroxidation include: i) structural derangement of the bilayer and altered membrane fluidity; ii) increased permeability of cytosolic constituents; iii) inactivation of intrinsic enzymes and transporters such as Na^+ and K^+-ATPase; iv) covalent cross-linking of lipids and proteins; v) polypeptide strand scission and vi) DNA damage and mutagenesis.

Data from chapter 6 showed that DNA and membrane protein damage occurred as a result of lethal photosensitisation. The activity of integral membrane proteins is sensitive to lipid bilayer dynamics and the physico-chemical state (Veld *et al.*., 1993) therefore changes in proteins embedded in the membrane result to a large extent from changes to the lipid environment (Sikkema *et al.*, 1995). Lipids stabilise the proteins through electrostatic interactions between the lipid head groups and charged amino acid residues and between the fatty acyl chains and the membrane-spanning segments. Any disruption between this interaction would cause loss of membrane activity. Transport of solutes may be affected, as the bacterial plasma membrane is a selective barrier in which the transport of solutes is catalysed by specific membrane proteins (Veld *et al.*, 1993). The decrease in membrane fluidity therefore is indicative of a number of adverse changes to the cell incurred by lethal photosensitisation.

The data provide strong evidence that lipid peroxidation occurs during lethal

photosensitisation. The fact that lipid peroxidation of *Por. gingivalis* could not be detected (chapter 5) may be explained by the observations made by Chen *et al.* (1994). They showed that the products of lipid peroxidation, 4-hydroxynonenal (HNE) and MDA influenced the decrease in fluidity (HNE more than MDA) by forming a complex with the membrane phospholipids. Therefore, if MDA itself formed cross-links with *Por. gingivalis* membrane proteins and/or lipids, it would not be detected in the supernatant when assaying for lipid peroxidation.

It was suggested in chapter 6 that protein-protein and protein-lipid crosslinking occurs within the membrane as a result of lethal photosensitisation. Figure 7.5a shows how a decrease in membrane fluidity may be due to aggregation of the membrane proteins caused by crosslinking between protein-protein, protein-lipid and lipid-lipid. Co-aggregation of *Por. gingivalis* cells (chapter 6) may also be due to lipid peroxidation. Almeida *et al.* (1994) using synaptosomes (nerve endings) and liposomes, suggested that the fusion (or co-aggregation) between the cells was due to an increase in intermembrane aggregation and protein hydrophobicity associated with lipid peroxidation.

It was found that TBO alone also causes a decrease in membrane fluidity, this may be due to some membrane damage incurred on exposure to white light as it was found that there is a decrease in trp fluorescence when sensitised cells are exposed to white light (chapter 6). An alternative explanation is outlined in figure 7.5b. TBO has a tendency to aggregate in the presence of bacterial cells (Philips, 1997) and these aggregates may tether the membrane proteins thereby decreasing membrane fluidity. The decrease in the fluidity, though significant in the presence of TBO, was not as significant as when sensitised

cells were exposed to laser light.

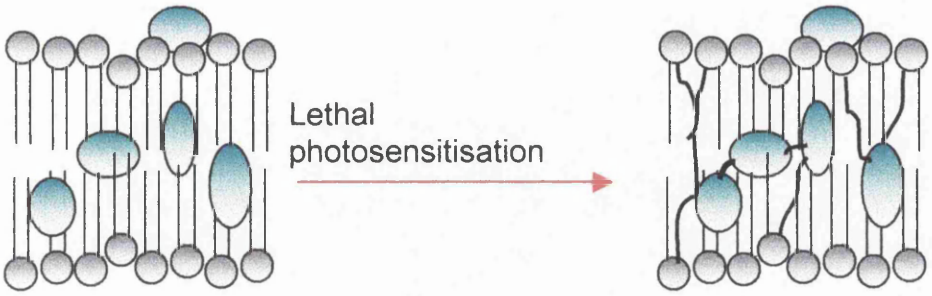


Figure 7.5a Lethal photosensitisation causes a decrease in membrane fluidity (plasma membrane shown) possibly due to protein-protein, protein-lipid and lipid-lipid cross-linking. Thick black lines show cross-links produced as a result of exposure of sensitised cells to laser light.

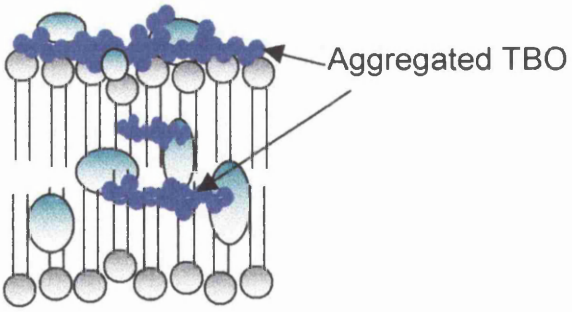


Figure 7.5b Decrease in membrane fluidity in the presence of TBO alone may be due to tethering of proteins and lipids by aggregated TBO.

The membrane fluidity was also affected by a change in pH. When the environment was slightly acidic (6.8) or slightly alkaline (8.0), there was an increase in the membrane fluidity compared to pH 7.4. It is known that the

fluidity of the membrane decreases when the lipids within the membrane convert from *cis* unsaturated fatty acids to *trans* fatty acids (Keweloh and Heipieper, 1996). At physiological pH values, *Por. gingivalis* may increase the amount of *trans* fatty acids in order to maintain a stable bilayer. A change in pH may prompt the bacterial cell to adapt to the new environment by converting *trans* fatty acids to *cis* fatty acids and hence cause an increase in membrane fluidity. Russell (1990) stated that the membrane fluidity causes an alteration in the permeability a less fluid membrane would lead to a decrease in uptake. As the fluidity of the *Por. gingivalis* membrane was found to be greater at pH 6.8 and 8.0 compared to 7.4, it was expected that there should have been an increase in uptake of TBO at these two pH values. The data from chapter 4 however, shows that the uptake is dependent upon both pH and TBO concentration. Photosensitiser uptake may be due to simple diffusion through the membrane porins that may not be affected by a change in membrane fluidity. Porins are thought to be controlled by electric fields and metabolic processes (Benz, 1985) and there may be disruptions in these properties when the pH and TBO concentration are altered leading to alterations in uptake.

7.5 SUMMARY

1. The fluidity of the *Por. gingivalis* membrane decreased as a result of lethal photosensitisation, which indicates that lipid peroxidation most probably occurred.
2. The membrane was most rigid at the physiological pH of 7.4 but may not influence the uptake of TBO at different pH values.

CHAPTER EIGHT

ANTIBODY-TARGETED LETHAL PHOTOSENSITISATION OF *POR. GINGIVALIS* IN THE PRESENCE OF *S. SANGUIS* OR HUMAN GINGIVAL FIBROBLASTS

8.1 INTRODUCTION

Lethal photosensitisation is not a specific modality and has been shown to be effective against a variety of tissues and cells such as neoplasm (Dougherty *et al.* 1990; Sheyhedin *et al.*, 1998), fungi (Paardekooper *et al.*, 1992, 1993, 1995a, 1995b; Wilson and Mia, 1994), viruses (Smetana *et al.,* 1998) and bacteria (Nitzan *et al.*, 1995, 1998; Orenstein, 1998, Merchat *et al.*, 1996a and 1996b; Bertoloni *et al.*, 1993, 1992, 1990; Millson *et al.*, 1996; Wilson and Pratten, 1994; Wilson *et al.*, 1992, 1993a and 1993b and 1993c). Work carried out by Wilson *et al.* (1994) using TBO as photosensitiser in combination with HeNe laser light, showed that in plaque samples containing *Por. gingivalis, Fusobacterium nucleatum, Streptococci,* black pigmented anaerobes and *Actinobacillus actinomycetemcomitans*, all the bacteria were susceptible to lethal photosensitisation. The fact that lethal photosensitisation is not specific is advantageous in one respect as this means it is possible to targ*et al.*l the bacteria in a mixed infection. However, this also means that commensal bacteria and host tissue could be affected by lethal photosensitisation.

Many therapeutic regimes used for oral infections eliminate both pathogenic and commensal organisms indiscriminately, thereby disrupting the natural ecosystem of the oral cavity (Wilson, 1994). Therefore it is important to develop a treatment that could specifically target the pathogenic organism without causing any adverse effects on the commensal oral flora or host tissue. In the case of PDT, one method of achieving this has been to conjugate the photosensitiser to antibody raised against the target organism or cell. Photosensitisers that have been conjugated to antibody include porphyrins (Papkovskii *et al.*, 1990), sulfonated aluminium phthalocyanine (Savitsky 1992;

Schultes *et al.*, 1992) and haematoporphyrin (Hamblin *et al.*, 1994). The target cells have mainly been neoplasms, however, Berthiaume *et al.* (1994) have shown that antibody against *Pseudomonas aeruginosa* (which they used to infect the dorsal skin of mice) conjugated with tin (IV) chlorin e_6 achieved 95 % reductions in viable bacteria when exposed to light at a wavelength of 630 nm.

To date, no work has been carried out to target oral bacteria using antibody conjugated to TBO. The aim of this study therefore was to specifically target *Por. gingivalis* to lethal photosensitisation when in the presence of *Streptococcus sanguis* or human gingival fibroblasts (HGF) using TBO conjugated to antibody against *Por. gingivalis* LPS.

8.2 MATERIALS AND METHODS

Por. gingivalis was maintained as described in chapter 2.

8.2.1 Preparation of Antibody-TBO conjugation

Antibody (2 mg) against *Por. gingivalis* lipopolysaccharide (kindly provided by Prof. Shepherd, Guy's Hospital) was added to 0.4 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Pierce Ltd.) which reacts with the carboxyl groups of the antibody molecule. This intermediate is unstable and therefore 1.1 mg of sulfo-N-hydroxysuccinimide (sulfo-NHS, Pierce Ltd.) was added (see figure 8.1).

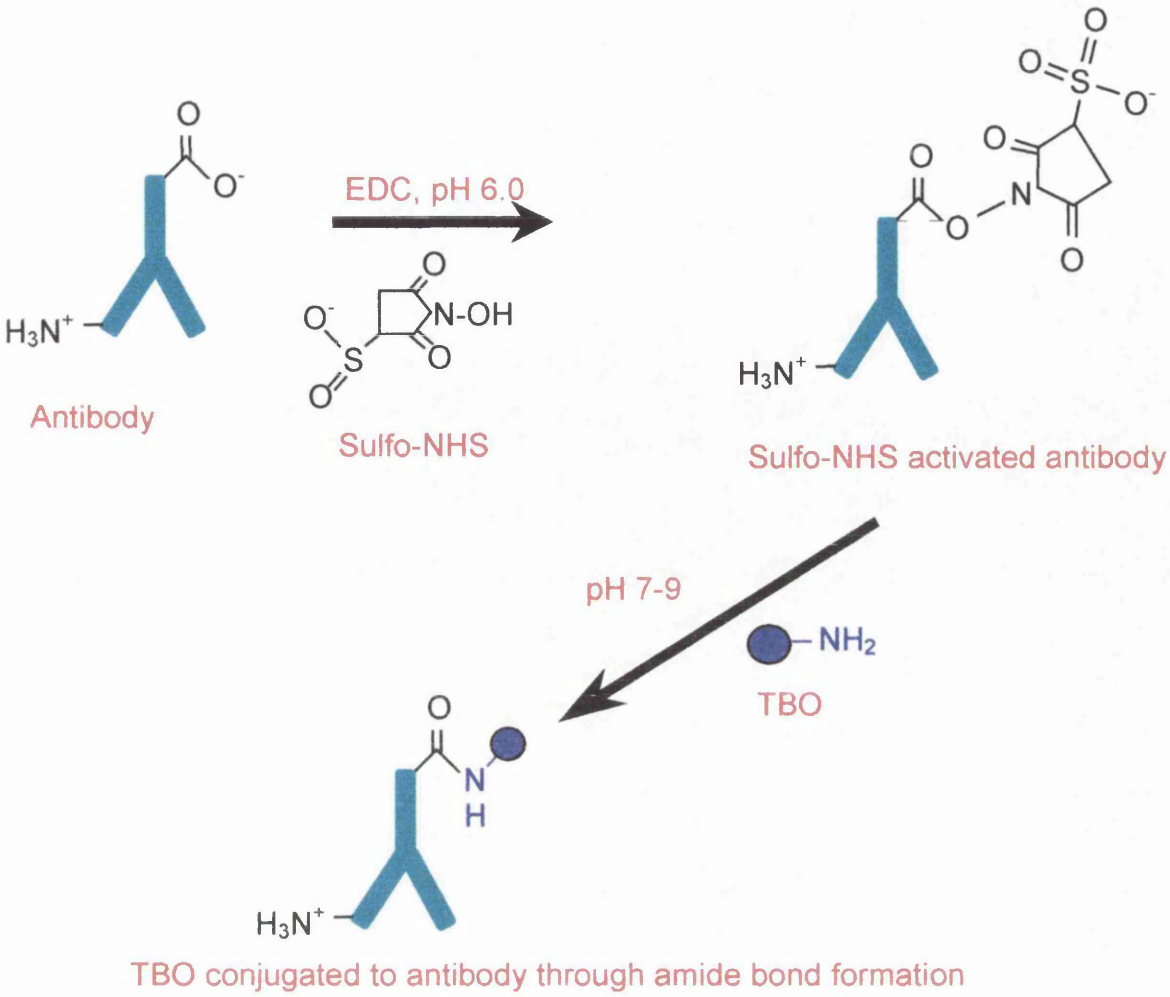


Figure 8.1 Schematic representation of the method used to conjugate antibody to TBO.

This was mixed continuously for 30 min at room temperature. TBO (5 mg) was added and the solution was mixed for a further 5 h at room temperature. The reaction was stopped by adding 20 mM ethanolamine. The solution was dialysed against buffer containing 0.15 M NaCl and 50 mM NaH_2PO_4 until the dialysis solution was no longer blue in colour. The Ab-TBO conjugate was concentrated using polyethylene glycol (PEG, Pierce Ltd.). In order to further remove any unconjugated TBO, the Ab-TBO solution was added to a filter unit (Sigma) and centrifuged at 1000 x g for 15 min. Dialysis buffer was added to the solution and the centrifugation was repeated. This procedure was carried out

until the filtrate was no longer blue. The absorbance of the TBO present in the conjugate was measured at 633 nm. It was found that 13 μM (4 μg) of TBO was conjugated to 2 mg of antibody.

8.2.2 Antibody-targeted lethal photosensitisation of *Por. gingivalis* and *S. sanguis*

Stationary phase cells of *Por. gingivalis* or *S. sanguis* were harvested and washed in sterile saline (refer to chapter 2, section 2.2 for maintenance of *S. sanguis*). The cells were resuspended in Ab-TBO conjugate and 100 μl of the suspension was aliquoted into a 96 well microtitre plate. Wells were exposed to laser light at a dose of 4.4 J (4.4 J/cm^2). The same procedure was carried out using free TBO (not conjugated to antibody) at a concentration of 13 μM. Control wells were neither sensitised nor exposed to laser light (L^-S^-). The same procedures were carried out on a suspension containing both *Por. gingivalis* and *S. sanguis*. Survivors were enumerated on FAA (*Por. gingivalis*) or tryptone soya agar (*S. sanguis*).

8.2.3 Antibody-targeted lethal photosensitisation of *Por. gingivalis* in the presence of human gingival fibroblasts

Human gingival fibroblasts (HGFs) were kindly provided by Dr Sajeeda Meghji (Department of Oral Maxillofacial Surgery, Eastman Dental Institute). The HGFs were maintained by fortnightly culturing in Dulbecco's Minimal Eagle's Medium (DMEM, Gibco UK) supplemented with 10 % fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin and incubated at 37 °C, 5 % CO_2. Antibody-targeted lethal photosensitisation of HGFs was carried out using the method below:

HGFs grown to confluence in 75 cm^3 flasks were washed twice with PBS.

↓

Attached cells were removed by treatment with 5 μg/ml trypsin

↓

Cells were seeded into 24-well plates at 15 000 HGFs/well

↓

Cells were incubated overnight at 37 °C at 5 % CO_2

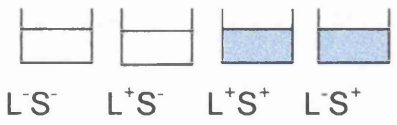
↓

Cells washed a number of times with PBS

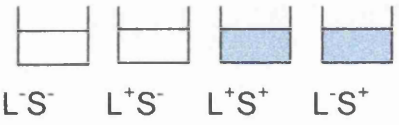
Por. gingivalis cells suspended in either saline or 13 μM free TBO (shown in blue) added

Por. gingivalis cells suspended in either saline or Ab-TBO (shown in blue) added

Laser light



Laser light



Por. gingivalis removed by washing HGFs thoroughly, and serial dilutions made. Survivors enumerated by plating on FAA agar

HGFs washed thoroughly with PBS and DMEM containing 18.5 kBq ^{3}H-Thymidine added to each well

Cells incubated overnight at 37°C.

↓

Cells washed thoroughly with PBS and TCA-insoluble material dissolved in 0.4 M NaOH. Incorporation of ^{3}H-Thy by HGFs measured by scintillation spectroscopy

8.3 RESULTS

Using free TBO, there was a 99.99 % (4 $\log_{10}$) reduction in the viable count of *S. sanguis* compared to a 97.5 % (1.5 $\log_{10}$) reduction in the case of *Por. gingivalis* using 4.4 J of light (figure 8.2). However, when the TBO was bound to antibody (Ab-TBO) against *Por. gingivalis* LPS, there was a 2 % (0.1 $\log_{10}$) reduction in viable counts of *S. sanguis*, and a 100 % (5 $\log_{10}$) reduction in the viable counts of *Por. gingivalis*.

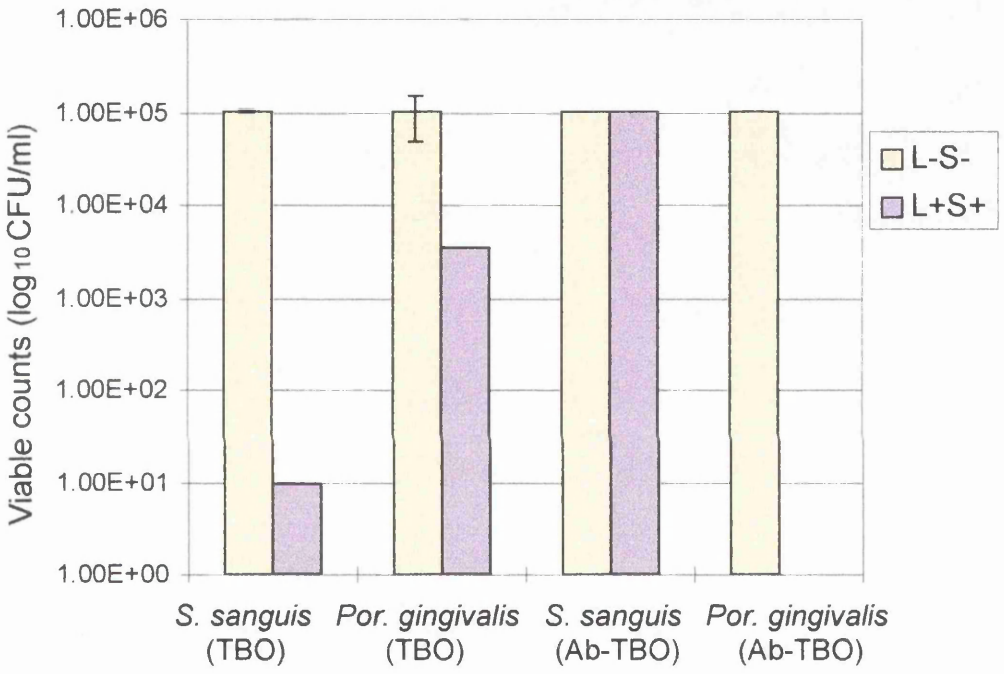


Figure 8.2 Viable counts of *Por. gingivalis* and *S. sanguis* sensitised with either free TBO or TBO conjugated to antibody (TBO-Ab) and exposed to 4.4 J of laser light. Stationary phase cells of *Por. gingivalis* or *S. sanguis* were resuspended in Ab-TBO conjugate or 13 μM free TBO and 100 μl of the suspension was aliquoted into a 96 well microtitre plate. Wells were exposed to laser light at a dose of 4.4 J. Control wells were neither sensitised nor exposed to laser light (L^-S^-).

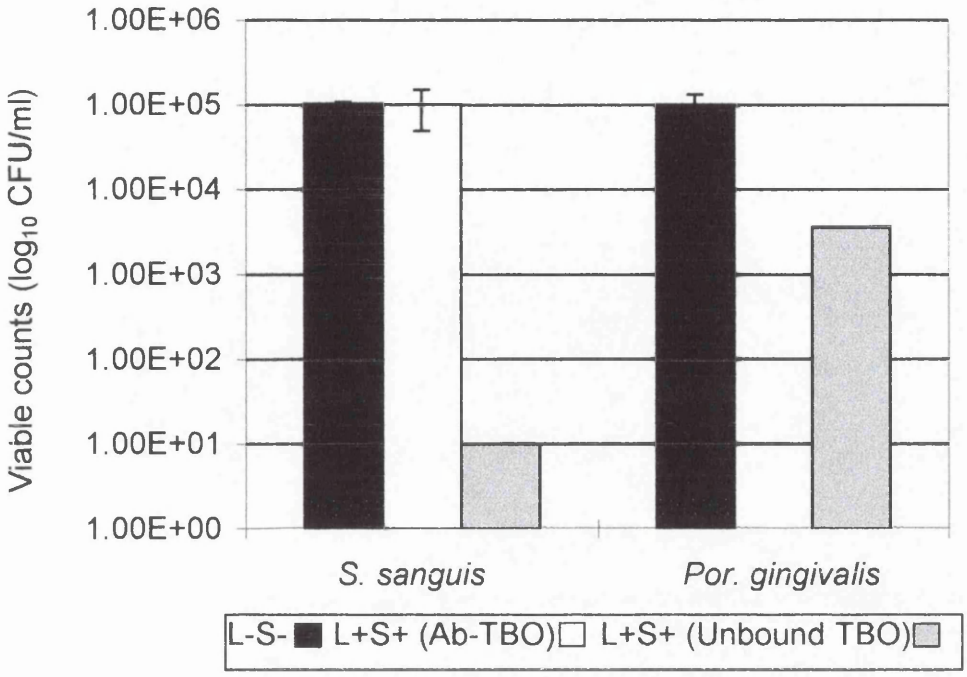


Figure 8.3 Viable counts of *Por. gingivalis* and *S. sanguis* treated with either free TBO or Ab-TBO and exposed to laser light when in mixed culture. Stationary phase cells of *Por. gingivalis* or *S. sanguis* were resuspended in Ab-TBO conjugate or free TBO and 100 μl of the mixed suspension was aliquoted into a 96 well microtitre plate. Wells were exposed to laser light at a dose of 4.4 J. The same procedure was carried out using free TBO (not conjugated to antibody) at a concentration of 13 μM. Control wells were neither sensitised nor exposed to laser light (L^-S^-).

Figure 8.3 shows the viable counts of *Por. gingivalis* and *S. sanguis* when in mixed suspension and sensitised with free TBO or Ab-TBO. There was a 4 log_{10} and a 1.5 log_{10} reduction in the viable count of *S. sanguis* and *Por. gingivalis*, respectively when sensitised with free TBO and exposed to laser light. When Ab-TBO was used, there was a 2 % decrease in the viable count of *S. sanguis*

and 5 $\log_{10}$ (100 %) decrease in the viable counts of *Por. gingivalis*.

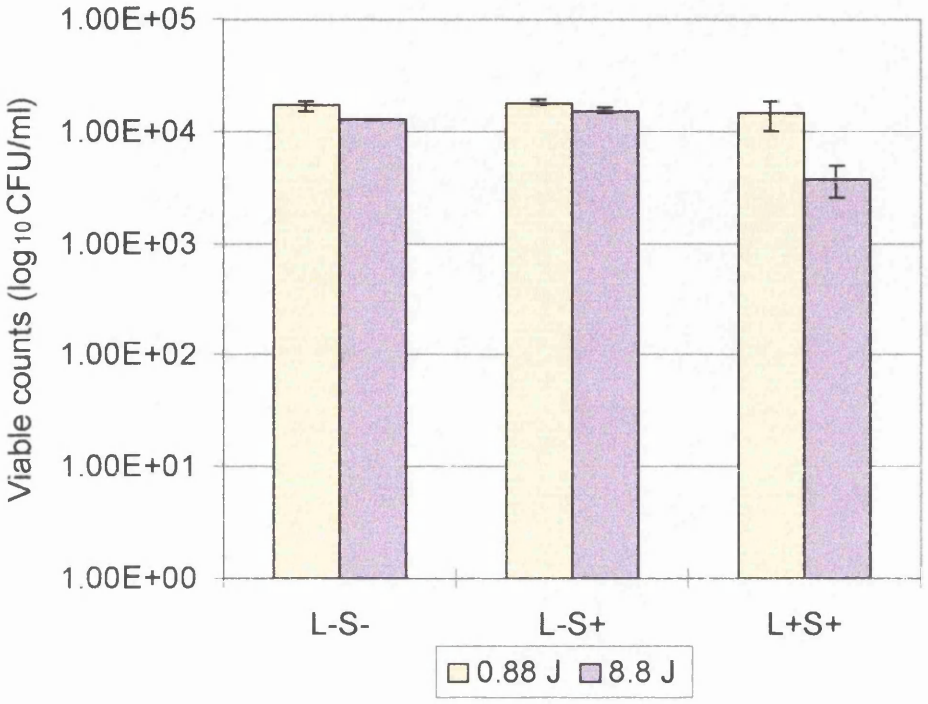


Figure 8.4 Viable counts of *Por. gingivalis* cells when sensitised with 13 μM TBO and exposed to laser light in the presence of HGFs. See section 8.2.3 for experimental details.

Figure 8.4 shows the reduction in viable counts of *Por. gingivalis* sensitised and exposed to laser light in the presence of HGFs. At a free TBO concentration of 4 μg/ml and a light dose of 0.88 J, there was no reduction in viable counts, and at a light dose of 8.8 J there was a 0.7 $\log_{10}$ reduction. However, in the presence of Ab-TBO, there was a 100 % kill at a light dose of 0.88 J (Figure 8.5).

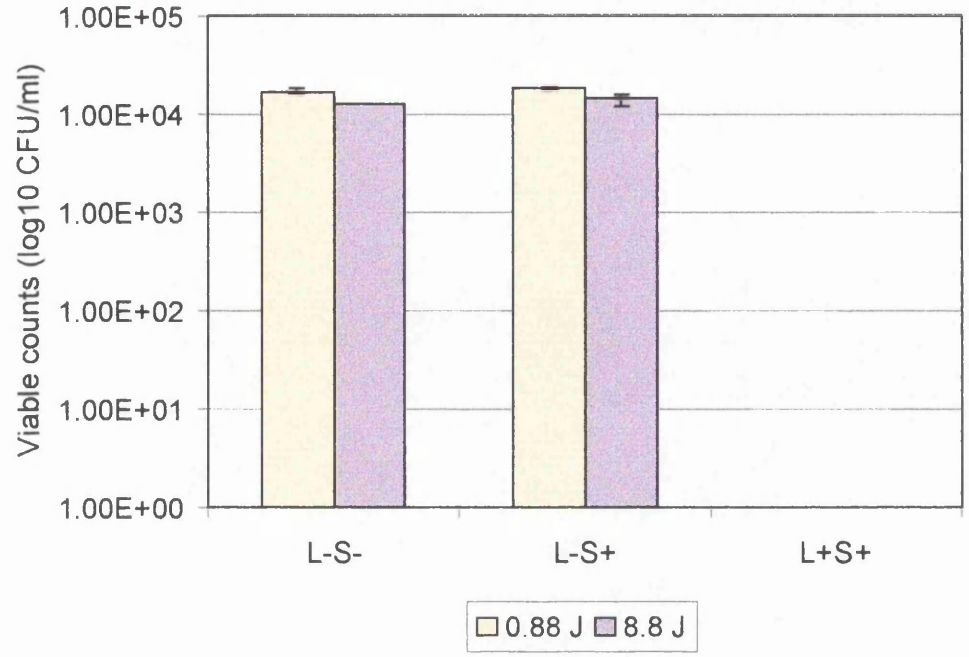


Figure 8.5 The viable counts of *Por. gingivalis* sensitised with Ab-TBO and exposed to increasing laser light in the presence of HGFs. See section 8.2.3 for experimental details.

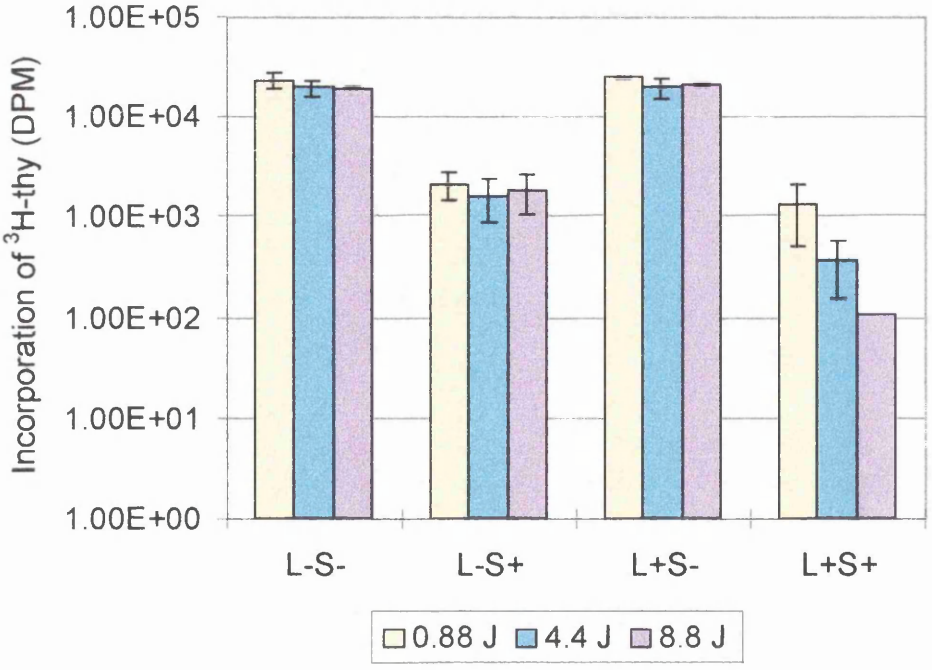


Figure 8.6 The incorporation of ^{3}H-Thy into HGFs sensitised with 13 μM TBO and exposed to increasing laser light doses in the presence of *Por. gingivalis*.

See section 8.2.3 for experimental details.

Figure 8.6 shows that in the presence of *Por. gingivalis*, at a free TBO concentration of 13 μM, there was a significant reduction in the incorporation of ^{3}H-thy into HGFs when exposed to increasing laser light doses compared to L^{-} S^{-}. There was a 94, 98 and 99 % reduction in incorporation of ^{3}H-thy into HGFs at 0.88, 4.4 and 8.8 J of laser light respectively. However, when Ab-TBO was used in combination with laser light, there were no significant reductions in the incorporation of ^{3}H-thy into HGFs (figure 8.7).

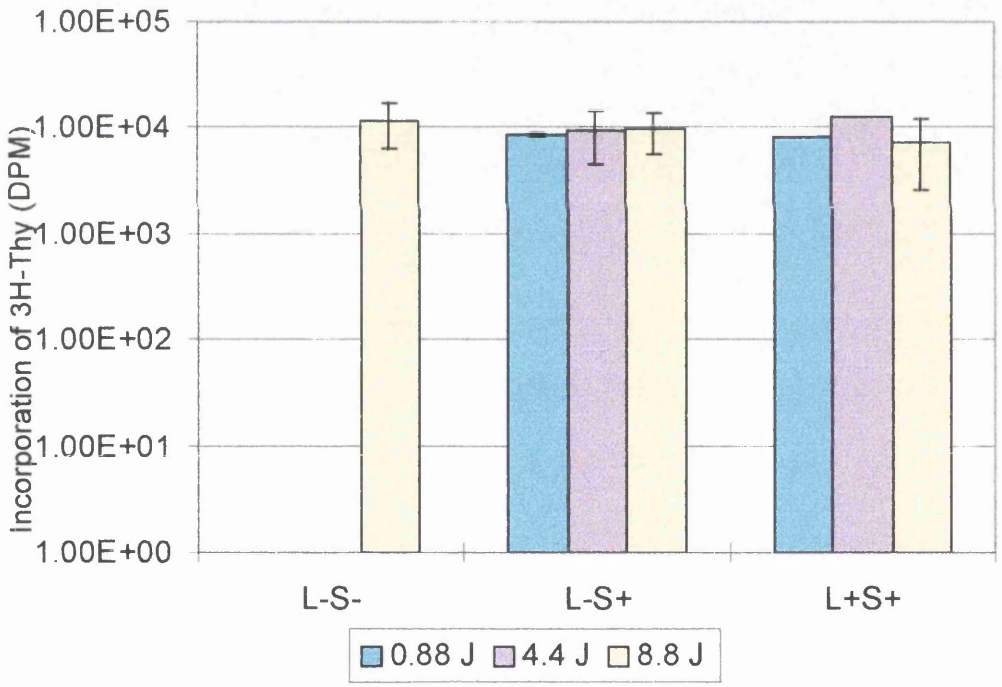


Figure 8.7 The graph shows the incorporation of ^{3}H-thy into HGFs after sensitising with Ab-TBO and exposing to increasing laser light doses in the presence of *Por. gingivalis*. See section 8.2.3 for experimental details.

8.4 DISCUSSION

When conjugating any photosensitiser to an antibody, it is desirable that on exposure to laser light, the cytotoxic yield should be the same as that when the photosensitiser is free (Strong *et al.*., 1994). Furthermore, as photosensitisers are sensitive to alterations in their structure (Dahl, 1992), conjugation of a photosensitiser to an antibody may alter the activity of the photosensitiser and specificity of the antibody (Dahl, 1992). The results show that in the presence of *S. sanguis* or HGFs, the reduction in viable counts of *Por. gingivalis* when sensitised with free TBO and exposed to laser light, was less than when sensitised with Ab-TBO. This suggests that if any alteration occurred to the structure of TBO on conjugation to antibody, it did not reduce the efficacy of the cytotoxic species produced.

The antibody used in the experiments was raised to *Por. gingivalis* LPS and it has been shown by Ní Eidhin and Mouton (1994) that these antibodies are highly specific to the bacteria. In fact, they found that antibody against *Por. gingivalis* LPS recognised 10 different *Por. gingivalis* strains but recognised none of the 34 non-*Por. gingivalis* strains of bacteria (22 *Prevotella* and 12 *Bacteroides* strains). This specificity is demonstrated well by the data within this chapter as *S. sanguis* was more prone to lethal photosensitisation than *Por. gingivalis* when using free TBO, however when Ab-TBO was used, there was a 100 % reduction in viable counts of *Por. gingivalis* cells compared to a 2 % reduction of *S. sanguis* on exposure to laser light. The 2 % kill may be due to some of the TBO dissociating from the antibody and binding to *S. sanguis* so that on exposure to laser light, there is some reduction in viable counts. Alternatively, it may be due to the binding of *S. sanguis* cells to TBO in the Ab-

TBO conjugate (see figure 8.8).

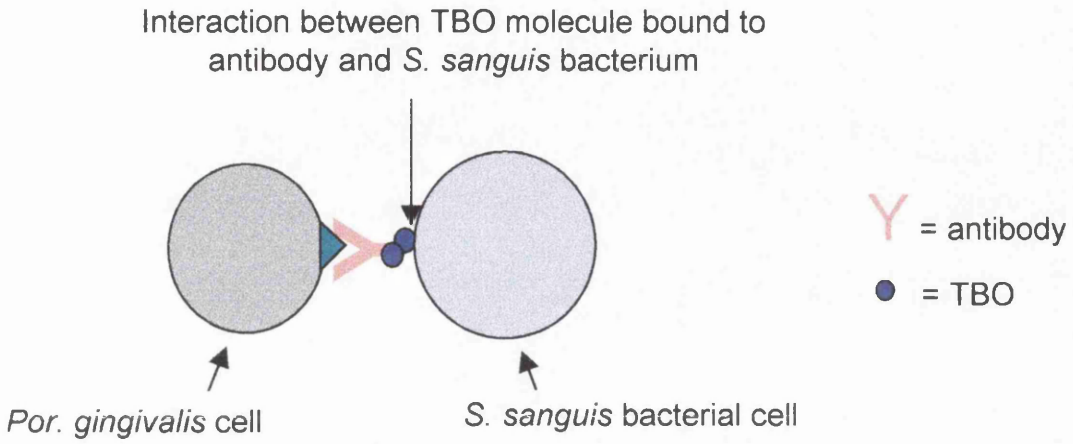


Figure 8.8 TBO conjugated to antibody against *Por. gingivalis* surface components can bind to *S. sanguis* bacterial cells which may lead to a small reduction in viable counts of *S. sanguis* on exposure to laser light.

The photosensitiser is linked to the antibody via the COOH terminus (figure 8.1) therefore cells in direct contact with this portion of the antibody should be more prone to cell death on exposure to laser light compared to the cell that the antibody is bound to. However, a possible reason why this does not occur is shown in figure 8.9. Photosensitisers and especially TBO have a tendency to aggregate, more so when in the presence of proteins (Philips, 1997). Therefore, TBO molecules in the conjugate aggregate, and as a consequence cause the aggregation of *Por. gingivalis* cells. This arrangement would not allow many *S. sanguis* cells to come into contact with the TBO bound to antibody.

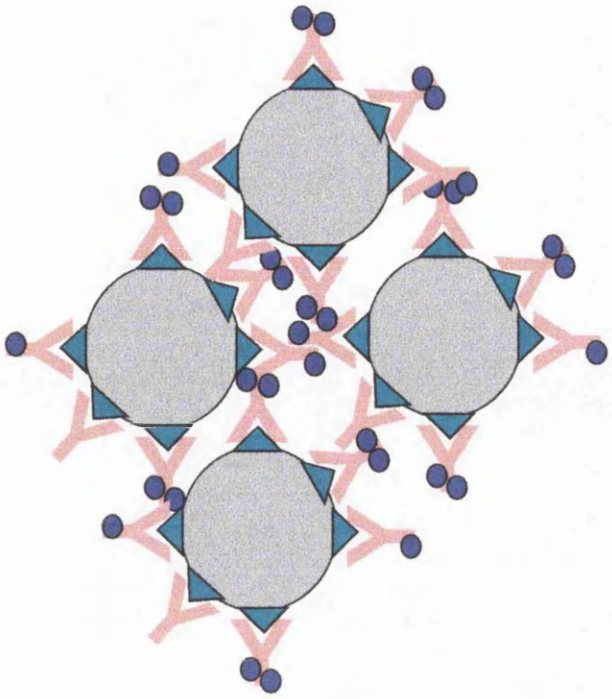


Figure 8.9 The TBO (blue dots) bound to antibody molecules (pink 'Y') aggregate causing aggregation of *Por. gingivalis* cells (grey circles, green triangles = antigen). The production of cytotoxic species is localised within the aggregate leading to higher kills of *Por. gingivalis* compared to *S. sanguis*.

In the presence of HGFs, the reduction in viable counts of *Por. gingivalis* sensitised with free TBO and exposed to 0.88 and 4.4 J laser light is much lower than when in pure culture (chapter 3). When HGFs (in the presence of *Por. gingivalis*) were sensitised with free TBO and exposed to increasing laser light doses, there was a light dose-dependent decrease in ^{3}H-thy incorporation. This would suggest that free TBO has a greater affinity for HGFs than for *Por. gingivalis*. Conjugation of the photosensitiser with antibody led to 100 % kills of *Por. gingivalis* at 0.88 J and no reduction in the incorporation of ^{3}H-thy by HGFs as the light dose was increased.

It was shown by Kontani *et al.* (1996) that a protease of *Por. gingivalis* 381 (molecular weight of 47 kDa determined by SDS-PAGE) enhanced the binding of fimbriae to HGFs and matrix proteins. The protease was found to degrade host proteins including collagen and fibronectin. They showed that treating HGF monolayers with the protease caused a significant increase in the binding of purified fimbriae to the cells compared to untreated HGF monolayers. As *Por. gingivalis* W50 possess fimbriae, it is highly probable that a similar mechanism to *Por. gingivalis* 381 is involved in binding to HGFs. It was shown in chapter 3 that lethal photosensitisation caused a decrease in *Por. gingivalis* protease activity, which suggests that if PDT was used *in vivo*, the binding capacity of *Por. gingivalis* to HGFs would be greatly compromised and yet no adverse effects would be incurred to HGFs.

8.5 SUMMARY

1. Specific killing of *Por. gingivalis* can be achieved in the presence of *S. sanguis* by binding TBO to antibody against *Por. gingivalis.*
2. *Por. gingivalis* can be killed when in the presence of HGFs without any effect on the viability of the HGFs when TBO is bound to antibody against *Por. gingivalis*

CHAPTER NINE

GENERAL DISCUSSION

9.1 AIMS

The aims of the study were:

(1). To compare the reductions in viable counts of *Por. gingivalis* when sensitised with TBO and exposed to light from a HeNe laser, or sensitised with $AlPcS_2$ and exposed to GaAs laser light.

(2). To study the effect on lethal photosensitisation of dosimetric and physiological factors most likely to be encountered in the periodontal pocket.

(3). To compare the uptake of TBO by *Por. gingivalis* under the dosimetric and physiological conditions studied in (2).

(4). To elucidate the mechanism of lethal photosensitisation; i.e. whether a type I and/or type II mechanism is/are involved.

(5). To elucidate the targets of lethal photosensitisation and effects on bacterial ultrastructure.

(6). To target *Por. gingivalis* to lethal photosensitisation when in the presence of *S. sanguis* and host cells by conjugating TBO to antibody against *Por. gingivalis*.

9.2 General findings

Por. gingivalis was found to be susceptible to lethal photosensitisation when sensitised with TBO and exposed to HeNe laser light and when sensitised with $AlPcS_2$ and exposed to GaAs laser light. Further studies using TBO showed that increasing the time that the bacteria were incubated with TBO before exposure to laser light did not cause an increase in the kills. As the periodontal pocket is bathed in a serum-like exudate (gingival crevicular fluid), lethal photosensitisation was

carried out in the presence of horse serum. There was a decrease in the numbers of *Por. gingivalis* killed in the presence of serum compared to saline. This may be because the TBO bound to serum proteins and the cytotoxic species produced were quenched before reaching the bacterial cells. The pH of the periodontal pocket is around 7.4, therefore lethal photosensitisation was carried out in the presence of increasing pH values (6.8, 7.3 and 8.0) and the highest kills were obtained at pH 7.3. Five different strains of *Por. gingivalis* were tested for their susceptibility to lethal photosensitisation, and it was found that all five strains were susceptible. One of the important virulence factors of *Por. gingivalis* is its protease activity. The protease activity of *Por. gingivalis* was reduced by TBO alone but to a greater extent by TBO plus laser light. The fact that protease activity can be a reduced by lethal photosensitisation is of significance *in vivo* as this may result in a reduction in degradation of host antibody and cytokines as a result of lethal photosensitisation.

As *Por. gingivalis* may be present *in vivo* in plaque as well as GCF, the lethal photosensitisation of biofilm-grown *Por. gingivalis* was compared to planktonic cultures. It was found that biofilm-grown *Por. gingivalis* was less susceptible to lethal photosensitisation compared to planktonic cultures as the energy dose required to obtain 100 % kill of biofilm-grown bacteria was 79 J compared to 6.6 J when cells were in the planktonic form. The reason for this may be that TBO and/or light cannot penetrate through the depth of the biofilm hence giving an overall reduction in the numbers of bacteria killed.

9.3 Uptake and mechanistic aspects

In order to study whether the uptake of the photosensitiser by *Por. gingivalis* influenced lethal photosensitisation, the uptake of ^{3}H-TBO was studied using the same conditions as those used for the lethal photosensitisation studies.

Parameters tested	Photosensitisation studies	Uptake studies
Concentration of TBO	Kills similar at 41-164 μM TBO and decreased slightly at 164μM	Uptake was increased as concentration of ^{3}H-TBO was increased from 82-656 μM
PIT	Similar kills were obtained as the PIT increased (1-15 min)	Of the total amount of ^{3}H-TBO taken up, 75 % was taken up within the first 60 s of incubation
PH	Kills greatest at pH 7.4 compared to 6.8 and 8.0	Uptake was greatest at pH 7.4 at 82 μM TBO
Serum	Kills were significantly decreased	Uptake was 2.5 fold lower in the presence of serum
Growth phase	Stationary phase cells were less susceptible than lag and log phase cells	Uptake by log phase cells was greater than the other two phases

Table 9.1 The table shows that on the whole, photosensitiser uptake did influence lethal photosensitisation.

As uptake of the photosensitiser by *Por. gingivalis* was found to influence lethal photosensitisation of the bacteria, and of the TBO distributed within the cell (most was bound to the outer membrane 86.7 %), the next question addressed was, is

the bound fraction essential for killing when exposed to laser light? It was found that this fraction may be responsible for most of the reduction in viable counts on exposure to laser light. This may in turn suggest that a type I reaction is involved in cell death. A type II reaction may also be involved in cell death as lethal photosensitisation in the presence of D_2O led to a 100 % decrease in viable counts compared to a 28 % decrease in the presence of H_2O at the same light dose. Figure 9.1 below summarises the data which may demonstrate that both type I and type II reactions are involved in lethal photosensitisation of *Por. gingivalis* using TBO and HeNe laser light.

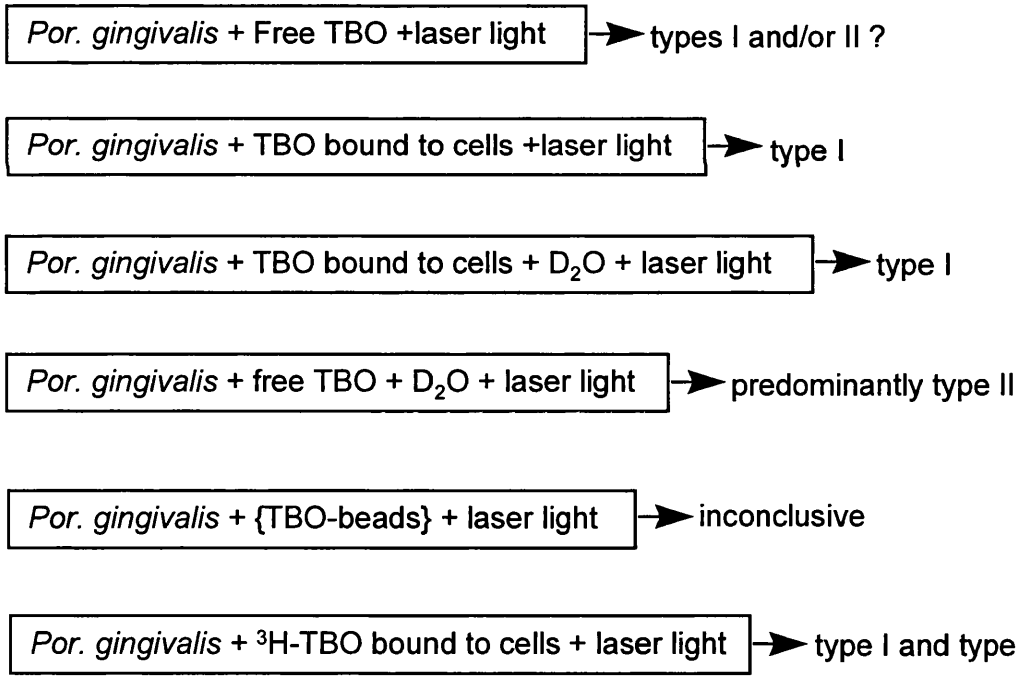


Figure 9.1 The diagram shows how the experiments carried out may provide evidence that both type I and type II reactions could be involved in lethal photosensitisation.

It is important to include the fact that there may be quenching of any oxygen species by iron protoporphyrin IX present in *Por. gingivalis* in the μ-oxo bishaem form. This may lead to a decrease in the overall efficiency of lethal photosensitisation.

There was a 33 % decrease in the amount of ATP present in cells sensitised with TBO and exposed to laser light compared to control cells. This decrease is most likely due to disruption of energy-generating mechanisms by cytotoxic species rather than merely reflecting cell death.

No lipid peroxidation as a result of lethal photosensitisation was detected. However, there was evidence of membrane damage as ^{3}H-TBO was internalised when cells sensitised with TBO were exposed to laser light.

The effect of lethal photosensitisation on *Por. gingivalis* proteins and DNA showed that high molecular mass proteins from the outer membrane, and high and low molecular mass proteins from the plasma membrane, were adversely affected. DNA from treated samples was damaged possibly due to single strand breaks.

The ultrastructure of *Por. gingivalis* was altered by lethal photosensitisation. There was contraction of the cytoplasm, and condensation of the membrane. SEM studies showed there was co-adherence of the cells and strand-like structures linking two cells together which may be evidence that cellular division is adversely affected by

lethal photosensitisation.

The fluidity of the *Por. gingivalis* membrane decreased as a result of lethal photosensitisation, which indicates that lipid peroxidation most probably occurred although it could not be detected by the usual method of detecting the production of the lipid peroxidation product, MDA. The membrane was most rigid at the physiological pH of 7.4; however membrane fluidity alone may not influence the uptake of TBO at different pH values.

Por. gingivalis was specifically targeted to killing by laser light by conjugating TBO to antibody against the bacterial surface components. It was found that sensitising a mixed culture of *Por. gingivalis* and *S. sanguis* using Ab-TBO conjugate and exposing to laser light, 100 % kill of *Por. gingivalis* was obtained whereas only a 2 % kill of *S. sanguis* was obtained. It was also found that *Por. gingivalis* can be killed when in the presence of HGFs without any effect on the viability of the HGFs when using Ab-TBO in combination with laser light.

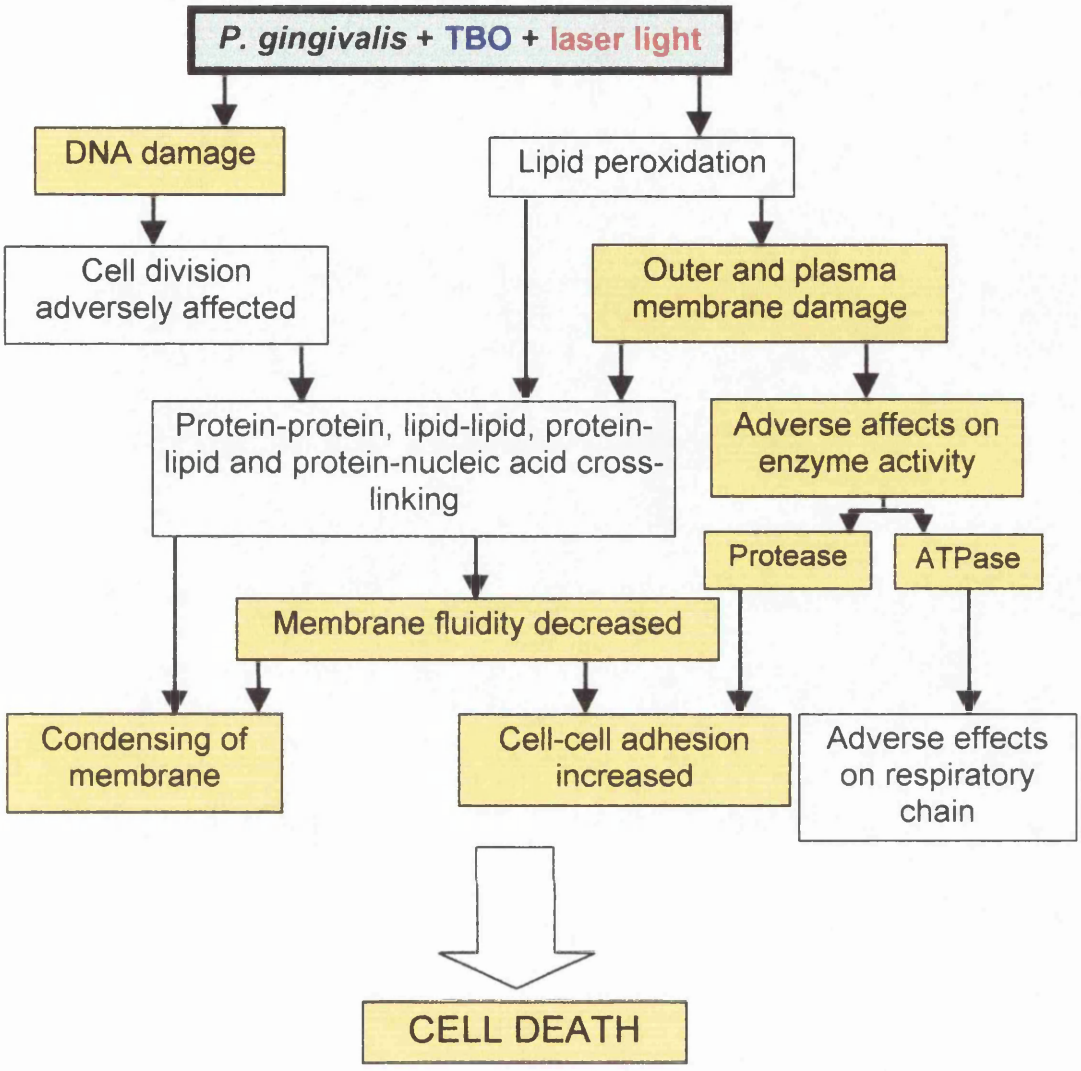


Figure 9.2 Flow chart summarising the cascade of events leading to cell death as a result of photosensitisation. Yellow boxes = results found through experimentation. Clear boxes = possible occurrences.

The flow-chart shows that alterations in the ultrastructure of the cells probably ensued as a result of the damage caused by cytotoxic species to the outer and plasma membrane proteins and DNA. For instance, condensing of the membrane seen by TEM may have been due to cross-linking between proteins and lipids. SEM

studies demonstrated abnormal bacterial clumping and dividing which might have been a consequence of DNA damage.

The lethal photosensitisation of *Por. gingivalis*, using both negatively and positively charged photosensitisers in combination with laser light, has lead to an understanding of the effect of lethal photosensitisation on Gram-negative bacteria and an insight into the mechanisms involved. As it was possible to kill the bacteria under various physiological conditions most likely to be encountered in the oral cavity, it is possible that this method could be applied *in vivo*. The main problems that need to be addressed when treating periodontitis using PDT are that firstly, the photosensitiser does not sensitise the surrounding healthy tissue and secondly, the balance of the commensal oral flora is not disrupted. In order to resolve these issues, the photosensitiser could be applied directly to the periodontal pocket with a blunt syringe. Blunt syringes have been used successfully in clinical trials for the direct application of tetracycline and chlorhexidine to the periodontal pocket (Unsal *et al.*, 1994). Laser light could then be delivered directly to the sensitised pocket by using an optical fibre or laser probe that is small enough to fit inside the pocket (Welch *et al.*, 1987). However, it is possible that the TBO could flow into the oral cavity and sensitise the surrounding tissue. One method of localising the photosensitiser to a specific treatment site may be to use the method employed by Jones *et al.*, (1996). Work they carried out on a syringeable bioadhesive containing tetracycline showed that the antibiotic was released into the periodontal pocket at a rate of 1.59-15.8 mg/h

depending on the amount of hydroxyethylcellulose (HEC) the tetracycline-containing bioadhesive contained. They found that the lower the amount of HEC in the bioadhesive, the more rapid the release of tetracycline. TBO could be incorporated into the bioadhesive containing a low concentration of HEC so that the photosensitiser is released over a short period of time. The advantage of using this type of delivery method is that the release would be localised to the pocket as the bioadhesive is flexible and syringeable and therefore takes on the shape of the pocket. However, as the authors point out, the flexibility may compromise the efficacy of antimicrobial release.

If either a solution of TBO or an immobilised delivery method is used, it is possible that in both cases, the serum-like exudate (gingival crevicular fluid) would reduce the uptake of TBO by the bacteria *in vivo*, which was demonstrated *in vitro* using horse serum. Also, if there is any blood present in the pocket, there may be quenching of any cyotoxic species produced. Rinsing the patients' pocket with water before applying the photosensitiser may circumvent this. Another physiological factor to be considered is the oxygen tension of the periodontal pocket. The oxygen tension is 1-2 % (Loesche, 1994), and the amount of oxygen would be further depleted by lethal photosensitisation as the singlet oxygen produced arises from ground-state oxygen. The rates of singlet oxygen depletion are high when both tissue photosensitiser levels and the light energy density are high (Dougherty *et al.*, 1998). A way of decreasing the oxygen depletion is to expose the sensitised area to short bursts of light. This would consist of short

periods of light and dark intervals (20-50 s) allowing regeneration of oxygen during the dark period (Foster *et al.*, 1991).

In order to avoid any possible damage to host tissue and commensal bacteria, it is pertinent to use a system that will only kill pathogenic bacteria and leave any surrounding tissue intact. The work in chapter 8 demonstrated that using Ab-TBO conjugate in combination with laser light effectively killed *Por. gingivalis* whereas there were no significantly adverse affects on *S. sanguis* or HGFs. As a variety of pathogenic bacteria may be present in the periodontal pocket, antibodies against each pathogen could be conjugated to TBO and a cocktail of Ab-TBO conjugates could be applied to the pocket thus ensuring selective killing of periodontopathogenic organisms.

9.4 Advantages of using PDT for the treatment of periodontitis

i) As it was found (*in vitro*) that *Por. gingivalis* is sensitised to laser light within 60 s, the length of time the patient has to sit with the TBO in the periodontal pocket before laser light exposure should not be a problem.

ii) Laser light alone has no adverse effects on host tissue or bacteria, cytotoxic species are only produced when TBO is used in combination with laser light. Therefore, if the method of localising the photosensitiser to the periodontal pocket is used as described above, any scattering of laser light within the oral cavity should not cause any damage to surrounding healthy tissue.

iii) One of the key host defence mechanisms against bacteria involves their killing by singlet oxygen, superoxide anions and superoxide radicals produced during phagocytosis by polymorphonuclear leukocytes. This defence mechanism has been used for millennia, implying that bacteria find it difficult to develop resistance to these cytotoxic species.

iv) With conventional treatments such as the use of orally administered antibiotics there is a perturbation of the intestinal microflora (Nord and Edlund, 1990). There are also many side-effects associated with antibiotics, such as hepatic damage, gastrointestinal disorders and cutaneous reactions (Westphal *et al.*, 1994). These types of adverse affects would be unlikely with PDT as the photosensitiser and laser light are applied directly to the periodontal pocket.

9.5 CONCLUSION

The work in this thesis has suggested that *Por. gingivalis*, an important periodontopathogen, can be killed effectively by lethal photosensitisation under various dosimetric and physiological conditions that may be encountered *in vivo*. It has also shown that damage to commensal and host cells can be avoided by sensitising the bacteria with TBO conjugated to antibody against *Por. gingivalis*. If this method works as well *in vivo* as it does *in vitro*, it may form a basis of a possible treatment for periodontal diseases.

9.6 FUTURE WORK

As *in vitro* studies have shown that PDT works well against *Por. gingivalis,* the next step would be to try this approach in the clinic. The main issues to be addressed would be: i) the concentration of photosensitiser and the method of application to the periodontal pocket; ii) laser light dose and method of light delivery. Once these issues had been addressed and the method refined, it may be possible to use PDT either in combination with mechanical debridement or on its own to successfully treat patients suffering from periodontal diseases.

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