Understanding the Interactions of Hydrogen Peroxide with Macromolecules and Microbial Components.

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The bactericidal mechanism of hydrogen peroxide is poorly understood, with most evidence being obtained from studies involving greatly reduced concentrations aimed at investigating the effects of stress. Current theory suggests that this mechanism is based on the oxidation of protein, DNA and lipids within the cell by the production of free hydroxyl radicals through the interaction of hydrogen peroxide and intracellular iron. The mechanism of vapour phase hydrogen peroxide treatment remains unstudied, despite evidence that it may be different to the liquid phase.

This study aimed to investigate the effects of bactericidal treatments of liquid and vapour phase hydrogen peroxide on the macromolecular components of a model organism, *Escherichia coli* strain K12. A set of treatment conditions producing a range of reductions in colony forming units was identified, and the effects of these conditions on the protein, DNA and lipid constituents of the cells assayed.

No effect on the lipid contents and membrane integrity of treated cells was found. Liquid hydrogen peroxide was found to reduce the thiol content of cytoplasmic protein, but this was not found to be a major mechanism of bactericidal action. Extensive fragmentation of DNA was found to result by treatment with both phases, the degree of which was correlated with a reduction in colony forming unit counts. No effect on bactericidal action was found on addition of a hydroxyl radical scavenger or an inhibitor of protein synthesis, showing that DNA damage was due to the primary action of hydrogen peroxide, and that this damage was not caused by the production of free radicals within the cell.

A modified mechanism of hydrogen peroxide bactericidal action is proposed, whereby lethality is due solely to DNA damage caused by the production of ferryl radicals by the interaction of hydrogen peroxide and iron associated with the DNA backbone.

Contents

Chapter 1

Introduction.

1.1   Properties of hydrogen peroxide.

Hydrogen peroxide (H_2O_2) was first produced by Louis Thénard in 1818 by reacting barium peroxide with nitric acid (Thénard, 1818). It is the simplest possible peroxide molecule, consisting of two hydrogen and two oxygen atoms bonded in an anti-clinal skewed confirmation, the oxygens joined by a single bond, rotationally restricted by non-bonding electrons, and the hydrogens each bonded to a single oxygen, as shown in figure 1.1.
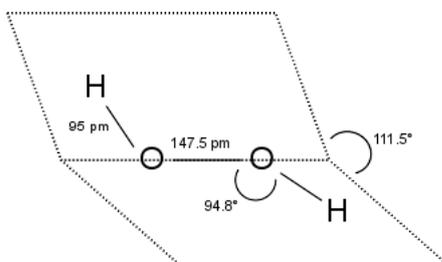
Figure 1.1: Structural formula of hydrogen peroxide

Having a wide-range of industrial applications, H_2O_2 is manufactured in large quantities (approximately 3 million tons per year) by the Riedel-Pfleiderer process. This commonly consists of the autoxidation of 2-ethyl-9,10-dihydroxyanthracene to the corresponding 2-ethyl anthraquinone and hydrogen by bubbling compressed air through a solution containing the anthracene. The H_2O_2 is then extracted and the anthracene regenerated from the anthraquinone by the addition of hydrogen gas in the presence of a

metal catalyst (Jones, 1999). H_2O_2 is considered an oxidizing agent, that is it generally reacts by accepting electrons, and the redox potential for the half-reaction of H_2O_2 to water (shown in figure 1.2) is 1.76 V.

$$H_2O_2 + 2H^+ + 2 \; e^- \quad 2H_2O$$
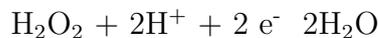
Figure 1.2: The half-reaction of hydrogen peroxide to water.

This relatively high redox potential (considerably higher than hypochlorous acid at 1.49 V and chlorine dioxide at 1.27 V, both potent oxidizing biocides) would suggest that H_2O_2 is a powerful oxidizing agent, however such is the stability of the molecule that a high activation energy input is required to start the oxidizing reaction. H_2O_2 is therefore only able to react directly with a limited number of compounds, and normally requires activation of some sort in order to react. One of the most common types of activation relies on the scission of the relatively weak O-O bond, which can be broken by thermal, photolytic, radiolytic and metal-redox methods to give hydroxyl radicals. These are extremely potent oxidizing agents, and only fluorine is known to have higher oxidizing power (Jones, 1999).

1.2   Fenton chemistry.

In 1876, H.J.H Fenton described a reaction between iron sulphate, H_2O_2 and tartaric acid, noting that a violet colour arose when ferrous (iron II) sulphate was used, but not when ferric (iron III sulphate) was substituted (Fenton, 1876). Consequently, this mixture of H_2O_2 and ferrous ions is referred to as Fenton's reagent, and the reactions dependent on it as Fenton chemistry. Haber and Weiss (1934) suggested that such reactions involving Fenton's reagent are driven by the one-electron reduction of H_2O_2 by Fe2+ to produce OH• radical (a radical is a species containing an unpaired electron, which is consequently unstable and reactive), and it is this extremely reactive radical which is responsible for further oxidations. Barb *et al.* (1951) modified the mechanism proposed by Haber and Weiss to give the set of reactions shown in figure 1.3:

1. $Fe^{2+} + H_2O_2$ $Fe^{3+} + OH\bullet + OH^-$
2. $H_2O_2 + OH\bullet$ $H_2O + HO_2\bullet$
3. $Fe^{3+} + HO_2\bullet$ $Fe^{2+} + O_2 + H^+$
4. $Fe^{2+} + OH\bullet$ $Fe^{3+} + OH^-$
5. $Fe^{2+} + HO_2\bullet$ $Fe^{3+} + HO_2^-$

Figure 1.3: The mechanism proposed by Barb *et al.* for the Fenton reaction.

Steps 1-3 of Barb's mechanism act as a cycle, with oxygen evolved and the ferrous ion regenerated. Steps 4 and 5 are the termination of the cycle. Barb *et al.* noted the importance of the relative ratios of ferrous ion and H_2O_2 for the oxidative effects of Fenton's reagent – if H_2O_2 is present in excess compared to ferrous ions, steps 1-3 will function as a cycle, with oxygen produced and the ferrous ion regenerated whilst an excess of ferrous ion will lead to termination of the reaction with ferric ion produced and no oxygen evolved. This mechanism, perhaps surprisingly for what appears to be rather simple chemistry that has been explored for well over a century, remains controversial. It has been suggested that the reactive product is not the hydroxyl radical, but rather the ferryl ion $[Fe(IV)O]^{2+}$. Further studies suggest that the reaction produces some unknown weakly acidic intermediate, and that the reactive product depends on the pH of the system with low pH resulting in the production of ferric ion and hydroxyl radical whilst high pH results in the production of ferryl ion (Barbusiński, 2009). Both species are powerful oxidants, however, and it has been suggested that Fenton or Fenton-like chemistry is important in biological systems. Due to the low concentrations of iron naturally present in biological systems, the presence of a functional metal redox-cycling mechanism is important to drive Fenton chemistry. The superoxide radical anion ($O_2^-\bullet$) has been proposed to play the role of the reducing agent in such a mechanism, as shown in figure 1.4 (Prousek, 2007).

1. $LFe^{3+} + O_2^-\bullet$ $LFe^{2+} + O_2$
2. $LFe^{2+} + H_2O_2$ $LFe^{3+} + HO\bullet + HO^-$
Or,
3. $LFe^{2+} + H_2O_2$ $L[FeO]^{2+} + H_2O$

Figure 1.4: Superoxide radical anion driven Fenton chemistry in biological systems. LFe^{n+} represents an iron ion co-ordinated with some biological ligand.

These reactive products are then able to participate in a number of classes of reaction, as summarized in figure 1.5 (Prousek, 2007).

i. Hydrogen abstractions:
$$R\text{-}H + HO\bullet \quad R\bullet + H_2O$$
ii. Addition reactions:
$$ArH + HO\bullet \quad HO\text{-}ArH\bullet \quad R_2C{=}CR_2 + HO\bullet \quad HO\text{-}C(R)_2\text{-}C\bullet(R)_2$$
iii. Oxidation reactions:
$$ArH + HO\bullet \quad HO^- + ArH{+}\bullet \quad Mn^+ + HO\bullet \quad HO^- + M(n{+}1)^+$$
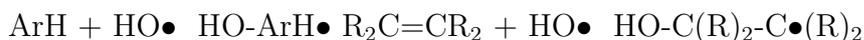
Figure 1.5: Classes of reaction undertaken by hydroxyl radical product of Fenton chemistry. Ferryl ions are believed to participate in similar reactions.

By these mechanisms, relatively unreactive H_2O_2 can oxidise diverse organic species in the presence of iron ions within the cell.

1.3 Hydrogen peroxide solution as a sterilant.

Throughout this thesis 'disinfectant' is taken to mean a biocide that kills all the vegetative bacteria on a surface (effectively, a greater than 6 log reduction), whilst 'sterilant' is taken to mean a biocide that kills all microorganisms including spores on a surface. The use of H_2O_2 solution as a disinfectant was first proposed by B. W. Richardson (1891) after he discovered its ability to neutralize foul odours. Its use as a general antimicrobial and disinfectant increased rapidly, peaking in the early decades of the twentieth century – C.B. Jordan (1913) wrote "The history of H_2O_2 is that of a chemical curiosity rapidly becoming so useful that it can be found in nearly every household, and so important that we have come to feel that it is next to impossible to get along without it." H_2O_2 solution remains in widespread use as a biocide in particular applications where its decomposition into non-toxic by-products (water and oxygen) is important. For example, the commercial dental disinfectants Dentasept (Muller Dental) and Oxfigenal (Kavo) use 1% and 0.4% H_2O_2 as an active ingredient respectively (Walker *et al.*, 2003), whilst many contact lens disinfectant solutions use H_2O_2 as an active ingredient; Concerto (Essilor), Oxysept 1 Step (Abbott), Multi (Sauflon) and AOSept 1-step (Ciba Vision) all use 3% H_2O_2 as the active ingredient (Hughes and Kilvington, 2001). The relative safety of H_2O_2 solutions has also meant that it had found extensive use in the food industry. For example, Sapers and Sites (2003) discuss a commercial postharvest wash (Biosafe) that uses H_2O_2 as an active ingedient at an in-use concentration of between 0.27 and 0.54%, and a surface disinfectant (Sanosil-25) with an in-use concentration of 0.24% H_2O_2 and their use in post-harvest washing of fruits and vegetables. They also demonstrate the efficacy of 1% H2O

as a wash to decontaminate apples. Perhaps the most important use of H_2O_2 solutions as a biocide is in packaging: Nikkah *et al.* (2008) state that "Hydrogen peroxide is the most commonly used packing sterilant in aseptic processing systems". Typically it is used at very high concentrations (35%) and often in combination with heat (Toledo, 1988).

Efficacy of H_2O_2 solution as a bacteridal agent has been demonstrated for a number of organisms, including vegetative bacteria, bacterial spores, fungi, yeasts, viruses and protozoa (Block, 2001). Table 1.1 presents a summary of the results of several studies investigating the bactericidal efficacy of H_2O_2 solution against vegetative bacterial species in suspension and table 1.2 presents a similar summary for spores.

1.4   Factors effecting hydrogen peroxide solution bactericidal efficacy.

The efficacy of H_2O_2 as a biocide is dependent on the concentration used. A kinetic study by Lambert *et al.* (1999) using *S. aureus* and *P. aeruginosa* exposed to H_2O_2 concentrations between 50 and 200 mM (0.17 % and 0.68 %) at pH 4 gave a value for the dilution coefficient of 1.247 for *S. aureus* analysed using the Chick-Watson model, 1.306 analysed using the Hom model, and 1.069 for *P. aeruginosa* analysed using both models. A study by Labas *et al.* (2008) using *E. coli* strain ATCC 8739 exposed to between 15 and 350 ppm (0.0015% and 0.035%) gave a value for the dilution coefficient of 0.293 when analysed using a Modified Events in Series model and 0.420 when analysed using a Modified Multitarget model. The effect of pH on the efficacy of H_2O_2 bactericidal activity has not been widely reported, however it appears to be relatively unimportant compared with some other bactericides. A study by Thomas *et al.* (1994) on *Streptococcus mutans* found that the ED_{50} (that is, the concentration required to produce a 50 % inhibition of metabolism, as measured by lactate dehydrogenase activity) values for H_2O_2 were 1.9, 1.9 and 2.7 mM (0.006, 0.006 and 0.009 %) at pH 6, 7 and 8 respectively. Sagripanti and Bonifacino (1996) found little or no effect of pH on sporicidal activity of H_2O_2 between pH 2 and 10. A study by Hughes and Kilvington (2001) investigating the activity of a number of H_2O_2-based contact lens disinfecting solutions against the amoeba *Acanthamoeba polyphaga* found no correlation between the pH of those solutions, which ranged from 3.25 to 6.5, and the antimicrobial activity measured. Baldry (1983) did find

Organism	H_2O_2	Temperature	Reduction in CFU/ml	Time (mins)	Reference
Micrococcus Sp.	3 %	25 °C	>6 $\log_{10}$	10	Wardle & Renninger (1975)
Staphylococcus epidermidis	3 %	25 °C	>6 $\log_{10}$	10	Wardle & Renninger (1975)
Listeria monocytogenes Scott A	3 %	20 °C	6 $\log_{10}$	10	Robbins *et al.*(2005)
Enterobacter cloacae IAL 1976	1.5 %	25 °C	6 $\log_{10}$	10.8	Mazzola *et al.* (2003)
Escherichia coli ATCC 25922	1.5 %	25 °C	6 $\log_{10}$	19.2	Mazzola *et al.* (2003)
Staphylococcus aureus ATCC 25923	1.5 %	25 °C	6 $\log_{10}$	20.4	Mazzola *et al.* (2003)
Legionella pneumophilia	1 %	20 °C	~7.5 $\log_{10}$	30	Domingue *et al.*(1988)
Pseudomonas aeruginosa ATCC 15442	0.68 %	20 °C	6 $\log_{10}$	30	Lambert *et al.* (1999)
Serratia marcesens	0.6 %	37 °C	>8 $\log_{10}$	60	Schaeffer *et al.* (1980)
Proteus mirabilis	0.6 %	37 °C	>8 $\log_{10}$	30	Schaeffer *et al.* (1980)
Streptococcus faecalis	0.6 %	37 °C	>8 $\log_{10}$	15	Schaeffer *et al.* (1980)
Tenacibaculum maritimum	0.012 %	20 °C	>6 $\log_{10}$	15	Avendaño–Herrera *et al.* (2006)

Table 1.1: Efficacy of hydrogen peroxide solution against various vegetative bacterial species in suspension.

Organism	H$_2$O$_2$	Temperature	Reduction in CFU/ml	Time (mins)	Reference
Bacillus subtilis var *globigii* ATCC9372	25.8 %	24 řC	~ 3.5 log$_{10}$	10	Toledo *et al.* (1973)
Bacillus subtilis SA 22	25.8 %	24 řC	2 log$_{10}$	17	Toledo *et al.* (1973)
Bacillus stearothermophilus 1518	25.8 %	24 řC	~ 2.5 log$_{10}$	6	Toledo *et al.* (1973)
Bacillus coagulans 56 186 A	25.8 %	24 řC	4 log$_{10}$	8	Toledo *et al.* (1973)
Clostridium sporogenes 3679	25.8 %	24 řC	4 log$_{10}$	2	Toledo *et al.* (1973)
Bacillus licheniformis	15 %	25 řC	>6 log$_{10}$	30	Wardle & Renninger (1975)
Bacillus subtilius var *niger*	15 %	25 řC	>6 log$_{10}$	60	Wardle & Renninger (1975)
Bacillus polymyxa	10 %	25 řC	>6 log$_{10}$	30	Wardle & Renninger (1975)
Bacillus atrophaeus ATCC 9372	7.5 %	Room temp.	>6 log$_{10}$	360	Enrique Acosta-Gío *et al.*(2005)
Alicyclobacillus acidoterrestris	4 %	23 řC	>5 log$_{10}$	10	Orr & Beauchat (2000)
Bacillus cereus	3 %	Room temp.	>6 log$_{10}$	240	Pinna *et al.* (2001)

Table 1.2: Efficacy of hydrogen peroxide solution against various sporaceous bacterial species in suspension.

that the minimum inhibitory concentration of H_2O_2 against *P. aeruginosa* at pH 5, 6.5 and 10 to be 5, 10 and 50 ppm (0.0005, 0.001 and 0.005 % respectively) but did not see a similar change with *Klebsiella pneumoniae*, *S. faecalis* or *S. aureus*. The exposure temperature is known to have a substantial effect on the sporicidal activity of H_2O_2 solution, for example Toledo *et al.* (1973) report that the z value (that is, the temperature change required to bring about a ten-fold change in D value) for *B. subtilis* var. *globigii* in 25.8 % H_2O_2 solution is 40 řC, whilst Xu *et al.* (2008) found that the time taken to inactivate 6 log CFU/ml of *B. anthracis* strain 9131 spores in milk by 1050 ppm (0.105 %) H_2O_2 solution was 253, 89 and 47 minutes at 72, 80 and 85 řC respectively. Strain ANR-1 gave times of 82, 16 and 11 minutes, and strain 7702 gave times of 14, 2 and 1.3 minutes at the same three temperatures. Brandi *et al.* (1987) have shown that killing of *E. coli* strain AB1157 by exposure to up to 18 mM (0.061 %) H_2O_2 for 15 minutes was greatly reduced at 4 řC compared with 37 řC.

1.5   Hydrogen peroxide solution bactericidal mechanism.

The mechanism of bactericidal activity is generally reported to be based on Fenton, and Fenton-like chemistry leading to the production of hydroxyl radicals, for example textbooks by Block (2001) and Ascenzi (1996) both report hydroxyl radical production as the mechanism of killing. It is known that *in vitro*, the hydroxyl radical acts as a potent oxidising agent, capable of reacting with many cellular macromolecules including lipids, proteins and nucleic acids (Liochev, 1999), and it is easy to imagine that such reactions can account for the bactericidal effects of H_2O_2.

Evidence for this mechanism in the action of H_2O_2 on bacterial cells was provided by Repine *et al.* (1981). They demonstrated that growing *Staphylococcus aureus* overnight in Bacto nutrient broth containing increasing concentrations of iron resulted in an increase in intracellular iron content. Incubation of harvested cells at 37 řC in Hanks' balanced salt solution containing 0.1 % bovine serum albumin and a range of H_2O_2 concentrations between 10 ţM and 0.01 M (3.4×10^{-5} to 0.034 %) showed that those cells with an elevated iron concentration had a greater susceptibility to H_2O_2, as measured by a decrease in H_2O_2 concentration required to kill 50% of cells after a 60 min exposure. An increase in iron concentration in growth

medium was shown to have no effect on growth rate or viability of *S. aureus* nor was any effect measured on catalase or peroxidase activity. Repine *et al.* (1981) also showed that the addition of thiourea, dimethyl thiourea, sodium benzoate and dimethyl sulphoxide inhibited the toxic effects of H_2O_2 in proportion to the effectiveness of the substances as hydroxyl scavengers. Again, addition of these substances had no effect on viability, nor were they found to directly react with H_2O_2. Further evidence was found by Mello Filho *et al.* (1984). They showed that the potent iron chelator 1,10-phenoanthroline could penetrate cultured mouse cells and protect them against killing by H_2O_2. The chelator alone had no effect on cell viability.

Studies that directly measure macromolecular damage caused by H_2O_2 have been performed using several different methods to estimate different types of damage. Ananthaswamy and Eisenstark (1977), Hagensee and Moses (1986), Rohwer and Azam (2000) and Fernàndez *et al.* (2008) have all shown direct evidence of DNA damage to *E. coli* cultures exposed to concentrations of H_2O_2 between 10 to 118 mM (0.034 to 0.401 %) for various exposure times at various temperatures. Tamarit *et al.* (1998) and Semchysyn *et al.* (2005) have shown protein oxidation in *E. coli* exposed to up to 2 mM (0.007 %) H_2O_2, Cabiscol *et al.* (2000) have shown protein oxidation in *Saccharomyces cerevisiae* exposed to 5 mM (0.017 %) H_2O_2. Semchysyn *et al.* (2005) also demonstrated lipid oxidation in *E. coli* exposed to up to 2 mM (0.007 %) H_2O_2, whilst Brandi *et al.*(1991) showed membrane damage as measured by loss of cell volume in *E. coli* exposed to 17.5 mM (0.0595 %) H_2O_2 and Baatout *et al.* (2006) showed membrane damage as measured by increased propidium iodide uptake in *Ralstonia metallidurans*, *E. coli*, *Shewanella oneidensis* and *Deinococcus radiodurans* cultures exposed to concentrations of H_2O_2 up to 880 mM (3 %).

1.6 Weaknesses with current evidence for bactericidal mechanism of hydrogen peroxide solution.

Despite the studies mentioned in section 1.5, there is remarkably little literature discussing the exact mechanism of biocidal action of H_2O_2, and in particular very few studies of such; with most mention of the mechanism occurring in general reviews of various biocidal mechanisms. This situation is

complicated by the importance of H_2O_2 as a physiological source of reactive oxygen species (ROS) and as a component of the innate immune system. The majority of studies investigating the toxic mechanism of H_2O_2 are therefore considering it as a source of oxidative stress in order to model chronic oxidative damage to cells, or to investigate the various killing mechanisms of leukocytes (Clifford and Repine, 1982). Whilst the studies mentioned in the section 1.5 all offer evidence for the *in vivo* oxidation of the three major classes of macro-molecular targets within the bacterial cell on exposure to H_2O_2 solutions, caution should be taken when drawing conclusions from these studies as to the bactericidal mechanism of H_2O_2. It is important to note that the majority of studies mentioned here are interested in oxidative stress effects, and criticisms of their applicability to the understanding of bactericidal action are not criticisms of their conclusions, however all studies mentioned suffer from one or more of the following problems if their findings are to be applied to elucidating the bactericidal mechanism of typical in-use solutions of H_2O_2:

i. Very low H_2O_2 concentrations. All the studies mentioned (apart from Baatout *et al.* (2006)) investigate concentrations several orders of magnitude lower than those most commonly used in sterilization applications, which are typically between 3 and 35 %. At these low concentrations, the bacterial cell's defenses against H_2O_2 become relevant, compared to the sterilization conditions (Block, 1991), so the effects on the cell are likely to be markedly different. Also, as discussed previously, Fenton and Fenton-like chemistry depends on the ratio between H_2O_2 and iron present within the system, obviously this will be far greater for sterilization conditions than those being investigated in the cited studies. Indeed, it is possible that at sufficiently high concentrations of peroxide, the Fenton reaction might become relatively less important as intra-cellular iron becomes limiting and the direct reaction between H_2O_2 and cellular targets (for instance, the direct reaction with protein thiols) becomes more important. Finally, the presence of reducing substances within the bacteria will be more important at lower H_2O_2 concentrations, and the effect therefore of ratio of H_2O_2 to cellular reductants is likely to be complex. This is because cellular reductants can act not only as a means of scavenging peroxide and decreasing susceptibility, for instance glutathione has been shown to protect *E. coli* against H_2O_2 (Chesney *et al.* (1996)), but also as a means of driving the Fenton reaction (thus increasing susceptibility), for example, Park and Im-

lay (2003) found that disrupting cysteine homeostasis and bringing about a eight-fold increase in intracellular cysteine in *E. coli* caused a substantial increase in susceptibility to 2.5 mM (0.009 %) H_2O_2.

ii. Long exposure times. Although treatment times for sterilization with liquid H_2O_2 are often in the range of hours (for example, contact lens treatments), little information can be gained about the cidal mechanism of a treatment if an excessively long exposure time is used. For example, Mazzola *et al.* (2003) suggest that an exposure time of ˜19 minutes is sufficient to bring about a 6-log reduction in viable cell count of *E. coli* treated with 1.5 % H_2O_2. If one were to study the damage caused cells after an eight-hour treatment, for the majority of that time all of the cells present will be dead and the peroxide will continue reacting with cellular components, leading to extensive damage and no indication of which damage lead directly to cell death. Indeed, even after the 19 minute exposure, extensive damage will have been caused to cells that are already dead, and thus the cause will be difficult to ascertain. This effect is potentially enhanced when sub-bactericidal concentrations are used on actively metabolising cells for long exposure times, as cells can both replace damaged molecules and divide. The doubling time of *E. coli* under optimal conditions is generally given as ˜20 minutes (Irwin *et al.*, 2010), so a 1 hour treatment with low concentrations of H_2O_2 will allow as many as three generations, thus replenishing the pool of macromolecular targets available for reaction. Damage to a target which is only a minor contributor to toxicity could therefore produce relatively high levels of measurable damage, leading one to suppose that such damage is more important than in reality. As an example, we might propose the hypothetical situation whereby specific peroxide damage to a particular metabolic enzyme is sufficient to cause cell death, whilst damage to other targets is not fatal. A study performed with a long exposure time, and a sub-bactericidal concentration of peroxide could lead to the accumulation of large amounts of non-specific damage, masking the real mechanism.

iii. Inconsistent exposure conditions. The studies described use a variety of exposure conditions with a number of different media including buffers such as Hank's balanced salt solution and phosphate buffered saline to rich growth media such as lysogeny broth. The presence of various organic substances has been shown to have an effect on the action of H_2O_2, for instance Link (1988) showed that pH dependence of H_2O_2 cytotoxicity on epithelial cells was greatly enhanced in Eagle's minimum essential medium compared

with phosphate buffered saline, and suggested that this was due to the presence of glucose and histidine in the media. Cultures were also exposed in different growth phases. These differences make comparison of studies difficult, and mean that an overall model of H_2O_2 cannot be compiled from them.

iv. Measurement of single target. Citations of studies designed to measure DNA damage by exposure to H_2O_2 as evidence that DNA damage is the major cidal mechanism of peroxide treatment might be considered to be begging the question if those studies only sought to measure DNA damage. It is necessary to measure damage to all likely targets, and to correlate this with reduction in viable cell numbers for a range of exposure times and/or concentrations in order to produce a model of bactericidal action. Merely demonstrating a correlation between damage to one target and cell killing is insufficient to demonstrate that this is the major cause of cell death – it might be the case that damage to another target is equally strongly correlated. Apart from the study by Semsychyn *et al.* (2005), which simultaneously measured damage to protein and lipid, all of the studies described in section 1.5 sought only to investigate damage to their targets of interest during H_2O_2 exposure (note again that this is not meant as a criticism of the studies in question, rather the application of their findings outside of the scope of the studies), and variations in testing conditions make it impossible to directly compare those studies that correlated reduction in viable cell count with measured damage. In conclusion, it is clear that whilst there exist a number of studies showing damage to various macromolecular components can occur following exposure to a range of H_2O_2 concentrations, the relative importance of this damage to the bactericidal mechanism is unknown, and, with the exception of an increase in membrane permeability (Baatout *et al.*, 2006) for which exposure times were too long to draw conclusions as to the importance, the occurrence of damage to these targets has not been demonstrated for the most commonly used sterilization conditions. In short, the mechanism of bactericidal action of H_2O_2 sterilization solutions remains unclear.

1.7   Vapourised hydrogen peroxide as a sterilant.

The use of vapourised H_2O_2 (VHP) as a sterilant was pioneered in the packaging industry by Wang and Toledo in the late 1980s. Its use in preference

to liquid solution of 35% (equivalent to 880 mM) H_2O_2 was initially investigated in order to avoid residual traces being retained on packaging, however use of a vapour phase also allows for far easier sterilization of large volumes, and of devices that might be damaged by exposure to water, compared to use of a solution. H_2O_2 vapour also offers the advantage over other commonly used vapour phase methods (such as formaldehyde or ethylene oxide) of low toxicity and spontaneous breakdown into completely harmless by-products (Wang and Toledo, 1986).

Since Wang and Toledo's initial work, commercial vapour phase H_2O_2 treatments have been developed and their efficacy investigated in several applications, including decontamination of laboratory and medical equipment, hospital wards and pharmaceutical manufacturing facilities. Hultman *et al.* (2007) describe two methods used to produce H_2O_2 vapour, those that evaporate H_2O_2 solutions by heating and those that flash vapourise the solution by direct application onto a hot surface. Hultman states that the second of these can produce higher concentrations of VHP due to the lower boiling point of water leading to a lower equilibrium concentration of gaseous H_2O_2 when the solution is heated, compared to flash vapourisation when both components are simultaneously vapourised.

1.8   Efficacy of vapourised hydrogen peroxide.

VHP sterilisation has been shown to be efficacious against a wide range of organisms including gram positive and gram negative vegetative cells, DNA and RNA viruses (Heckert *et al.*, 1997), bacterial endospores (Kokubo *et al.*, 1998) and fungi (Hall *et al.*, 2008). A study by Otter and French (2009) demonstrated efficacy of a VHP system against a number of different organisms. Inocula of 10^6 cells of *Klebsiella pneumonia* strains K41, K2 and NCTC 9633 were completely inactivated by exposure to >1000 ppm (0.1 %) vapourised H_2O_2 with micro-condensation after 90 minutes, similarly two clinically isolated strains of *Acinetobacter* were inactivated after 90 minutes, and five methicillin-resistant strains of *S. aureus* were inactivated after 75 minutes. A study by Hall *et al.* (2007) showed that a 30 minute exposure to >2000 ppm (0.2 %) vapourised H_2O_2 was sufficient to completely inactivate 103 CFU of *Mycobacterium tuberculosis*, a highly resistant organism.

1.9 Vapour phase hydrogen peroxide bactericidal mechanism.

Despite the increasing use of such decontamination methods, and the growing body of literature detailing validation of these methods for use in various applications, it appears that no work has been done on understanding the mechanism(s) of biocidal activity of the vapour form of H_2O_2. Indeed, one early study of a vapour system to sterilised centrifuges performed by Klapes and Vesley in 1990 concludes "The application of VPHP (Vapour Phase Hydrogen Peroxide) as a potential sterilant is still clearly in its infancy: definitive knowledge of the mechanism(s) of cidal action, and the factors which influence it, is lacking." (Klapes & Vesley, 1990) whilst a study of the use H_2O_2 vapour (HPV) to deactivate *Mycobacterium tuberculosis* performed by Hall *et al.* in 2006 states in the conclusion "...the exact mechanism of action of HPV remains to be fully elucidated...." (Hall *et al.*, 2007)

The lack of investigation into vapour-phase H_2O_2 biocidal mechanism appears more remarkable in the light of a study performed by Yan *et al.* (2004) which showed that of several decontamination methods tested, only gaseous H_2O_2 prevented the exhibition of spongiform pathologies in hamsters following intercerebral inoculation with steel wires contaminated with infectious brain matter and then treated with the decontamination methods. A follow-up study performed by Fichet *et al.* (2007) reproduced this destruction of infectivity with gaseous H_2O_2 but not liquid H_2O_2. *In vitro* studies showed unfolding and degradation of the prion proteins by gaseous but not liquid H_2O_2.

These studies suggest that the ability of H_2O_2 to oxidatively degrade protein is greatly enhanced in the vapour phase compared with the liquid phase, but as yet there exists no studies comparing the *in vivo* bactericidal action of the two phases.

1.10 Project aims.

This project aims to study the effects of H_2O_2 treatment, both in liquid and vapour phase, on proteins, nucleic acids, lipids and the cell membrane of *E. coli* K12. In order to do this, bacterial cultures will be treated and components extracted and assayed to determine the extent of oxidative damage caused.

By characterising the damage caused to these macromolecular cell components in a well-studied Gram-negative model organism, it is hoped that a deeper understanding of the mechanism of biocidal action of H_2O_2 will be gained, and in particular any differences in this mechanism between the two phases identified.

Chapter 2

Cell enumeration and biocide efficacy.

2.1 Introduction.

Understanding the effects of H_2O_2 treatment on bacterial cell components, and how these relate to the bactericidal mechanism requires that this damage is correlated with lethal effects of the treatment. Thus, the reduction in viable cells caused by a treatment must be measured alongside the damage caused to cell components. For the purposes of this study, we define viable cells as units capable of forming colonies when cultured. As discussed in chapter 1, in order to be able to compare results between and within studies, it is important to ensure that culture and exposure conditions are standardised. If measurement of one kind of damage requires certain conditions, it is necessary to ensure that these conditions are used throughout the study to ensure that measured effects are due solely to the H_2O_2 treatments, and not due to differences in culturing and/or exposure. Before measurement of damage to macromolecules could proceed then, it was therefore necessary to identify, optimise and validate methods of treating bacterial cultures and measuring their viability.

2.1.1 Experimental considerations.

2.1.1.1 Viable cell enumeration methodology.

In order to measure the effect of treatments on the viability of bacterial cells, a method had to be identified to allow the total number of viable cells in a sample to be counted. In this way the cidal effect of a treatment could

be assessed by estimating the change in colony forming units (CFU) with respect to an untreated control. The Miles and Misra drop count is one of the standard methods used in the microbiology laboratory (Miles *et al*, 1938). The method is relatively facile, convenient and well-understood, so it was proposed as the main method to be used to determine differences in bacterial cell viability. The Miles and Mira method uses small volumes of a serial dilution of bacterial suspension dropped onto agar plates. Whilst relatively rapid, as a single plate can be used to measure multiple dilutions, the small volumes used mean the method is inherently prone to error due to variance in pipetting volumes being a relatively high percentage of the total volume added. An alternative method is the spread plate method whereby a larger volume of a single dilution is added to a single plate and spread over the surface of the agar, either by agitation or by spreading with a glass rod (Anderson and Stuart, 1935). This has the disadvantage of being more time consuming, and requiring very many more plates, but potentially reduces some of the errors due to pipette variance and clumping of bacterial cells.

In order to establish a testing regimen to investigate differences between liquid and vapour phase biocide efficacy, it was necessary to determine whether the proposed methods gave sufficient performance to detect interesting differences in viable cell counts. Requirements for the method were decided and a study designed to determine whether the method met these requirements. The results obtained from the study had to meet several predefined acceptance criteria in order to demonstrate that the method met these requirements. If any proposed method did not meet those requirements, an alternative method was identified and similarly tested.

For initial studies, relatively large differences (in the order of a 1 $\log_{10}$ drop in CFU/mL count) were measured over a number of treatments. The chosen method therefore had to be able to reliably detect differences between treatments of this magnitude with a minimum number of replicates in a convenient format. These requirements are stated formally below:

- The CFU counting method shall be able to identify changes in CFU per mL of sample of 0.5 $\log_{10}$ or less.
- The CFU counting method should be able to identify these changes with no more than three replicates per condition.

Counts performed using this method were to be analysed statistically using a two-sample t-test on occasions when a single treatment was to be compared to a control and ANOVA when multiple levels of a treatment were

to be compared to a control. The data from the samples being tested must be approximately normally distributed, this was tested using the Anderson-Darling test for normality to check that they did not show significant evidence of departure for normality.

Power of a statistical test refers to the probability that the test will reject the null hypothesis. By using power analysis, the chance that a test will reject a false null hypothesis that two means are not different at a given confidence level (usually 95%) can be calculated by reference to size of the samples, the variance of the sample means being compared and the size of the true differences being measured (Park, 2008). In this study, we wished to demonstrate that the cell counting method can detect a difference of 0.5 $\log_{10}$ with three replicates per treatment, therefore, the measured variance could be used in power analysis to demonstrate this.

In order to test sub-lethal treatment conditions, inhibition of growth rather than reduction in viability needs to be measured. To do this, a method based on the use of a Bioscreen instrument (Oy Growth Curves Ab, Helsinki) to produce growth curves was used. The Bioscreen is a combined incubator and plate absorbance reader – cultures can be incubated under a variety of conditions simultaneously by inoculating 100 well plates in which wells containing different media have been prepared, and the growth monitored by use of broad-range absorbance measurement. This instrument has been used successfully to measure the minimum inhibitory concentration of biocidal substances (e.g. Lambert *et al.* 1998).

2.1.1.2 Methodology for rapid estimation of cell counts.

It was often necessary to make an estimate of cell count in order that cell suspensions could be adjusted to the desired CFU/mL before treatment to ensure that approximately equal numbers of cell were treated. A method that gives an estimate of cell number strongly correlated with the $\log_{10}$ CFU/mL values is to measure the optical density of a sample of the bacterial suspension using a spectrophotometer, and this method has long been used to estimate bacterial population density as for most species there is a linear relationship between optical density and cell count for some range of optical densities (e.g. Contois, 1959), and it is a simple matter to dilute a sample from a cell suspension to give an optical density within this range. The measured optical density can then be corrected for this dilution factor to obtain an estimate of the cell density in the original sample.

Alternative methods include direct counting of cells under a microscope, or flow cytometric methods using fluorescent stains (Monfort and Baleux, 1992), however the turbidometric method using a spectrophotometer was preferred due to convenience.

The accuracy of the method tested could be assessed by plotting estimated values using this method against Miles and Misra cell counts made from the same samples. These requirements are formally stated below:

• The method shall give an accurate (that is, unbiased) estimate of CFU/mL in a suspension.

• The method shall give values having a linear relationship to $\log_{10}$ CFU/mL measured using the Miles and Misra drop count method.

• The method shall be quick to perform with no incubation step.

2.1.1.3 Vapourised hydrogen peroxide exposure methodology.

A major aim of this project was the comparison of the modes of action of vapourised and liquid H_2O_2, a testing protocol was therefore required that was able to produce comparable results from the two different treatment systems. The vapourised H_2O_2 treatment was to be effected by means of a Steris Vaprox VHP1000 unit, which is described in the schematic in figure 2.1.
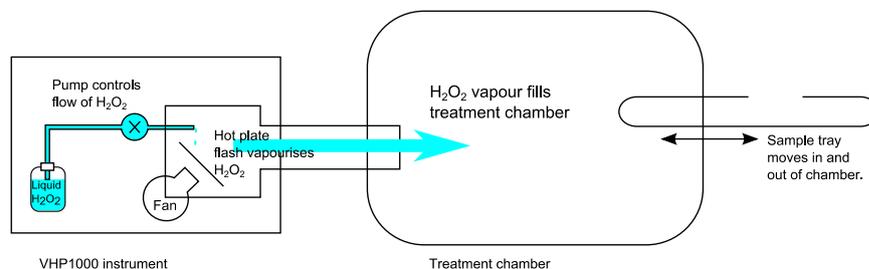
Figure 2.1: Schematic showing VHP1000 vapourised hydrogen peroxide treatment system.

In order to compare performance of liquid and vapourised H_2O_2 (VHP) systems, it was first necessary to have some means of exposing samples of *E. coli* culture to the VHP treatment. Exposing liquid suspensions to the treatment was not appropriate as the vapourised H_2O_2 will dissolve in the suspension medium, therefore, some method of attaching the bacterial cells in a solid phase to a surface was required. This method was also required to cause minimal cell mortality – any cell damage due to the attachment would be a source of experimental error, as effects due to this damage would

confound effects due to the vapourised H_2O_2 treatment. Mortality of cells
of 10% or less due to the method was considered acceptable. The method
had to be compatible with the Vaprox treatment system - the surface had to
fit through the inlet port into the treatment enclosure and both the surface
and attachment method had to survive the treatment. The method also
had to allow treatment with liquid H_2O_2 in order that both phases could be
compared. The standard method of affixing bacteria to a solid surface for
biocide treatment is the carrier test as described in BS:EN 1276, whereby
samples of bacterial suspensions are dried onto small stainless steel coupons
which are then dried at 37ºC for 1 h and treated with the biocide under
investigation. Cells thus treated are recovered by agitation in a releasing
solution and counts performed on the resulting suspension. This method
might present problems when used with vegetative cells as the drying process
may cause damage to those cells; for instance, Wendt *et al.*(1997) found an
approximately 2 $\log_{10}$ reduction in colony forming units when *E. coli* strain
11775 was dried onto various surfaces including stainless steel whilst Hirai
(1991) determined a D value (time taken for a 1 $\log_{10}$ reduction in CFU
count) of 2.4 hours for *E. coli* K12. Methods to stabilise and thus increase
the tolerance of *E. coli* K12 suspensions to the drying process involving
the addition of various solutes to the suspension buffer before drying have
been reported, with the greatest success seen when non-reducing sugars are
added to the cell suspension immediately before inoculation onto coupons in
order to stabilise the cells. Leslie *et al.* (1995) found that resuspending *E.
coli* in 100 mM sucrose or trehalose before freeze-drying increased survival
rates to 60 and 70% respectively, compared to 8% for water alone. Louis
et al. (1994) also found that sucrose protects *E. coli* cells during air dry-
ing. However, there are potential drawbacks to this method as these sugars
are likely to cause substantial changes to normal membrane chemistry (it
is hypothesised that sugars such as trehalose protect cells during drying
due to the replacement of water in the membrane). Additionally, drying
in a high-sugar solution may cause the formation of a 'glass' which the
vapourised H_2O_2 will be unable to penetrate. Another alternative method
involves embedding the cells to be tested in an artificial biofilm, rather than
drying, in order to attach them to a surface. Shackleford *et al.* (2006) have
demonstrated the use of an alginate gel to simulate a biofilm in order to test
the efficacy of biocides. Such a gel could be used to affix cells embedded
within it to a surface, and should have sufficiently large pores to allow easy

penetration of H_2O_2 vapour. However, it is possible that such a film could interfere with H_2O_2 action. Studies were performed to test the recovery of cells using these methods, and to optimise the recovery and compatibility of the method. The chosen method was validated in a similar manner to the cell counting method in section 2.1.1.1 to ensure that results were sufficiently repeatable.

2.1.1.4   Determination of neutraliser efficacy.

It was also necessary to have an effective system for neutralising the H_2O_2 in order that exposure times could be controlled. This was less important for vapour treatment, where samples could simply be removed from the treatment enclosure (though condensed H_2O_2 might continue to affect bacteria, even when the samples are removed from the enclosure), but vital for liquid treatment where surface-attached and suspended cells had to be compared and there was no such rapid way of instantaneously removing the cells from the H_2O_2 solution. Johnston *et al.*(2002) discussed the importance of using the correct quenching agent, especially when using compounds with low dilution coefficients such as H_2O_2, as simple dilution of biocide is often not sufficient to effect a cessation of bactericidal activity. Both sodium thiosulphate (see for example Hilgren *et al.*, 2007) and catalase (see for example Boateng *et al.*, 2011) are commonly reported as neutralisers of H_2O_2. Since a major component of the project was the study of proteins, it was decided to avoid the use of a protein neutraliser and instead investigate the concentrations of sodium thiosulphate necessary to neutralise various amounts of H_2O_2 and the toxicity of those concentrations.

2.1.1.5   Determination of exposure conditions.

In order to elucidate the mechanism of bactericidal action of H_2O_2, it was planned to extract macromolecules for assay after treatments of varying lethality. Since high concentrations or long exposure times to powerful oxidising agents are likely to lead to cell lysis and uniform oxidation of macromolecules, a range of concentrations and exposure times were to be employed in order to examine differentially progressive macromolecule oxidation in liquid and vapour systems. A series of studies was therefore performed in order to determine a range of H_2O_2 concentrations and exposure times with which to perform initial testing. The aim of these studies was to identify concentrations and exposure times of liquid H_2O_2 in suspension that

produce effects from a small retardation of cell proliferation to a substantial (2 $\log_{10}$) reduction in CFU/mL count. In order to investigate and compare the cidal mechanisms of the two H_2O_2 phases, it was necessary to have some means of treating cells with comparable exposures of both. The most obvious way to achieve this was to expose cells to a similar amount of H_2O_2 for a similar time using either method, however this is complicated by the profound differences between the two means of delivery. Vapourised H_2O_2 delivery systems inject a stream of H_2O_2 and water vapour to a chamber or room containing equipment to be decontaminated, this stream constantly replenishes the supply of H_2O_2 within the sealed chamber, maintaining the concentration at a steady state. Watling *et al.*(2002) have produced a theoretical analysis of the concentration of H_2O_2 present in the vapour phase (vH_2O_2) within a decontamination chamber and and have shown that for any given temperature and humidity, the concentration of vH_2O_2 reaches a steady-state equilibrium with condensed, liquid H_2O_2 such that increasing the rate of injection will no longer increase the concentration of vH_2O_2. Work by the Steris Corporation using real-time monitoring shows that the maximum vH_2O_2 concentration produced in a 22 cubic foot enclosure is about 2.7 mg/L. Typically, decontamination times for VHP treatments are measured in hours. In contrast, liquid systems use far higher concentrations of H_2O_2 than vapour systems, typically 3% (30 g/L) or about 1000 times that used in a VHP treatment when considered weight for volume. Substantially lower volumes of liquid H_2O_2 are used as surfaces are generally sprayed or wiped, and contact times are greatly reduced, generally measured in seconds compared to the hours used for VHP treatments. Also, there is no simple way to maintain a steady state concentration of H_2O_2 in a liquid system – if large numbers of cells are exposed to very low concentrations of liquid H_2O_2 for long periods, catalase produced by the bacteria will catalyse the break-down of the H_2O_2 causing a reduction in concentration greater than that expected from stochiometric reaction alone. A range of concentrations and exposure times were therefore investigated in both systems to determine which of these approaches to adopt, and to identify the best conditions to use during testing.

2.1.1.6   Effects of hydroxyl radical scavengers.

In order to gain an understanding of the importance of Fenton chemistry, and the production of free hydroxyl radicals to the biocidal mechanism

of liquid H_2O_2, the effect of adding a scavenger of such free radicals on the biocidal or bacteriostatic efficacy of liquid H_2O_2 was tested. Several substances have been reported as scavengers of hydroxyl radicals. For instance, Suthanthiran *et al.* (1984) report on the use of dimethyl sulphoxide (DMSO), thiourea, dimethyl urea, tetramethyl urea, benzoic acid, ethanol, methanol and ethylene glycol to scavenge hydroxyl radicals in natural killer cells; Smirnoff & Cumbes (1989) report that sorbitol, mannitol, myo-inositol and proline were effective hydroxyl radical scavengers in *in vitro* radical generating systems based on ascorbate-hydrogen peroxide or xanthine oxidase-hypoxanthine-hydrogen peroxide; Billany *et al.* (1996) report that some alcohols and ethanolamines demonstrated hydroxyl scavenging activity in an *in vitro* system, with diethanolamine being particularly potent; whilst several studies report that the common buffer component tris(2-amino-2-hydroxymethylpropane-1,3-diol)(Tris) is an efficient scavenger of hydroxyl radicals (e.g. Hicks and Gebicki, 1986). Care must be taken in the selection of a suitable scavenging agent as three important criteria have to be met before such a study can be carried out, namely the reagent must not cause damage to bacterial cells at the concentration required, must not react directly with H_2O_2, and must be membrane permeant. Of the most commonly used hydroxyl scavengers, DMSO is potentially toxic at concentrations equal to those of H_2O_2 tested; a 5 % v/v H_2O_2 solution is equal to approximately 1 M solution and would require the addition of 8.8 % DMSO to scavenge all radicals potentially produced, whilst Markarian *et al.* (2002) report that DMSO concentrations of greater than 5 % are strongly inhibitory of *E. coli* K12 growth. Benzoic acid and ethanol are known biocides with a long history of use as antiseptic and disinfectant agents. Mannitol (Bálint *et al.*, 2007) and sorbitol (van den Bogaart *et al.*, 2007) are cell-impermeant, and so will only be effective at scavenging radicals attacking the exterior of the cell. Many of the other potential hydroxyl radical scavengers have been shown to react directly with H_2O_2, being reasonably strong reducing agents, for example, Farmer *et al.*, (2006) demonstrated the direct scavenging of H_2O_2 by thiourea, Curtis *et al.*, (1988) demonstrated the direct reaction of dimethyl urea with H_2O_2. Tris fulfills all of the desired criteria being cell permeant, non-toxic and not known to react with H_2O_2, and was therefore chosen as the initial candidate for testing.

2.1.2 Aims and objectives.

- Validate a colony-forming unit counting method.

- Validate a method to rapidly estimate cell counts in a culture in order to standardise the number of cells treated.

- Identify and validate a method of exposing cultures to VHP treatment.

- Identify and validate a neutralising substance so that exposure times to H_2O_2 treatment can be controlled.

- Identify treatment conditions that give a range of reductions in cell viability and obtain reliable estimates of CFU/ml counts under these conditions.

- Test the effect of addition of a hydroxyl radical scavenger on bactericidal efficacy.

2.2 Materials and methods.

2.2.1 Materials.

Tryptone soya agar, tryptone soya broth, tryptone, sodium thiosulphate, sodium citrate, calcium chloride, trehalose, sucrose, tris(2-amino-2-hydroxymethylpropane-1,3-diol) (Tris) and sodium chloride were purchased from Sigma (Poole, UK).

Sodium alginate was purchased from Acros Organics (Loughborough, UK).

Escherichia coli K12 cultures (accession number 10218) were purchased from NCIMB (Aberdeen, UK).

Bioscreen plates were purchased from Fisher (Loughborough, UK).

35% hydrogen peroxide stock was provided by Steris (Basingstoke, UK).

E. coli Top10 cultures were a gift from Cardiff University School of Biosciences.

2.2.2 Methods.

2.2.2.1 Cell propagation.

Freeze-dried *E. coli* K12 cultures were reconstituted by inoculating into 10 mL tryptone soya broth (TSB) and incubated overnight at 37°C. This culture was inoculated a second time into 10 mL TSB, again incubated overnight at 37°C and used to produce freezer stocks by adding 1 mL culture in TSB to 1 mL glycerol. Freezer stocks were stored at -80°C until use.

E. coli K12 freezer stocks were regenerated as required by inoculating into 10 mL tryptone soya broth (TSB) and incubated overnight at 37°C. This culture was inoculated a second time into 10 mL TSB, again incubated overnight at 37°C, and used to generate working slopes by inoculating 10 mL tryptone soya agar (TSA) with culture in TSB and incubating overnight at 37°C. Following incubation, slopes were stored at 2-8°C before use, for a maximum of 4 weeks.

Bulk *E. coli* cultures were produced by inoculating 100 mL TSB in a 250 mL Erlenmeyer flask with culture from working slopes using a culture loop. These were then incubated overnight at 37°C with shaking (100 rpm) and used immediately.

E. coli Top10 cultures were similarly produced.

2.2.2.2 Validation of drop count method.

An *E. coli* cell suspension was produced from a working slope by washing with 5 mL tryptone sodium chloride solution (TSC). The cell suspension was centrifuged at 4000 x g for 15 min, and the supernatant discarded. The pellet was washed three times with deionised water, and resuspended in deionised water. Ten dilution series from 10-1 to 10-8 of the suspension in TSC were prepared and the 10-5 to 10-8 dilutions from each series were plated and the mean CFU/mL calculated according to the equation:

$$\log CFU/mL = \log \left(\tfrac{count}{dilution} \right)$$

The variance due to the measurement method was estimated by preparing and counting 10 dilutions from a single suspension. That is to say, the repeatability of the method was measured. As treatments were always compared with their controls prepared and tested on the same occasion, and as there was only a single operator, reproducibility was not measured. Power

analysis was performed to determine the minimum difference detectable using this method when analysed with a two-sample t-test of alpha of 0.95 and beta of 0.95, assuming the standard deviation calculated was an accurate estimate of the standard deviation of the method. That is to say, the smallest true difference between two samples that could be detected on 95% of occasions when data were analysed using a two-sample t-test with 95% confidence.

Before the study was performed, the acceptance criteria for the results were determined. These are formally stated below:

- The distribution of the mean $\log_{10}$ (CFU/mL) values of the 10 dilution series must not show significant evidence of departure from normality according to an Anderson-Darling test (i.e. P > 0.05).

- Power analysis should determine a minimum detectable difference between two samples run in triplicate of not more than 0.5 $\log_{10}$ (assuming data are analysed using a two-sample t-test with alpha 0.95 and beta 0.95), based on the measured standard deviation of the 10 dilution series.

- Power analysis shall determine a minimum detectable difference between two samples run with four replicates of not more than 0.5 $\log_{10}$ (assuming data are analysed using a two-sample t-test with alpha 0.95 and beta 0.95), based on the measured standard deviation of the 10 dilution series.

2.2.2.3   Validation of the turbidometric method of estimating CFU counts.

A 5 mL suspension of *E. coli* culture in TSC was prepared from a TSA working slope as described in section 2.2.2.1. Dilutions of 1 in 2, 1 in 4, 1 in 8, 1 in 10, 1 in 20, and 1 in 100 in TSC were made of the suspension. Samples of 1 mL each dilution were taken and the absorbance at 500 nm measured using a spectrophotometer (Unicam Helios) blanked against TSC. Miles and Misra counts were made in duplicate of each dilution as detailed in section 2.2.2.2. The $\log_{10}$ CFU/mL values as calculated by the Miles and Misra counts were plotted against the A500 readings and a linear trend line fitted to this plot. The gradient of the trend line gave the extinction coefficient to convert A500 to CFU/mL.

Before the study was performed, acceptance criteria were determined. These are stated formally below:

- The plot of $\log_{10}$ CFU/mL vs. A500 shall display a linear trend.

- The best fit linear trend shall have an R^2 goodness-of-fit value of at least 0.90.

2.2.2.4   Investigation of VHP exposure methods.

An initial study was performed to check the reduction in CFU/mL due to drying onto coupons according to the method of BS:EN 1276. Twenty ţL of ˜10^8 CFU/mL *E. coli* (as determined by OD500 measurement) was suspended in TSC and inoculated onto a total of nine coupons. These coupons were dried at 37°C for 1 h then transferred to 100 mL glass bottles containing 10.1 mL deionised water and 5g borosilicate glass beads. The bottles were incubated at room temperature for 5 min, followed by agitation on a shaker at 150 rpm for 1 min. Miles and Misra counts were performed in duplicate for samples taken from each bottle. The inoculant was serially diluted to 10^{-8} and the four highest dilutions plated in duplicate.

Lower dilutions of recovered sample were counted to determine whether any CFU could be recovered, as the previous experiment had a lower limit of detection of 10^6 CFU/mL. Lower dilutions were tested by inoculating three coupons with 20 ţL of *E. coli* K12 culture suspended in TSC (approximately 10^8 CFU/mL). These were dried and recovered as previously detailed. Samples were taken from the three recovery bottles and serial dilutions to 10^{-3} prepared. The neat recovery solution and the dilutions were counted according to the Miles and Misra method.

Larger stainless steel coupons that have previously been used successfully with other organisms were compared to the new small coupons by inoculating two of each coupon type with 20 ţL of *E. coli* suspension as before. Coupons were dried and recovered, and CFU counts made as before.

An alternative *E. coli* strain (Top 10) was tested to determine whether it would be more resistant to drying that the K12 strain. Small stainless steel coupons were inoculated with 20 ţL of a ˜10^8 CFU/mL suspension in TSC of each strain in triplicate. Coupons were dried and recovered, and CFU counts made as before.

Recovery in disposable plastic containers was compared to glass containers by inoculating small stainless steel coupons with K12 suspension as

before. Coupons were dried as before. Cells were recovered as before in 100 mL glass bottles or 25 mL plastic containers. Conditions were tested in triplicate and CFU counts were made as before.

Recovery in deionised water was compared to recovery in TSC by inoculating small coupons as before. Coupons were dried as before. Cells were recovered in glass bottles as before, using 10 mL deionised water or 10 mL TSC. Recovery was tested in duplicate and CFU counts were made as before.

Recovery from deionised water inoculated with culture was also tested to determine whether use of deionised water was toxic (possibly through osmotic shock) or ineffective due to hydrophobicity of cells. This was done by inoculating 20 ţL *E. coli* K12 suspension directly into 10 mL deionised water. Recovery was tested in duplicate and CFU counts were made as before.

Drying of cells at 37řC was compared to drying at room temperature in laminar flow hood for longer times to determine if a gentler drying process would reduce cell loss. Coupons were inoculated as before and dried at 37°C for 1 h or at room temperature in a laminar flow hood for 90 min. Drying was performed in duplicate. Cells were recovered in glass bottles as before and CFU counts were made as before.

Alternative recovery processes (gentler or more vigorous) were tested to see if this would increase cell recovery or reduce cell damage. More vigorous recovery was tested by inoculating small coupons as before. Coupons were dried at 37řC as before. Two coupons were recovered as before (shaking at 150 rpm for 1 min), two coupons were recovered under more vigorous conditions (shaking at 200 rpm for 10 min). CFU counts were made as before. Gentler recovery was tested by inoculating small coupons as before. Coupons were dried as before. Coupons were recovered as before, or by incubating at room temperature for 30 min followed by gently pipetting 10 times to mix. CFU counts were taken as before.

The addition of reducing sugars to the inoculant was testing by preparing three *E. coli* K12 suspensions from TSA working slopes grown overnight at 37°C. The slopes were washed with 5 mL of TSC, and the TSC centrifuged at 4000 g for 15 min. The supernatant was discarded and the pellets resuspended in 2 mL TSC, 4 mL 100 mM trehalose in TSC or 4 mL 100 mM sucrose in TSC. Twenty ţL of each of the sugar suspensions was inoculated onto small coupons in duplicate. The coupons were dried at room

temperature in a laminar flow cabinet for 90 min and cells were recovered by incubating the coupons in 10 mL TSC at room temperature for 30 min, followed by gently pipetting 10 times to mix. Samples were taken from each bottle, serial dilutions to 10^{-5} prepared in TSC and CFU counts were taken using the Miles and Misra method.

Artificial biofilm embedding was tested by preparing alginate gels in duplicate according to the following procedure: 2 mL of the TSC suspension was added to 2 mL 4% sodium alginate solution in deionised water and the two were mixed by pipetting. Twenty ţL aliquots of the mixture were transferred to small steel coupons by pipetting. The alginate gels were cured by addition of 100 ţL 2% calcium chloride solution and incubated at room temperature for 5 min. The resulting gels were dissolved in 3 mL McIlvaine buffer at pH 7.4 (4 parts 0.2 M disodium hydrogen orthophosphate to 1 part 0.1 M citric acid). Samples were taken from the dissolved gels, serial dilutions prepared in TSC and CFU counts were taken using the Miles and Misra method. CFU counts were also taken of the inoculant using the Miles and Misra method to provide a control. Mean $\log_{10}$ CFU/mL values for each condition were calculated as before.

For all tests, CFU/mL counts of original inoculant were made and test conditions compared to these to calculate $\log_{10}$ CFU/mL recovery. Mean $\log_{10}$ CFU/mL for each condition was calculated and subtracted from the mean $\log_{10}$ CFU/mL for the inoculant used to give a value for the reduction in mean $\log_{10}$ CFU/mL.

2.2.2.5 Validation of alginate gel recovery repeatability.

Ten replicate gels were made and cells resuspended as in section 2.2.2.4, with the slight modification that cured gels were rinsed twice with 1 mL deionised water following addition of CaCl$_2$. Dilutions were prepared and plated and results analysed as for section 2.2.2.2.

2.2.2.6 Determination of working liquid hydrogen peroxide exposure and neutralisation conditions.

A Bioscreen study was performed in order to identify the range of H_2O_2 concentrations that retard cell growth, and the degree to which they do so. An *E. coli* K12 suspension was prepared by washing a working slope inoculated with K12 and incubated overnight at 37ºC with 5 mL of TSC. The TSC was decanted and centrifuged at 4000 g for 15 min. Following

centrifugation, the supernatant was discarded and the pellet resuspended in 2 mL TSC. A series of H_2O_2 solutions were prepared from 35% Steris working stock at concentrations of 3.75, 1.5, 0.75, 0.5, 0.15, 0.06, 0.03, and 0.12% in deionised water. A Bioscreen plate was prepared with 370 ţL TSB in each well. Test wells were prepared by the addition of solutions in the amounts shown in table 4.1 (1 whole column of 10 wells were prepared for each condition).

Column	K12 suspension (ţL)	H_2O_2 conc	H_2O_2 vol (ţL)	TSC (ţL)
1 (Blank)	0	-	0	30
2 (Control)	20	-	0	10
3	20	3.75 %	10	0
4	20	1.5 %	10	0
5	20	0.75 %	10	0
6	20	0.5 %	10	0
7	20	0.15 %	10	0
8	20	0.06 %	10	0
9	20	0.03 %	10	0
10	20	0.012 %	10	0

Table 2.1: Layout of Bioscreen test plate for hydrogen peroxide concentration test.

The Bioscreen instrument was then operated overnight at 37 řC with measurements taken every 15 minutes. Data were collected from the overnight run, mean ODs calculated (the Bioscreen measures broad-range absorbance, filters are not used) from the ten wells run for each condition and the corresponding CFU count was estimated from the equation determined in section 2.2.2.3.

In order to determine the efficacy and toxicity of sodium thiosulphate as a neutraliser when used with the alginate gel system, a starting concentration of 7.5% hydrogen peroxide was used to treat *E. coli* K12 embedded in Ca-alginate gels. Gels were prepared by adding 2 mL of ˜10^8 CFU/mL K12 suspension in TSC (prepared as before) to 2 mL 4% sodium alginate solution. Twenty ţL aliquots of the K12/alginate mixture were pipetted onto small steel coupons and the gels cured by addition of 100 ţL 2% $CaCl_2$ solution followed by incubation at room temperature for 5 min. Three gels were then dissolved in each the following solutions:

- 5 mL McIlvaine buffer (Control).

- 5 mL McIlvaine buffer containing 5 g/L sodium thiosulphate (neu-

traliser toxicity).

- 5 mL McIlvaine buffer containing 5 g/L sodium thiosulphate and 50 ţL 7.5% H_2O_2 (neutraliser efficacy).

Samples were taken from each dissolved gel and counts taken using the Miles and Misra method as previously described. Plates were incubated overnight at 37ºC overnight and mean $\log_{10}$ CFU/mL calculated for each condition.

A range of hydrogen peroxide concentrations (0.019, 0.038, 0.075, 0.375, 0.75, 1.5, 3, 3.75, and 4.5%) were tested to evaluate biocide and neutraliser efficacy. All tests were performed in triplicate as follows (a control condition was run on each occasion of testing):

- K12/ca-alginate gels were prepared as described above.

- Control gels were treated with 50 ţL deionised water for 30 s then dissolved in 5 mL McIlvaine buffer and 5 g/L sodium thiosulphate.

- Neutraliser efficacy test gels were treated with 50 ţL deionised water for 30 s then dissolved in 5 mL McIlvaine buffer + 5 g/L sodium thiosulphate and 50 ţL H_2O_2 solution (not all concentrations of hydrogen peroxide were tested for neutraliser efficacy).

- Biocide efficacy test gels were treated with 50 ţL H_2O_2 solution for 30 s then dissolved in 5 mL McIlvaine buffer and 5 g/L sodium thiosulphate.

Samples were taken from each dissolved gel and Miles and Misra counts taken as described above.

Sodium thiosulphate concentrations of 7.5, 10 and 12 grams per litre were tested for toxicity and efficacy with 4.5% H_2O_2 in duplicate as follows:

- K12/Ca-alginate gels were prepared as described above.

- Control gels were treated with 50 ţL deionised water for 30 s then dissolved in 5 mL McIlvaine buffer and 5 g/L sodium thiosulphate.

- Neutraliser toxicity test gels were treated with 50 ţL deionised water for 30 s then dissolved in 5 mL McIlvaine buffer and the concentration of sodium thiosulphate being tested.

- Neutraliser efficacy test gels were treated with 50 ţL deionised water for 30 s then dissolved in 5 mL McIlvaine buffer and 5 g/L sodium thiosulphate + 50 ţL H_2O_2 solution.

Samples were taken from each dissolved gel and Miles and Misra counts taken as described above. All tests were run in duplicate.

Exposure times of 60 s, 90 s, 2 min, 5 min and 10 min were tested in duplicate to evaluate biocide efficacy of 0.75% hydrogen peroxide with longer exposure times as follows: K12/ca-alginate gels were prepared as described above. Control gels were treated with 50 ţL deionised water for the exposure time under test then dissolved in 5 mL McIlvaine buffer and 5 g/L sodium thiosulphate. A separate control was run for each exposure time to control for any loss or death of cells due to exposure to deionised water. Biocide efficacy test gels were treated with 50 ţL H_2O_2 solution for the exposure time under test then dissolved in 5 mL McIlvaine buffer and 5 g/L sodium thiosulphate. Samples were taken from each dissolved gel and Miles and Misra counts taken as described above.

2.2.2.7 Determination of working VHP exposure conditions.

The ability to control the vH_2O_2 concentration in the VHP 1000 instrument was tested by testing various injection rates. The instrument was allowed to equilibrate for 30 min, and then a temperature reading taken.

The effect of VHP exposure time was tested using a cell suspension containing approximately 10^9 CFU/mL prepared from an overnight 50 mL TSB. Alginate gels were prepared and exposed as in section 2.2.2.4. Exposure times of 30, 60, 90 and 120 s were tested at a constant injection rate of 3.2 g/min.

2.2.2.8 Effect of hydroxyl radical scavenger on liquid hydrogen peroxide efficacy.

A bulk *E. coli* K12 culture was produced by inoculating a 250 mL Erlenmeyer flask containing 100 mL TSB with culture from working slopes using a culture loop. This was then incubated overnight at 37ºC with shaking (100 rpm) and used immediately. The cell suspension was transferred into two 50 mL centrifuge tubes, centrifuged at 4000 x g for 15 min, and the supernatant discarded. The pellets were washed three times with deionised water, and resuspended in 2.5 mL deionised water or 2.5 mL 1 M Tris-HCl, pH 7.4. Stock 35 % H_2O_2 solution was diluted in deionised water or 1 M Tris-HCl pH 7.4 in microcentrifuge tubes to give 1 mL of 1.7, 6 and 20 % H_2O_2 solutions. Cells were exposed to H_2O_2 by adding 50 ţL of H_2O_2solution to a 50 ţL aliquot of cell suspension to give the desired final

concentrations of 0.85, 1.5, 3 and 10 %. H_2O_2 diluted in water was added to cells suspended in water, and H_2O_2 diluted in Tris-HCl was added to cells suspended in Tris-HCl. Controls were prepared by adding 50 ţL of deionised water or Tris-HCl alone. The treatment mixtures were incubated for 45 s before the H_2O_2 was quenched by addition of 5 volumes of 10 g/L sodium thiosulphate and 0.2 M sodium citrate solution. The mixtures were then centrifuged at 5000 x g for 15 min, the supernatant discarded and the pellets washed three times with deionised water. The washed pellets were resuspended to an optical density of 2 in deionised water for further treatment. Samples were taken from each suspension and Miles and Misra counts taken as described before.

A bulk *E. coli* culture was produced as before, and a Bioscreen study was performed in order to identify the effect of 1 M Tris-HCl on the inhibitory concentration of H_2O_2. A Bioscreen plate was prepared with 370 ţL TSB or TSB + 1 M Tris-HCl in each well. Test wells were prepared by the addition of H_2O_2 stock to the first well to give a concentration of 880 mM (3%). A 1:10 serial dilution was then prepared to give concentrations of 88 mM, 8.8 mM, 880 ţM, 88 ţM, 8.8 ţM, 880 nM, 88 nM, 8.8 nM and 880 pM with three wells for each concentration in each medium (TSB or TSB + Tris-HCl). Each well was then inoculated with 30 ţL of *E. coli* culture, the lid replaced on the plate and the Bioscreen instrument run overnight. Data were collected from the overnight run, mean ODs calculated from the three wells run for each condition and the corresponding CFU count was estimated from the equation determined in section 2.2.2.3.

2.3   Results.

2.3.1   Validation of drop-count method.

The mean $\log_{10}$ CFU/mL values of the ten dilution series tested did not show evidence of significant departure from normality. The Anderson-Darling test gave a value for P of 0.196, this is not considered significant. This means that data so produced can be tested using parametric tests that assume a normal distribution, and that the method was considered to have met the first acceptance criterion.

The power analysis was performed using the standard deviation calculated for the ten dilution series, assuming a sample size of 3 and a confidence level of 95% and the true difference in means that would give a significant

result in 95% of tests was calculated to be 0.236. This is well within the 0.5 value required, meaning that this method can detect differences of the size desired.

The method, having met all acceptance criteria, was considered to have passed validation and was used for further studies.

2.3.2   Validation of turbidometric method of estimating

CFU/mL counts.

Figure 2.2 shows the plot of absorbance at 500 nm as against $\log_{10}$ CFU/mL. A linear best-fit line was plotted with a goodness-of-fit value of 0.94. This shows that there is a strong linear relationship between absorbance at 500 nm and $\log_{10}$ CFU/mL. The line had a gradient of 0.94 and an intercept of 6.
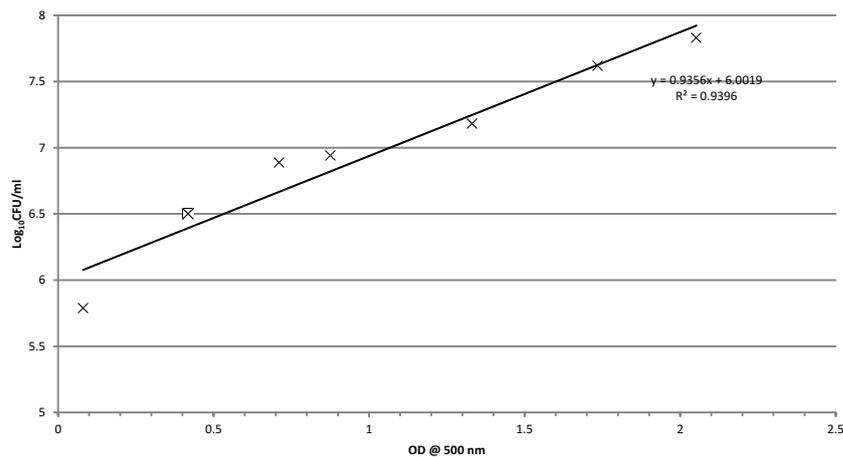
Figure 2.2: A500 (x axis) vs. $\log_{10}$ CFU/mL showing linear trendline and equation.

The highest concentration of cells tested gave a $\log_{10}$ CFU/mL value of 7.83 and an absorbance of 2.05. This absorbance is towards the highest end of the linear range of the spectrophotometer, so samples of higher concentration should not be measured using this method. The lowest concentration of cells tested gave a $\log_{10}$ CFU/mL value of 5.79 and an absorbance of 0.08. This absorbance is at the lowest end of the linear range of the spectrophotometer, so samples of lower concentration should not be measured using this method.

Measuring absorbance at 500 nm therefore allows estimation of $\log_{10}$ CFU/mL between 6 and 8 (i.e. CFU/mL between 10^6 and 10^8) and $\log_{10}$ CFU/mL can be calculated from absorbance using the following equation:

$$\log CFU/mL = 0.94 \times A_{500} + 6$$

This method met the acceptance criteria and thus the requirements and was used in further testing as a means of quickly estimating cell density in samples.

2.3.3   Investigation of VHP exposure methods.

No colonies were present for any of the samples recovered from dried coupons in the first study of the standard carrier test (Figure 2.3). Colonies were present for the inoculant corresponding to a count of approximately 10^8 CFU/mL. The drying and recovery process therefore caused a greater than 3 $\log_{10}$ CFU/mL reduction in cells (no colonies were present at the 10^{-2} dilution which means that less than 2 x 10^5 CFU were recovered from the coupons).
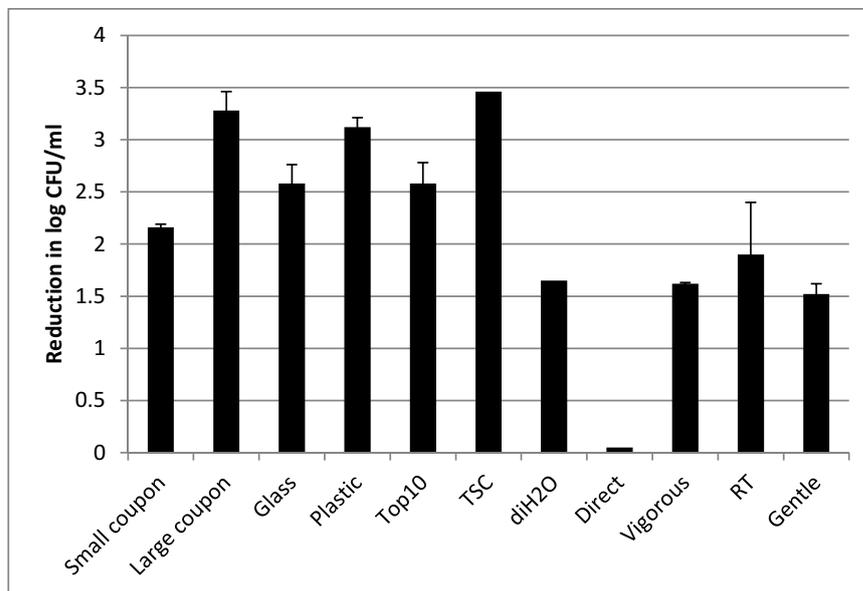
Figure 2.3: Reduction in mean $\log_{10}$ CFU/mL for test conditions compared to the inoculum used. Error bars show standard error of mean.

As can be seen from Figure 2.3, none of the adaptations to the carrier test conditions tested gave a significant improvement on recovery of CFUs compared to the standard conditions (small coupons, inoculated with K12

strain, dried for 1 h at 37°C, recovered in deionised water in a glass bottle, shaken at 150 rpm for 1 min with glass bead), apart from the direct inoculation into deionised water. This demonstrated that the cause of the large drop in CFU count is probably due to the sensitivity of *E. coli* to drying, as changing the recovery procedure had no effect. This sensitivity could not be ameliorated by drying at lower temperature.

As can be seen from Figure 2.4, the trehalose and sucrose solutions both gave >1 $\log_{10}$ reduction in CFU/mL compared to their respective inoculants when dried and recovered. This is possibly better than water alone but insufficient replicates were performed to fully assess this. It is possible that use of such sugars could give further improvements with modifications to the method. However, the alginate gel gave a less than 0.2 $\log_{10}$ reduction compared to the inoculant. This is not statistically significant for the number of replicates tested (P = 0.51) and it seems likely that ˜100% of CFUs are recovered using this method. Based on these results, it was decided to adopt the alginate attachment method for further testing.
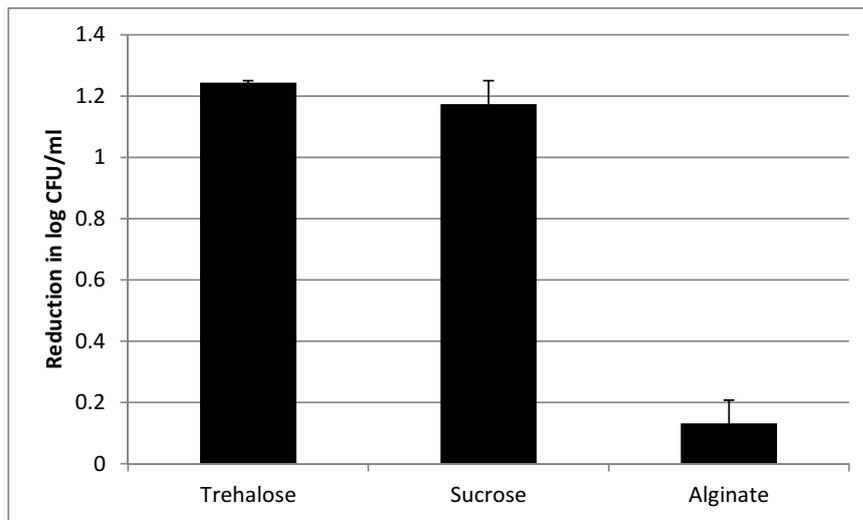
Figure 2.4: Reduction in mean $\log_{10}$ CFU/mL for the three alternative surface attachment methods compared with the original inoculant. Error bars show standard error of the mean.

2.3.4 Validation of artificial biofilm recovery repeatability.

The mean $\log_{10}$ CFU/mL values of the ten dilution series tested did not show evidence of significant departure from normality. The Anderson-Darling test gave a value for P of 0.196, this is not considered significant. This means that data so produced can be tested using parametric tests that assume a

normal distribution, and that the method was considered to have met the first acceptance criterion.

The results of the power analysis performed using the standard deviation calculated for the ten dilution series, assuming a sample size of 3 and a confidence level of 95% gave a value for the true difference in means that would give a significant result in 95% of tests of 0.236. This is well within the 0.5 value required, meaning that this method can detect differences of the size desired.

2.3.5 Determination of working liquid hydrogen peroxide exposure and neutralisation conditions.

Figure 2.5 shows the results of the Bioscreen evaluation of various H_2O_2 concentrations. It can be seen that an effective concentration of 0.0938% H_2O_2 was sufficient to completely prevent growth, whilst lower concentrations produced varying degrees of retardation. These results were used to establish a minimum H_2O_2 concentration to for further investigation of 0.0375%.
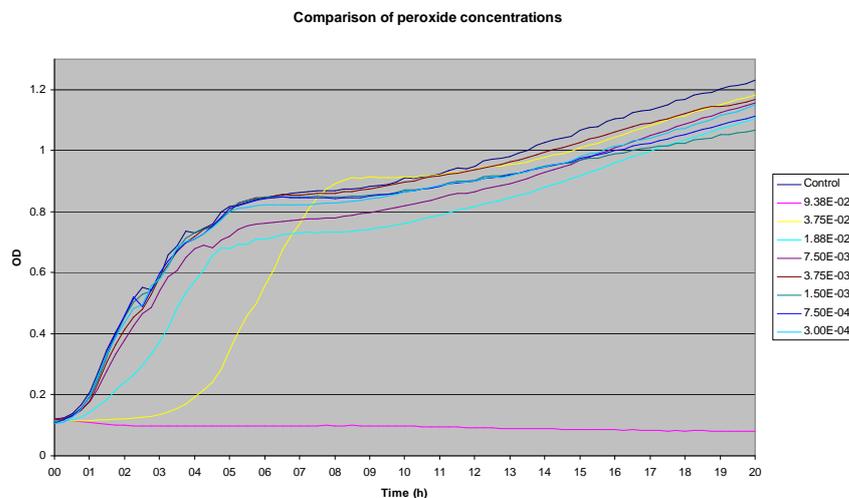
Figure 2.5: Optical density versus time for *E. coli* cultures incubated with various concentrations of hydrogen peroxide.

It can be seen from Figure 2.6 that no H_2O_2 concentration below and including 1.5% gave a significant reduction in CFU count with an exposure time of 30 s. Above this concentration, CFU/mL reduction increased with H_2O_2 concentration.
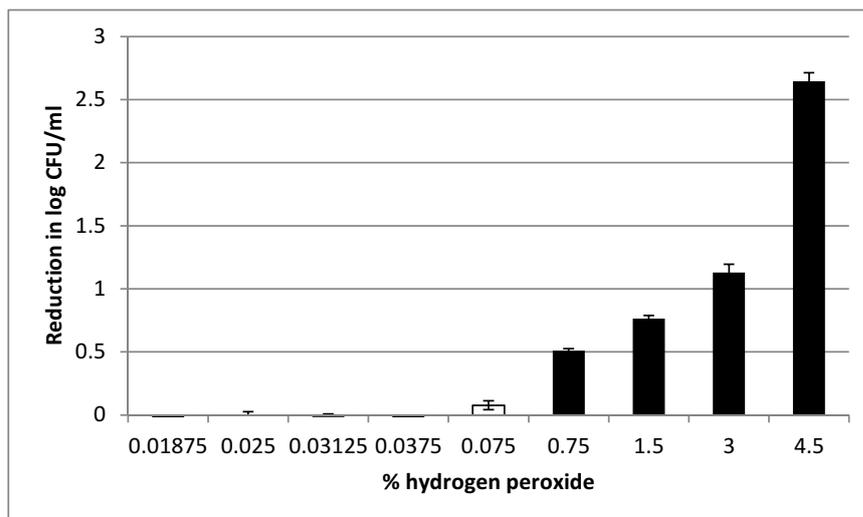
Figure 2.6: Reduction in $\log_{10}$ CFU/mL of various hydrogen peroxide concentrations compared to deionised water control. Solid bars shows conditions that gave a statistically significant reduction (as determined using ANOVA, P<0.05). Error bars show the standard error of the mean.

It can be seen from Figure 2.7 that 5 g/L sodium thiosulphate did not completely neutralise any of the tested concentrations of H_2O_2. It can also be seen from Figure 2.7 that the presence of 5 g/L sodium thiosulphate in the gel solvent was more effective at neutralising higher H_2O_2 concentrations, in that the higher H_2O_2 concentrations gave a significantly smaller reduction in cell count when added to gel solvent containing 5 g/L thiosulphate rather than directly to the coupon.
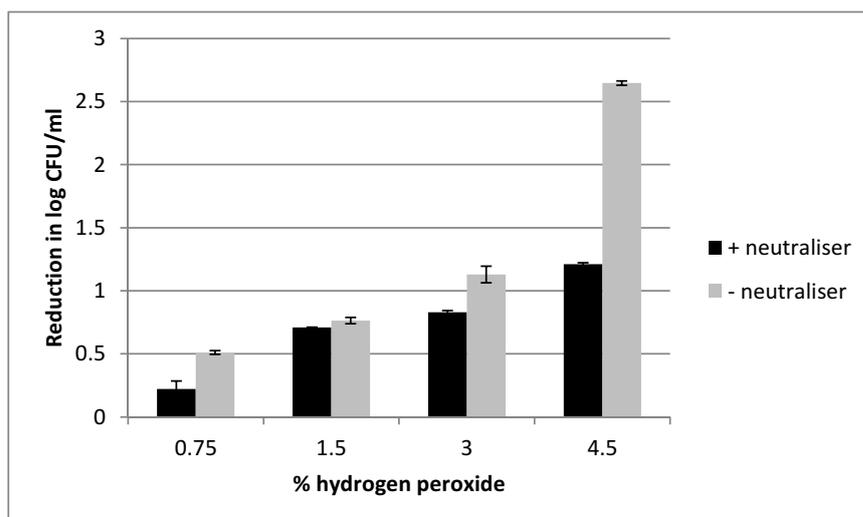
Figure 2.7: Comparison of mean $\log_{10}$ CFU/mL reduction at various hydrogen peroxide with (black bars) and without (grey bars) 5g/L sodium thiosulphate in gel solvent. Error bars show standard error of the mean.

The efficacy of the sodium thiosulphate at neutralising 4.5% H_2O_2 did not improve when the thiosulphate concentration was increased, as shown by Figure 2.8. This suggests that the concentration of H_2O_2 is the rate-limiting factor in the reaction between H_2O_2 and neutraliser.
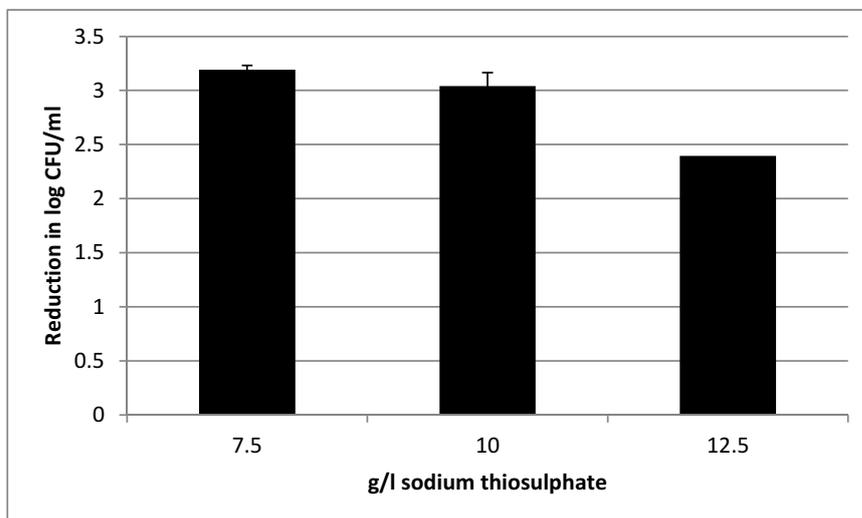
Figure 2.8: Comparison of mean $\log_{10}$ CFU/mL reductions due to addition of 50 ţL 4.5% hydrogen peroxide to gel solvent containing various concentrations of sodium thiosulphate. Error bars show the standard error of the mean.

It can be seen from Figure 2.9 that none of the concentrations of thiosulphate tested had toxic effects as none resulted in a significant reduction in CFU count.
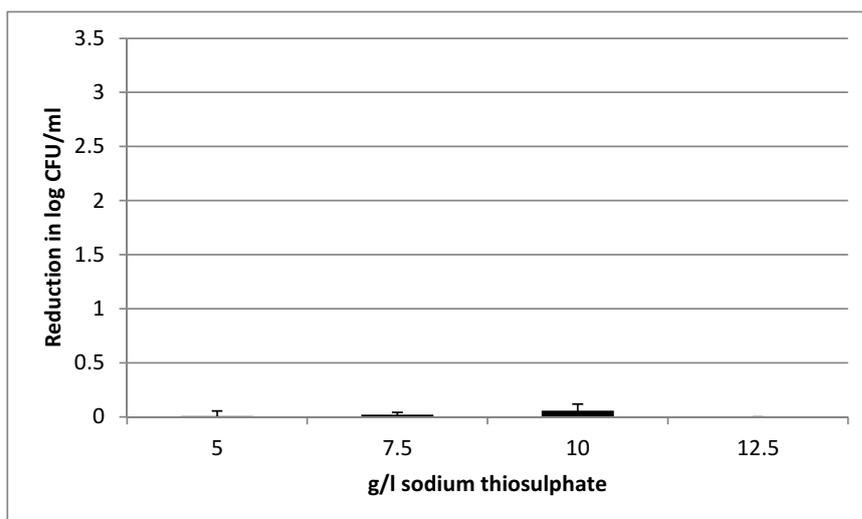
Figure 2.9: Comparison of mean $\log_{10}$ CFU/mL reduction due to addition of various concentrations of sodium thiosulphate to gel solvent. Error bars show standard error of the mean.

Increasing the exposure time of gels to 0.75% H_2O_2 had a dramatic effect on the biocide efficacy. Increasing exposure time to 5 min did not appear to have any further effect, but it is possible that a further increase to 10 min might have some effect (it is not possible to directly compare the 60 s and 10 min exposure times as they were run on different occasions), as shown in Figure 2.10. This suggests that the presence of the gel might have an effect on biocide activity as it takes >30 s to fully penetrate the gel and react with the majority of bacterial cells. The effect of exposure time also suggests that sodium thiosulphate can effectively neutralise 0.75% H_2O_2 (otherwise such an effect would not be seen as exposure would continue on addition of gels to solvent).
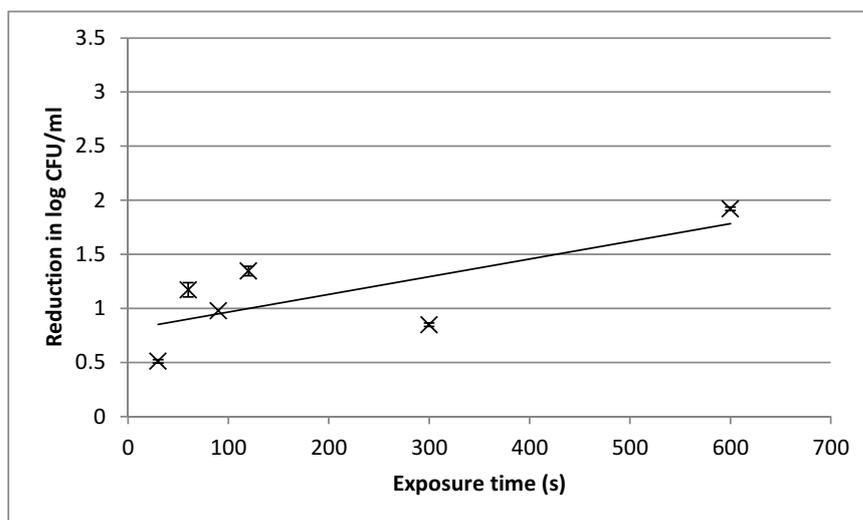
Figure 2.10: Comparison of mean $\log_{10}$ CFU/mL reduction due to exposure to 0.75% hydrogen peroxide for various times. Error bars show standard error of the mean.

These results suggest that the best results will be obtained using low concentrations of H_2O_2 and increasing the exposure time. These two factors can be varied simultaneously in order to achieve a range of biocidal efficacy, whilst also allowing effective use of sodium thiosulphate as a neutraliser.

2.3.6 Determination of working VHP exposure conditions.

Table 2.2 shows the results of varying the H_2O_2 injection rate on the temperature of VHP chamber. As can be seen, varying the injection rate produced a large increase in temperature. Based on these results, an injection rate of 3 g/min was selected for further study, and this rate was not varied as the

effect on the concentration of vapourised H_2O_2 in the chamber is complex due to the change in temperature.

Injection rate (g/min)	Measured temperature
2.0	18 °C
2.4	23 °C
2.8	27 °C
3.2	30 °C
3.6	36 °C
4.0	40 °C

Table 2.2: Measured chamber temperature at various injection rates.

Figure 2.11 shows the decrease in mean $\log_{10}$ CFU/mL after various VHP exposure times compared to control gels incubated at 37řC for 120 s. All conditions gave a significant decrease in CFU count as determined by ANOVA (P<0.05).
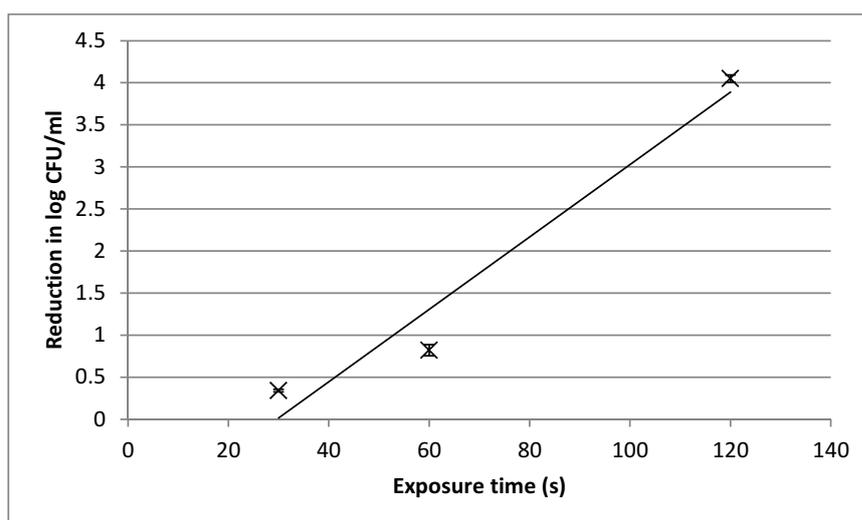
Figure 2.11: Comparison of $\log_{10}$ CFU/mL reduction due to exposure to vapourised hydrogen peroxide at an injection rate of 3 g/min for various times.

2.3.7 Effect of hydroxyl radical scavenger on liquid hydrogen peroxide efficacy.

Figure 2.12 shows the change in $\log_{10}$ CFU/mL for *E. coli* suspensions exposed to increasing concentrations of H_2O_2 in Tris buffer or deionised water. It can be seen that the addition of Tris buffer gave no difference in biocidal efficacy between 0.85 and 10 % H_2O_2, with very similar results being obtained for both Tris and water.
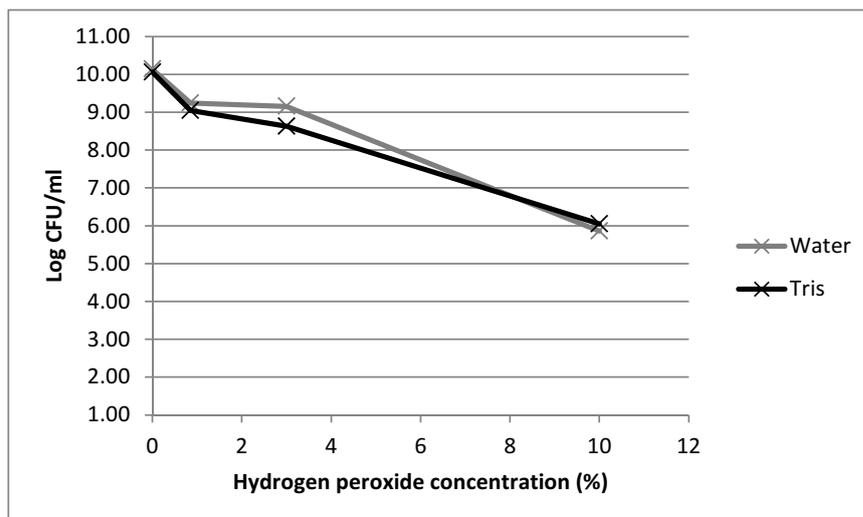
Figure 2.12: Reduction in $\log_{10}$ CFU/mL *E. coli* in suspensions exposed to increasing concentrations of hydrogen peroxide in water or Tris buffer.

Figure 2.13 shows the increase in optical density with time for cultures incubated in a Bioscreen plate with decreasing concentrations of H_2O_2 in TSB with or without the addition of 1 M Tris-HCl. An increase in optical density represent an increase in viable cell count, as previously discussed. No growth was seen with H_2O_2 concentrations of 88 mM and higher in either medium, and no inhibition was seen with concentrations of 8.8 ţM and lower in either medium.
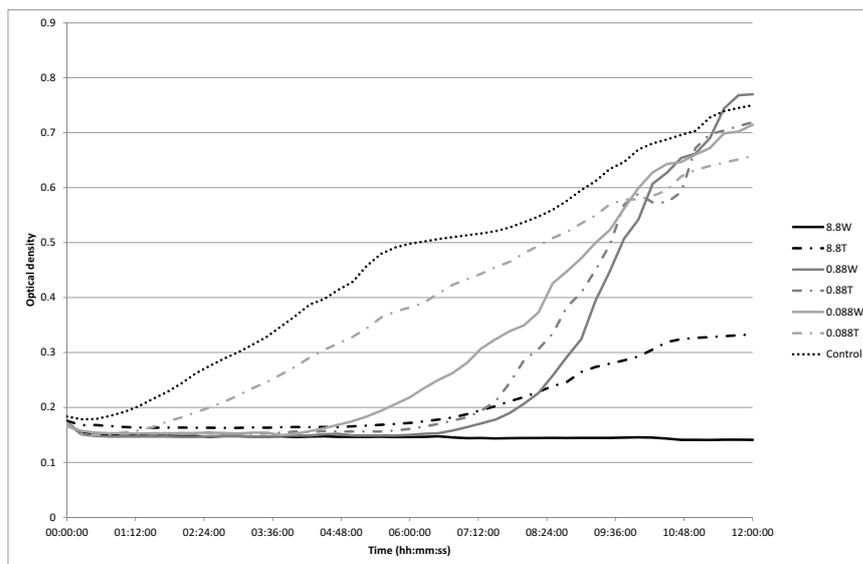
Figure 2.13: Growth curves for *E. coli* cultures incubated in the presence of decreasing concentrations of hydrogen peroxide in tryptone soy broth with or without the addition of 1 M Tris-HCl. In the legend, the first set of numbers gives the concentration of hydrogen peroxide (mM) whilst 'W' indicates water as the diluent and 'T' indicated Tris-HCl.

It can be seen that the addition of Tris-HCl has an effect on inhibition of growth by H_2O_2 at concentrations between 88 ţM and 8.8 mM. No growth was seen when 8.8 mM H_2O_2 was added to TBS in water, but growth was observed after 6 hours at the same H_2O_2 concentration in the presence of Tris-HCl. Growth was seen after 8 hours in cultures containing 880 ţM H_2O_2 in TBS + water, but started at 7 hours in cultures containing the same concentration of H_2O_2 in the presence of Tris-HCl. Growth started after 4 hours and 30 minutes in cultures containing 88 ţM H_2O_2 in TBS + water, but no retardation was seen in the presence of Tris-HCl.

2.4   Discussion.

The standard carrier test investigated gave a greater than 2 $\log_{10}$ reduction in CFU/mL compared to the original inoculum. This suggests that either the process does not efficiently recover dried cells from the coupons, or that either the drying or recovery processes are toxic. Either scenario is intolerable for testing the effects of H_2O_2 on cells; in the first, the vast majority of cells will have been killed by means other than H_2O_2, whilst in the second, such inefficient recovery would render any test too insensitive to be practical. Adaptations to the method did not give a substantial improvement

in cell recovery, and suggested that the drying process was actually toxic to cells. This is supported by the previously mentioned study by Louis *et al.* (1994), who saw 1.5 to 2 $\log_{10}$ CFU/mL reduction in E. coli cell count immediately following air-drying. However, in the present study addition of reducing sugars did not sufficiently stabilise the cells to allow the carrier method to be used.

Embedding cells in an alginate gel gave effectively complete recovery of cells. Testing of 10 replicates demonstrated that this method does not significantly impair repeatability compared to direct Miles and Misra counts from a bacterial suspension, and it was therefore adopted for future testing. It is however possible that this method will have some effect on results, either by changing the manner in which H_2O_2 contacts the cells, or by introducing additional impurities which could themselves interfere with H_2O_2 action. In all subsequent studies, three conditions were used whenever comparisons were to be made between liquid and vapour phases; bacterial suspensions treated with liquid H_2O_2, alginate gels treated with liquid H_2O_2, and alginate gels treated with vapourised H_2O_2. Where substantial differences were seen between the alginate gels and bacterial suspensions treated with the liquid phase, these will be mentioned and taken into consideration when comparing the effects of the two phases.

Investigation of liquid H_2O_2 concentrations and exposure times showed an approximately 1 $\log_{10}$ drop in CFU/mL count when cells embedded in alginate gels were exposed to 0.85% H_2O_2 for 45 s. A similar drop in CFU/mL count was seen when gels were exposed to vH_2O_2 for 60 s with the VHP100 instrument set at an injection rate of 3 g/min. Investigations into changing the injection rate of the VHP 1000 instrument showed a sharp increase in temperature as the injection rate was increased; theory predicts that such an increase will be accompanied by an increase in relative humidity and condensation rate. The effects of condensation and humidity are somewhat controversial – Watling *et al.* (2002) and Marcos-Martin (1996) maintain that invisible "microcondensation" is necessary for biocidal effects of vapourised H_2O_2, whilst Hultman *et al.* (2007) suggest that VHP decontamination is purely a dry vapour process and is impaired by excessive condensation. Support for the position of Watling and Marcos-Martin is provided by a study of the effects of humidity, H_2O_2 concentration and condensation on inactivation of *Geobacillus stearothermophilus* spores by vH_2O_2 which shows that increased humidity was associated with a decrease in D-value at low

vH_2O_2 concentrations and that increased microcondensation was associated with decreased D-values at all tested concentrations of vH_2O_2. Whichever position is correct, however, both agree that condensation has some effect on the efficacy of VHP biocide action, and as the only way to increase vapour H_2O_2 concentration in the VHP 1000 system is to increase injection rate with an associated rise in temperature, it was decided that the possibility of complicated confounding effects made testing at varying vH_2O_2 concentrations impractical. It was also considered advantageous that the desired reduction in CFU/mL count could be obtained with a short, 60 s exposure, comparable to the time of 45 s required to produce a similar reduction with 0.85% liquid H_2O_2. This should minimise effects due to the constant replenishment of fresh H_2O_2 in the vapour system.

Investigation of sodium thiosulphate as a neutraliser suggests that 10 grams per litre of sodium thiosulphate is sufficient to neutralise up to 10 % H_2O_2 and allow some control over exposure times. Unfortunately, the reaction between peroxide and thiosulphate does not appear to be instant, however the fact that a measurable effect was seen due to exposure time to liquid H_2O_2 shows that sodium thiosulphate does reduce the cidal effect. Increasing the concentration of sodium thiosulphate had no observable effect, nor did any of the concentrations tested have a toxic effect on cells. The requirement to neutralise H_2O_2 as quickly as possible, and release cells from alginate gels lead to the development of a neutralisation procedure based on the addition of 10 volumes 10 g/L sodium thiosulphate + 0.2 M sodium citrate solution immediately following exposure. This solution, along with unreacted H_2O_2 and dissolved sodium alginate was then removed as quickly as possible by centrifugation, followed by washing of the cell pellet. It was thought that this method offered the best compromise between stopping the effects of H_2O_2 exposure at the desired time, and keeping the methods used to treat with liquid and vapour phase as similar as possible.

Interestingly, it appears that the effect of adding Tris as a hydroxyl radical scavenger varies according to the concentration of H_2O_2 present. The maximum concentration tested was 10 % H_2O_2 which corresponds to a concentration of 2.94 M, although the concentration of Tris tested was only 1 M, this is sufficient theoretically to react with more than one third of the total hydroxyl radicals that could be produced from such a peroxide concentration. The concentration of Tris tested was more than sufficient, theoretically, to completely react with all the hydroxyl radicals produced

by the other two concentrations of peroxide tested (0.85 %, corresponding to 0.25 M; and 3 %, corresponding to 0.88 M), however no effect was seen on H_2O_2 toxicity by the addition of Tris to cell suspensions before H_2O_2 treatment. Addition of Tris to growth medium did have an effect on growth inhibition by H_2O_2 concentrations of 8.8 mM and lower (0.03 %), with Tris appearing to reduce the inhibitory effect.

These results suggest that the production of free hydroxyl radicals either outside or inside the cells is less important to the biocidal mechanism of H_2O_2 as the concentration used to treat increases. In particular, at concentrations typically used a liquid sterilant, addition of a hydroxyl radical scavenger has no effect on bactericidal efficacy against *E. coli* suspensions, which would imply that the production of free hydroxyl radicals has no part in the bactericidal mechanism of such concentrations, with toxicity instead being caused either by non-radical mediated reactions, or by the localized production of hydroxyl, or some other radicals such that these radicals can immediately *react* with macromolecular targets without chance for scavenging substances to react.

Chapter 3

Protein.

3.1 Introduction.

3.1.1 Protein oxidation by hydrogen peroxide.

Proteins, being complex molecules made up of a chain of amino acid residues, are susceptible to damage by oxidation in a variety of ways, and these have been studied quite extensively *in vitro*, with the chemical mechanisms and products of oxidation of individual amino acids residues characterised. Most simply, H_2O_2 has been shown to be able to directly react with the two sulphur-containing residues, cysteine and methionine.

3.1.1.1 Direct reaction of hydrogen peroxide with cysteine.

The reaction of H_2O_2 with cysteine residues has been particularly well-studied, due to the importance of cysteine residues in many enzyme active sites (such as protein tyrosine kinases) and its presence in cellular antioxidants (such as glutathionine), and the mechanism is most commonly described as a two-step nucleophilic substitution to give a disulphide as shown in equations 1, 2 and 3:

1. CSH CS$^-$ + H$^+$
2. CS$^-$ + H_2O_2 CSOH + HO$^-$
3. CSOH + CS$^-$ CSSC + HO$^-$

The overall stoichiometry of the reaction is shown in equation 4:

4. 2RSH + H_2O_2 RSSR + 2H_2O
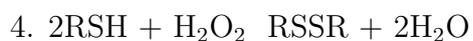
That is, one single molecule of H_2O_2 reacts with two cysteine residues to form a single disulphide and two molecules of water. This reaction has

been demonstrated to occur to free cysteine *in vivo* in the absence of metal irons (Barton *et al.*, 1973; Luo *et al.*, 2005).

Within the cytoplasm, most protein cysteine residues are preserved as thiols, and the importance of the preservation of the oxidation state of protein thiols is demonstrated by the maintenance of a reducing environment within the cell. The formation of disulphides is damaging to protein function in two ways: first, the destruction of thiols in enzyme active sites leads to the inhibition of those enzymes, and second, the uncontrolled formation of disulphide bonds between proteins can lead to denaturation and aggregation. The formation of disulphide bonds is reversible by cellular reductants such as glutathionine, and so damage of this nature can be repaired, providing the cell possesses sufficient reducing power.

It has also been shown that, when the ratio of H_2O_2 to cysteine greatly exceeds the stoichiometric ratio, H_2O_2 can compete with cysteine for the reactive sulphenic acid (CSOH) intermediate, leading to the production of cysteine sulphinic acid (CSO2H) , shown in equation 5:

5. $CSOH + H_2O_2 \ CSO_2H + H_2O$
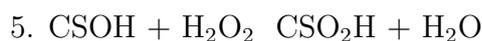
And this sulphinic acid can further react with H_2O_2 to give the sulphonic acid, cysteic acid (CSO_3H), as shown in equation 6 (Luo *et al.*, 2005):

6. $CSO_2H + H_2O_2 \ CSO_3H + H_2O$
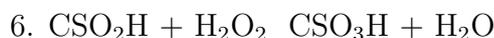
Unlike disulphide formation, the oxidation of cysteine to give sulphinic and sulphonic acids is not always reversible, and production of these groups can affect protein function either by permanent removal of an active thiol, or by denaturation brought about by the negative charge and steric requirements of the highly oxidised sulphur species (Reddie and Carroll, 2008). The oxidation of protein thiols by H_2O_2 to give protein sulphenic acids has been observed *in vivo* (Saurin *et al.*, 2004).

3.1.1.2   Direct reaction of hydrogen peroxide with methionine.

H_2O_2 oxidises methionine residues to give methionine sulphoxide, as shown in equation 7:

7. $MetS + H_2O_2 \ MetSO + H_2O$ This reaction is reversible, and most cells contain reductase enzymes that catalyse the reduction of methionine sulphoxide to methionine e.g. *E. coli* contains the enzymes methionine sulphoxide reductase A + B, responsible for reducing the two enantiomeric forms of methionine sulphoxide (Ezraty *et al.*, 2005).

The oxidation of methionine to methionine sulphoxide is the most common oxidative damage occurring to proteins, and H_2O_2 will readily oxidise methionine residues in proteins even at acidic pH. The readiness with which methionine can be oxidised, combined with the reversibility of this reaction, and the seeming lack of specific function of methionine residues (these can generally be replaced with any other hydrophobic residue, such as leucine) has lead some commentators to propose that its general function might be to act as an antioxidant to defend the cell from oxidative attack, and in particular as an internal antioxidant within the protein molecule to prevent oxidation of more sites (Levine *et al.*, 2000).

Oxidation of methionine residues is not without consequence as the modification to methionine sulphoxide is effectively replacing a hydrophobic residue with a charged, hydrophilic one. This is likely to have effects on the structural and functional properties of the protein, and methionine oxidation has been suggested to be of importance for regulating cell function (Hoshi and Heinemann, 2001). Nonetheless, oxidation of methionine residues alone seems unlikely to be the major fatal lesion of H_2O_2 on cells.

3.1.1.3   Oxidation of other amino acid residues.

Unlike cysteine and methionine, which contain sulphur moieties rendering them susceptible to oxidation, the other amino acids found in *E. coli* proteins are not readily oxidised by H_2O_2 alone, and can only be oxidised by Fenton chemistry in the presence of transition metal ions. In the presence of a suitable metal ion, H_2O_2 is reduced leading to the formation of the hydroxyl radical (OH•) and it is these radicals which go on to attack the amino acid. Amino acid residues that chelate metals (e.g. histidine) are most susceptible to this form of oxidation, and it has been suggested that *in vivo* metal-catalysed protein oxidation is a site-specific process, that is only certain residues on a protein are oxidised with other residues remaining undamaged, though these undamaged residues might be susceptible to oxidation as free amino acids in an *in vitro* system. Table 3.1 summarises the reaction products of amino acids on exposure to hydroxyl radicals (Dean *et al.*, 1997).

Amino acid	Product
Tyrosine	Dopa
Tyrosine	Dityrosine
Phenylalanine	*o*- and *m*-tyrosine
Phenylalanine	Dimers of hydroxylated amino acids
Tryptophan	*N*-Formylkynurenine; kynurenine
Tryptophan	5-Hydroxytryptophan; 7-hydroxytryptophan
Histidine	2-oxohistidine
Glutamic acid	Glutamic acid hydroperoxide
Leucine	Leucine hydroperoxides and hydroxides; -ketoisocaproic acid; isovaleric acid; isovaleraldehyde; isovaleraldehyde oxime; carbonyl compounds
Valine	Valine hydroperoxides and hydroxides; carbonyl compounds
Lysine	Lysine hydroperoxides and hydroxides; carbonyl compounds
Proline	Proline hydroperoxides and hydroxides; 5-hydroxy-2-aminovaleric acid; carbonyl compounds
Arginine	5-Hydroxy-2-aminovaleric acid
Isoleucine	Isoleucine hydroperoxides; isoleucine hydroxides; carbonyl compounds

Table 3.1: Reaction products of amino acid residues on exposure to hydroxyl radicals.

It can be seen from Table 3.1 that metal-catalysed oxidation of amino acid residues commonly results in the formation of new carbonyl groups (aldehydes and ketones) on side-chains.

3.1.2   Methods for measuring protein oxidation.

3.1.2.1   Thiol oxidation.

Due to the importance of protein thiol oxidation both as a lesion of damage, and a means of redox signaling within the cell, many methods to measure this have been developed and described. Eaton provides a thorough overview of methods of measuring protein thiol oxidation in complex mixtures of protein, with particular focus on disulphide formation (Eaton, 2006). He covers methods of derivitising protein thiol groups using various chemical labels; commonly-used thiol labels include derivatives of maleimide, iodoacetamide, iodoacetate, and thiosulphates. These are typically labeled with affinity tags such as biotin, fluorophores, radiolabels, or molecular weight markers such as PEG (which cause large changes to the mobility of proteins

during electrophoresis). For example, Kim *et al.* (2000) describe a method for identifying proteins containing cysteine residues sensitive to oxidation by hydrogen peroxide based on the use of biotin-conjugated iodoacetamide (BIAM). BIAM and H_2O_2 selectively and competitively react with cysteine residues with a low pKa, thus exposure of cells to H_2O_2 reduces the pool of protein cysteines available for labeling with BIAM. Proteins were immobilised on 96-well plates, and incubated with HRP-conjugated streptavidin. The conjugated streptavidin bound to BIAM-labelled proteins and was detected with the colorimetric reagent 3,3',5,5'-tetramethyl benzidine, the intensity of colour produced being inversely proportional to the number of cysteine residues oxidised by exposure to H_2O_2 (Kim *et al.*, 2000).

Other agents used to label thiols described by Eaton (2006) include alkyl halides, arylating agents, disulphide compounds, and thiosulphates. Eaton states that the most well-known of these is the disulphide DNTB (5,5-dithiobis-2-nitrobenzoic acid), also known as Ellman's reagent. The use of this reagent was first described by Ellman in 1959 (Ellman, 1959), and is based on the reaction of DNTB with protein thiols to give a yellow product, the production of which can be monitored directly using a spectrophotometer. Many studies can be found that make use of this method, for instance, Thomas and Aune describe the use of Ellman's reagent to measure thiol oxidation in *E. coli* by the lactoperoxidase, peroxide, thiocyanate antimicrobial system (Thomas and Aune, 1978), Turner *et al.* described the use of Ellman's reagent to measure tellurite-mediated thiol oxidation in *E. coli* (Turner *et al.*, 1999), and Vollenweider *et al.* describe its use in the study of the antimicrobial action of the hydroxypropionaldehyde system (Vollenweider *et al.*, 2010).

Eaton (2006) also describes methods for measurement of thiol oxidation in specific proteins, and for determining the nature of the thiol oxidation. Such specificity is far beyond the scope of this study however (indeed, given the ease with which protein thiols can be oxidise, as discussed earlier, such information is likely to be irrelevant to a study in which bacteria are exposed to high concentrations of oxidant. These methods were developed to investigate damage to particular proteins at a low level in disease states and aging, not acute damage to cells), and these methods will not be discussed further. Due to the established nature, wide acceptance and relatively facile method for protein thiol assay using Ellman's reagent, this method was initially chosen to investigate for use in determining the level of thiol oxidation

in *E. coli* cells treated with H_2O_2.

3.1.2.2   Methionine oxidation.

Methionine oxidation results in the production of methionine sulphoxide as discussed earlier. Measurement of methionine oxidation is therefore usually performed by measuring the amount of methionine sulphoxide present in a sample. Several methods have been developed to do this, most depending on the ability of cyanogen bromide (CNBr) to cleave proteins at methionine residues, but not at methionine sulphoxide residues. For instance, Melkani *et al.* describe a method based on CNBr cleavage and SDS-PAGE analysis of the resulting fragments to estimate the number of oxidised methionine residues in a single *E. coli* protein (Melkani *et al.*, 2006). However, such a method is unsuited for analysis of complex protein mixtures which would not give a simple fragment pattern that could be easily analysed. Additionally, the CNBr method has been criticised for itself causing oxidation of methionine and so introducing artifacts into results (Joppich-Kuhn *et al.*, 1982).

Studies investigating methionine sulphoxide formation in complex mixtures generally rely on amino acid analysis using mass spectroscopy or similar methods. For instance, Rosen *et al.* describe a study to investigate killing by the myeloperoxide system of neutrophils wherein methionine and methionine sulphoxide content of *E. coli* cell extracts treated with myeloperoxide, hypochlorous acid or H_2O_2 was analysed using liquid chromatography coupled with electron spray ionization and tandem mass spectrometry (LC-ESI-MS/MS) and compared to untreated controls, methionine sulphoxide showing as a 16 AMU shift relative to the methionine peak (Rosen *et al.*, 2009).

As previously discussed, methionine residues are easily and reversibly oxidised by hydrogen peroxide and might actually provide some measure of antioxidant protection, so the formation of methionine sulphoxide is probably not relevant to the bactericidal action of high concentrations of H_2O_2. As there exists no rapid and simple method to measure methionine oxidation, it was decided not to measure this as part of the study.

3.1.2.3   Other amino acid oxidation products.

There are numerous studies reporting on various markers of protein oxidative damage, most often as indicators of aging. For example, Leeuwenburgh

et al. (1998) describe the mass spectroscopic analysis of amino acids purified from rat tissues to detect the oxidative products o-tyrosine (from phenylalanine) and 3-nitrotyrosine (from tyrosine) as a means of assessing long-term damage from hydroxyl radical and reactive nitrogen species; Morin *et al.* (1998) report on the HPLC detection of 2-hydroxylysine, a product of hydroxyl radical attack of lysine residues, in proteins subjected to -radiolysis in the presence of oxygen; Davies *et al.* (1999) review the detection of DOPA, *o-* and *m-*tyrosine, di-tyrosine, 5-hydroxyleucine, 3-hydroxyvaline, 3-chlorotyrosine, 3-nitrotyrosine, aminomalonic acid, and 5-hydroxy-2-aminovaleric acid using various HPLC and GC/MS methods.

By far the most widely used measure of protein oxidation though is the non-specific accumulation of protein carbonyl groups. As previously discussed, carbonyl groups are C=O moieties present in unmodified proteins but which are also produced in greater numbers by many oxidative reactions of various amino acid residues, and they can be detected by derivitisation with dinitrophenyl hydrazine. Figure 3.1 shows the derivitisation reaction:
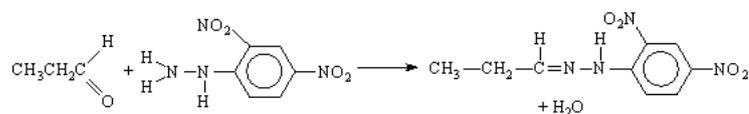
Figure 3.1: Derivitisation reaction of dinitrophenyl hydrazine with carbonyl groups

This gives a yellow label to the protein which can be detected colourimetrically (Levine *et al.*, 1990).

Antibodies against the DNP hapten on keyhole limpet haemocyanin (KLH) are commercially available and so ELISA techniques have been developed to quantify protein oxidation (Buss *et al.*, 1997). Both of these techniques have seen widespread use for studies of aging effects in various tissue and cell cultures and are well-established (Halliwell and Whiteman, 2004).

The colourimeteric method is relatively simple and involves derivitising precipitated protein with DNPH dissolved in hydrochloric acid and then removing unreacted DNPH with a series of washes in hydrochloric acid and methanol. The derivitised protein is redissolved in guanidine hydrochloride and the absorbance read using a spectrophotometer. This gives a direct measurement of carbonyl content by reference to an extinction coefficient for the DNP chromophore, so the method does not require a calibration

with a series of standards. However, the method is relatively insensitive, requiring a minimum of 1 mg of protein and the requirement for multiple washing steps results in high variance of results. As protein is lost during the process, it is also necessary to simultaneously prepare control samples with no DNPH present to estimate the concentration of protein in samples tested. This increases the protein requirement and introduces further sources of error as the protein concentration of DNP-derivitised samples cannot be directly measured. (Levine *et al.*, 2000).

The ELISA method is substantially more complicated, and requires calibration using a series of oxidised protein standards. Proteins are derivitised with DNPH in guanidine hydrochloride solution, diluted and then bound to an ELISA plate which can be probed with a primary and secondary antibody. As would be expected from an ELISA method, it is considerably more sensitive than the colourimeteric method (requiring only 60 ţg of protein) and the lack of a requirement for multiple washing steps gives more reproducible results. As no protein is lost during the process, concentration of samples can be measured before ELISA and used to calculate the carbonyl content per unit of protein (Buss *et al.*, 1997).

The availability of the anti-KLH-DNP antibodies has also allowed the development of Western blot techniques to identify individually oxidised proteins. These involve the separation of DNP-derivitised proteins by SDS gel electrophoresis and transfer of the separated protein to a membrane for probing with anti-DNP antibodies (Shacter *et al.*, 1994). As previously mentioned, Tamarit *et al.* (1998) have used this technique to reveal that individual proteins can be selectively oxidised in *E. coli* subjected to hydrogen peroxide stress. Talent *et al.* (1998) described modifications to this method to provide a double stain for both total protein and derivitised protein following 2-dimension separation. This involved once again derivitising the proteins before isoelectric focusing, second-dimension SDS-PAGE, blotting, staining of the blot using a commercial reversible protein stain, and finally probing the blot using anti-DNP antibody. Although they state in this study that derivitisation of the protein before 2D electrophoresis is required due to high backgrounds from post-blotting derivitisation, a further study by the same group reveals that use of a PVDF membrane allowed post-electrophoretic derivitisation of the proteins on the blot (Conrad *et al.*, 1999). The technique has been used in several studies since (Sultana *et al.*, 2006; Murtaza *et al.*, 2008; Perluigi *et al.*, 2009).

3.2   Materials and methods.

3.2.1   Materials.

Tryptone, sodium chloride, sodium thiosulphate, sodium citrate, calcium chloride, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), phenylmethylsulphonyl fluoride (PMSF), ethanol, bicinchoninic acid (BCA), bovine serum albumin (BSA), phenol, ammonium sulphate, methanol, acetone, sucrose, tris(hydroxymethyl)aminomethane (Tris), urea, guanidine hydrochloride, 5 and 2 N hydrochloric acid solution, sodium dihydrogen orthophosphate, disodium hydrogen phosphate, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4,4-disulfonic acid sodium salt (Ferrozine), methanol, trichloroacetic acid (TCA), potassium phosphate, phosphate-buffered saline (PBS) tablets, amidosulfobetaine-14, 3-[N,N-Dimethyl(3-myristoylaminopropyl)ammonio]propane-sulfonate (ASB-14), 3,3,5,5-Tetramethylbenzidine (TMB), Tween-20, sodium borohydride, trichloroacetic acid (TCA), hypochlorous acid ,urea, thioglycolic acid, potassium ferricyanide, iron (III) chloride, glacial acetic acid, sodium acetate, citric acid, ethylenediaminetetraacetic acid (EDTA), glycine, polyvinyl pyrrolidone (PVP), agarose, bromophenol blue, ProteoPrep Reduction and Alkylation kits, sodium dodecyl sulphate (SDS) and goat anti-rabbit IgG polyclonal antibody were purchased from Sigma (Poole, UK).

Sodium alginate and tryptone soya buffer (TSB) were purchased from Acros Organics.

SuperSignal West Pico chemiluminescent reagent, PVDF membrane and Maxisorb 96-well ELISA plates were purchased from Fisher (Loughborough, UK).

Immobilon Chemiluminescent Western reagent was purchased from Millipore (UK).

Dinitrophenyl hydrazine (DNPH) was purchased from Camlab (Cambridge, UK).

Rabbit anti-KLH-DNP polyclonal antibody was purchased from Molecular Probes/Invitrogen (Paisley, UK).

35% hydrogen peroxide stock was provided by Steris (Basingstoke, UK).

Criterion TGX precast 10-20 % gradient gels, 11 cm IPG strips, Transblot Turbo PVDF membrane packs and Biolytes 3/10 ampholytes solution were purhased from Bio-Rad Laboratories (Hemel Hempstead, UK).

Non-fat dried milk was purchased from a supermarket.

3.2.2   Method development.

3.2.2.1   Development of methods for purifying and assaying protein.

In order to determine the effects of liquid and vapour H_2O_2 treatment on proteins within *E. coli* cells and elucidate the bactericidal mechanism, methods were developed to selectively purify outer membrane and cytoplasmic protein from treated cultures. As a process of embedding cells in alginate gel had been developed to compare the liquid and vapour phases, modifications were required to the alginate solvents and purification procedures in order that they should be compatible. This required devising a method of separating dissolved sodium alginate following dissolution of gels. Various methods of selectively precipitating either the protein or the alginate were tested.

Extracting outer membrane and cytoplasmic proteins from *E. coli* cells presents two distinct challenges – the first is the selective extraction and solubilisation of hydrophobic outer membrane proteins and the second is the removal of large amounts of DNA which is likely to interfere with separation and measurement of cytoplasmic protein. Extraction and purification of outer membrane proteins from *E. coli* has been reported using a number of methods: an early review by Hall and Silhavy (1981) compares results using spheroplast formation followed sucrose density gradient centrifugation with those obtained using French press disruption followed by selective dissolution in various detergents. They report that detergent use can miss some minor outer membrane proteins, though the procedure is far less time consuming. Molloy *et al.* (2001) reported a comprehensive proteomic analysis of the outer membrane proteins using a French press to disrupt cells and sodium carbonate to selectively dissolve the inner membrane and cytoplasmic proteins allowing outer membranes to be collected by centrifugation. Work by Hobb *et al.* (2009) with *Campylobacter jejuni* suggests that of several techniques, disruption by French press followed by removal of non-OMP components with N-lauroylsarcosine (Sarkosyl) gave the purest outer-membrane fraction. Because this study was less concerned with complete representation of the total outer-membrane proteome, and more with ensuring that only outer-membrane proteins are represented in this fraction, it was decided to use the more practical Sarkosyl method. No French press was available, so disruption using sonication was used instead, Benov and Al-Ibraheem (2002) having shown that both methods give

comparable results when disrupting *E. coli* cells.

Various methods have been used for separation of DNA and protein from crude cell extracts. Classically, phenol:chloroform mixtures are used to remove protein from nucleic acid preparations (Chomczynski and Sacchi, 1987): addition of these mixtures into cell extracts causes the partitioning of protein into the organic layer and nucleic acids into the aqueous layer. Other methods include protein precipitation by TCA or various alcohols, and DNase treatment. Antonioli *et al.* (2009) compared these methods, as well as a modification to the normal phenol:chloroform method that used alkaline (pH 9.5 – 10) conditions, to remove nucleic acids from crude *E. coli* cell extracts before 2d gel electrophoresis. They concluded that alkaline phenol:chloroform gave the clearest 2d image, so this method was investigated to determine its suitability.

A 50 mL culture of *E. coli* was grown overnight in TSB, pelleted, washed and resuspended in 5 mL deionised water. This suspension was divided into two, half diluted 1:1 with deionised water and half mixed 1:1 with 4% sodium alginate in a 15 mL centrifuge tube. The alginate was cured by addition of 5 mL 2% $CaCl_2$ and rinsed twice with 5 mL deionised water. Ten mL McIlvaine buffer was added to each tube and the tubes mixed until the alginate gel had broken apart. Cells were recovered by centrifugation at 4000 x g for 15 min, the supernatant discarded and the pellet washed twice with deionised water.

Washed pellets were resuspended in 5 mL deionised water and crude cell extracts prepared as follows: PMSF dissolved in ethanol to a concentration of 100 mM was added to cell suspensions to give a resulting PMSF concentration of 0.4 mM. Cells were then broken apart by three cycles of 60 s sonication on ice separated by 30 s intervals. Intact cells and debris were removed by centrifugation at 4000 x g for 15 min at 4řC, the supernatant was retained and the pellet discarded.

Outer membrane proteins were purified from the crude extracts by addition of Sarkosyl solution in deionised water to give a final volume of 10 mL and a final Sarkosyl concentration of , the mixture was then incubated at room temperature for 30 min with agitation every 10 min. Following incubation, the mixture was centrifuged at 26000 x g for 1 h at 4řC, the supernatant was discarded and the pellet washed three times with 1 mL of deionised water. The pellet was then dissolved overnight at 4řC in 150 L collection buffer containing 7 M urea, 1% ASB-14, 40 mM Tris, 0.5 %

Ampholytes pH 3-10. Dissolved protein was divided into 50 L aliquots and stored at -80řC until use.

This first attempt at purifying outer-membrane proteins from alginate samples was unsuccessful as incompletely dissolved alginate was retained, leading to the final pellet being contaminated with large amounts of alginate, so alternate solvent buffers were tested in order to better dissolve the alginate gel.

A 50 mL culture of *E. coli* was grown overnight in TSB, pelleted, washed and resuspended in 6 mL deionised water. This suspension was divided into three, and each aliquot mixed 1:1 with 4% sodium alginate in a 15 mL centrifuge tube. The alginate was cured by addition of 5 mL 2% $CaCl_2$ and rinsed twice with 5 mL deionised water.

Five mL McIlvaine buffer was added to one tube, 5 mL 0.5 M tetrasodium EDTA added to the second tube and 0.25 M sodium citrate added to the third tube. The tubes mixed until the alginate gel had broken apart, and the tubes were centrifuged at 4000 x g for 15 min. The presence of alginate gel particles before and after centrifugation was judged by eye. Of the alternative solvent buffers tested, the 0.5 M EDTA buffer gave the best results with complete dissolution of the gel occurring within s and no visible particles of alginate remaining before or after centrifugation. Sodium citrate (0.25 M) was slightly inferior as it took several minutes to completely dissolve the alginate. No visible alginate particles were found before or after centrifugation. EDTA is known to disintegrate the outer membrane and increase cell permeability (Vaara, 1992) so it was decided to use 0.25 M sodium citrate as the gel solvent. Tests showed no detectable toxicity to *E. coli* of 0.25 M sodium citrate combined with 5 g/lg/L sodium thiosulphate (results not shown).

A second attempt to purify outer-membrane protein was made using the new gel solvent solution, as follows: a 50 mL culture of *E. coli* was grown overnight in TSB, pelleted, washed and resuspended in 5 mL deionised water. This suspension was divided into two, half diluted 1:1 with deionised water and half mixed 1:1 with 4% sodium alginate in a 15 mL centrifuge tube. The alginate was cured by addition of 5 mL 2% $CaCl_2$ and rinsed twice with 5 mL deionised water. Ten mL 0.25 M sodium citrate + and 5 g/L sodium thiosulphate buffer was added to each tube and the tubes mixed until the alginate gel had completely dissolved. Cells were recovered by centrifugation at 4000 x g for 15 min, the supernatant discarded and the

pellet washed twice with deionised water.

Washed pellets were resuspended in 5 mL deionised water and crude cell extracts prepared as before. Outer membrane proteins were purified from the crude extracts as before.

The protein concentration of the redissolved pellets was measured as follows: a 10 mg/mL solution of BSA in PBS was prepared and then diluted in PBS to give a series of protein standards of concentration 0.1, 0.2, 0.4, 0.5, 0.75, and 1.0 mg/mL. Two aliquots of 190 L of each standard was taken and 10 L of collection buffer (7 M urea, 1% ASB-14, 40 mM Tris, 0.5 % Ampholytes pH 3-10) added to each. Negative standards were prepared by adding 10 L of collection buffer to 190 L PBS. Samples to be measured were defrosted and two 10 L aliquots were taken from each sample and added to 190 L PBS to give a 1 in 20 dilution.

BCA working solution was prepared by adding 50 parts of the BCA solution to 1 part of 4% copper sulphate solution. A 1 mL volume of BCA working solution was added to each 200 L aliquot of standard or sample and mixed by vortexing. These solutions were then incubated at 37řC for 15 min and immediately transferred to ice before having absorbance at 462 nm read using a spectrophotometer blanked against the negative standards.

A standard curve was produced by plotting the mean absorbance at 462 nm of the duplicate standards against the concentration. A straight line was fitted to these points using least-squares regression and the equation of this line calculated. This equation was then used to convert the absorbance at 462 nm of the samples to a concentration in mg/mL. This was corrected for the dilution by multiplying by 20.

This second attempt at purifying outer membrane proteins using sodium citrate as the gel solvent was successful, and produced concentrations of 3.1 and 2.8 mg/mL for the suspension and alginate samples respectively. Further investigations were performed to determine the suitability of the total protein purification method.

A 50 mL culture of *E. coli* was grown overnight in TSB, pelleted, washed and resuspended in 2.5 mL deionised water. This suspension was mixed 1:1 with 4% sodium alginate in a 15 mL centrifuge tube. The alginate was cured by addition of 5 mL 2% $CaCl_2$ and rinsed twice with 5 mL deionised water. Ten mL 0.25 M sodium citrate + and 5% sodium thiosulphate was added to each tube and the tubes mixed until the alginate gel had dissolved. Cells were recovered by centrifugation at 4000 x g for 15 min, the supernatant

discarded and the pellet washed twice with deionised water. Washed pellets were resuspended in 5 mL deionised water and crude cell extracts prepared as described in before. The crude cell extract was divided into ten 500 ţL aliquots and stored at -20ºC until use.

A 10 mL volume of phenol:chloroform:isoamyl alcohol::25:24: 1 was mixed thoroughly with 10 mL of 50 mM tris base pH 9.5 and incubated for eight hours. Three of the aliquots prepared above were taken and prepared as described by Antonioli: 1 mL of the alkaline phenol/chloroform/isoamyl alcohol solution was added to the crude extract in a microcentrifuge tube. The tubes were thoroughly vortexed and the aqueous and organic phase were allowed to separate for 5min at room temperature. The tubes were then centrifuged at 22000 x g for 10 min at 4ºC . The upper-aqueous phase saturated with isoamyl alcohol (containing the DNA) was carefully removed and lower organic phase was discarded. 1.5 mL of ice-cold acetone was then added to precipitate protein and the tubes centrifuged at 22000 x g for 10 min at 4ºC. Following centrifugation, the pellet was washed twice with cold acetone, and dissolved in 6 M guanidine hydrochloride.

The first attempt at using the phenol:chloroform method described by Antonioli was not successful as attempting to remove both the aqueous and organic layers and retain the interface proved to be too difficult, so the method was modified as described. Chloroform was removed as this was responsible for the precipitation of protein at the interface, and sonication steps introduced to resuspend the pellet following precipitation in methanol/ammonium sulphate. Three of the aliquots prepared for testing Antonioli's procedure were processed using these modifications. The modified method proved to be successful with good protein yields.

3.2.2.2   Testing of effect of alginate on protein oxidation.

A 10 mg/mL BSA in PBS solution was prepared and four 20 ţL aliquots of BSA were taken. Two aliquots were diluted 1:2 with deionised water and two were mixed with equal volumes of 4% sodium alginate solution. The alginate was cured by the addition of 100 ţL 2% $CaCl_2$ and rinsed twice with 100 ţL deionised water. 17.2 ţL of 35% H_2O_2 stock was added to the suspension samples to give a final concentration of 7.5 %. Fifty ţL of 7.5 % H_2O_2 solution was added to the alginate samples. Samples were incubated for 60 s and the reaction stopped by addition of 200 ţL of 0.25 M sodium citrate and 5g/L sodium thiosulphate. Samples were mixed until the gels

were completely dissolved. Samples were then added to an equal volume of SDS-PAGE sample buffer (10 % glycerol, 62.5 mM Tris-HCl pH 6.8, 2% SDS, 0.01 mg/mL bromophenol blue, 5 % -mercaptoethanol) and boiled for 7 min. Samples were cooled and loaded onto a precast homogenous 12.5% Phastgel (GE Healthcare) before running and silver-staining on a Pharmacia (GE Healthcare) Phast Gel System according to the manufacturer's protocol. Figure 3.2 shows the Phastgel run with alginate and suspension samples treated with H_2O_2.
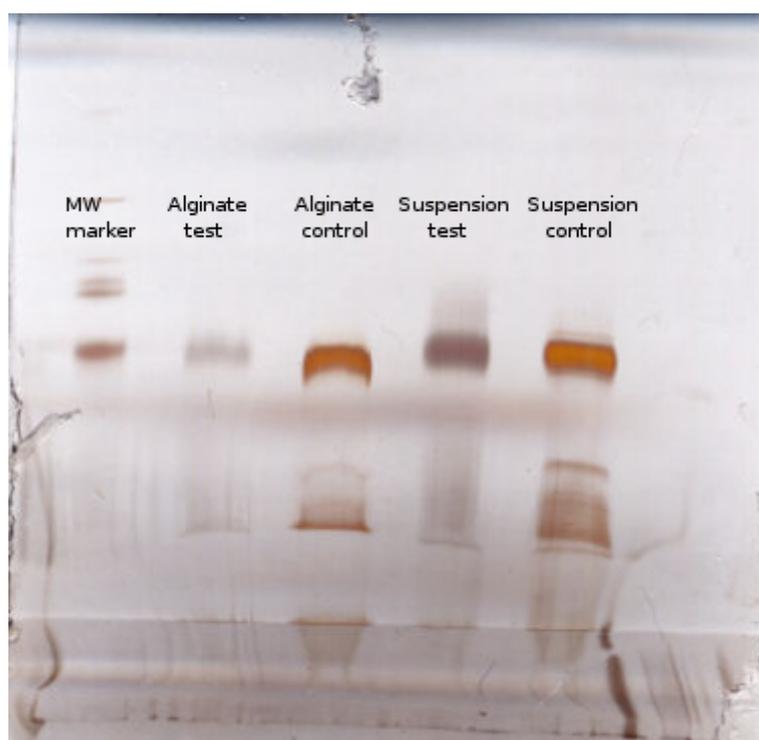
Figure 3.2: Silver stained Phastgel showing comparison of hydrogen peroxide treated BSA in alginate gel or suspension.

As can be seen, no degradation of BSA was visible using H_2O_2 alone. In order to test the effect of alginate on protein oxidation, the test was repeated using Fenton's reagent in place of H_2O_2 alone.

Suspension and alginate gel samples of BSA were prepared as before. 8.6 ţL of 35% H_2O_2 stock and 10 ţL of 0.1 M iron (II) chloride solution was added to the suspension samples to give a final H_2O_2 concentration of 7.5 %. Fifty ţL of 7.5 % H_2O_2 and 10 ţL of 0.1 M iron (II) chloride solution were added to the alginate samples. Samples were incubated for 60 s and the reaction stopped by addition of 200 ţL of 0.25 M sodium citrate and 5g/L sodium thiosulphate. Samples were run on a Phastgel as before. Figure

3.3 shows the Phastgel image for BSA samples treated with Fenton reagent (H_2O_2 and FeCl).
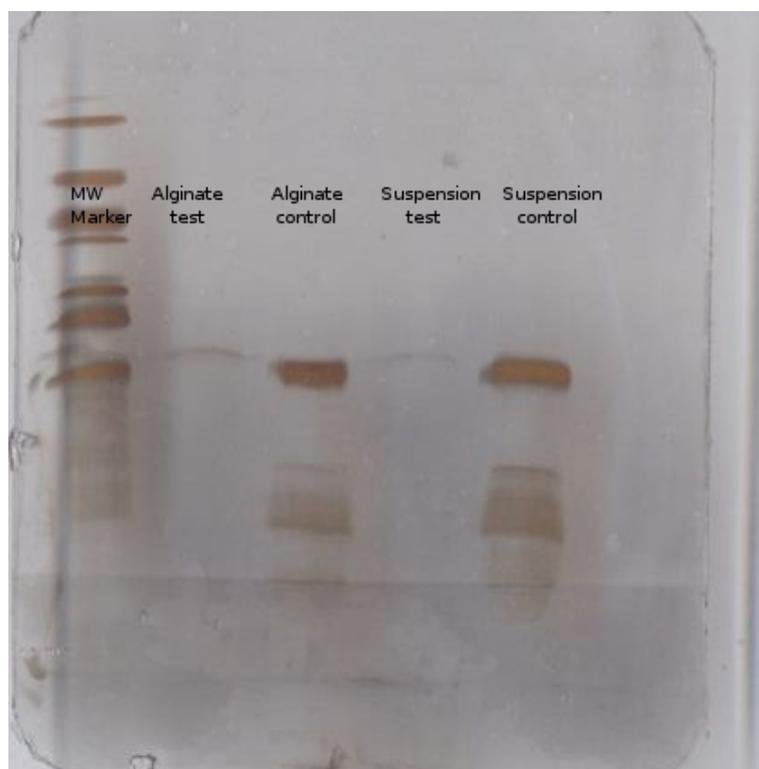
Figure 3.3: Silver stained Phastgel showing comparison of Fenton reagent treated BSA in alginate gel or suspension.

Degradation of the BSA can be seen clearly by the reduction in intensity of the two test bands. This shows that the use of an alginate gel does not prevent oxidation of protein by H_2O_2. It was decided therefore to proceed using this method.

3.2.2.3   Testing of colorimetric method for measuring protein carbonyl content.

Measurement of *in vitro* oxidation of BSA.   Twelve 50 ţL aliquots of 20 mg/mL BSA in PBS were taken. Six aliquots were diluted 1:2 with deionised water and six were mixed with equal volumes of 4% sodium alginate solution. The alginate was cured by the addition of 100 ţL 2% $CaCl_2$ and rinsed twice with 200 ţL deionised water. 21.9 ţL of 35% H_2O_2 stock was added to the suspension samples to give a final concentration of 7.5 %. One hundred and twenty ţL of 7.5 % H_2O_2 solution was added to the alginate samples. Samples were incubated for 60 s and the reaction stopped by

addition of 500 ţL of 0.25 M sodium citrate and 5 g/L sodium thiosulphate. Samples were mixed until the gels were completely dissolved, alginate was precipitated as described previously (this procedure was carried out for both the alginate and the suspension samples). Precipitated protein pellets were not redissolved. Five hundred ţL of 10 mM DNPH in 2 N hydrochloric acid was added to three of the suspension protein pellets and three of the alginate proteins pellets, the others were retained as controls and had 500 ţL 2 N hydrochloric acid added. Samples were mixed thoroughly by vortexing and incubated at room temperature in the dark for 1 h. Unincorporated DNPH was removed by centrifuging at 22000 x g for 20 min at 4řC, the supernatant was discarded and the pellet washed by resuspending in 500 L 2 N hydrochloric acid with thorough vortex mixing. The resuspended protein was incubated at room temperature for 5 min, pelleted by centrifugation at 22000 x g for 20 min at 4řC and the supernatant discarded. This washing procedure was repeated for a total of three washes with 2 N hydrochloric acid and three washes with methanol. Following the final washing step, the pellet was air dried at room temperature for 15 min and the pellets dissolved in 1 mL 6 M guanidine hydrochloride by incubating at 37º C for 15 min and thoroughly vortex mixing.

Incorporated DNPH was measured by taking the absorbance at 375 nm with a 6 M guanidine hydrochloride blank, whilst the protein concentration of the control samples was measured by taking the absorbance at 280 nm with a 6 M guanidine hydrochloride blank. The amount of carbonyl groups present in each derivitised sample was calculated using the extinction coefficient of 22000 M^{-1}, and the concentration of protein present in each control sample was calculated using the extinction coefficient 1.35 mg^{-1} mL. The means of these calculated values for all the replicates were used to calculate mean nanomoles of carbonyl groups present per microgram of protein. Figure 3.4 shows the results of the colorimetric assay of protein oxidation.
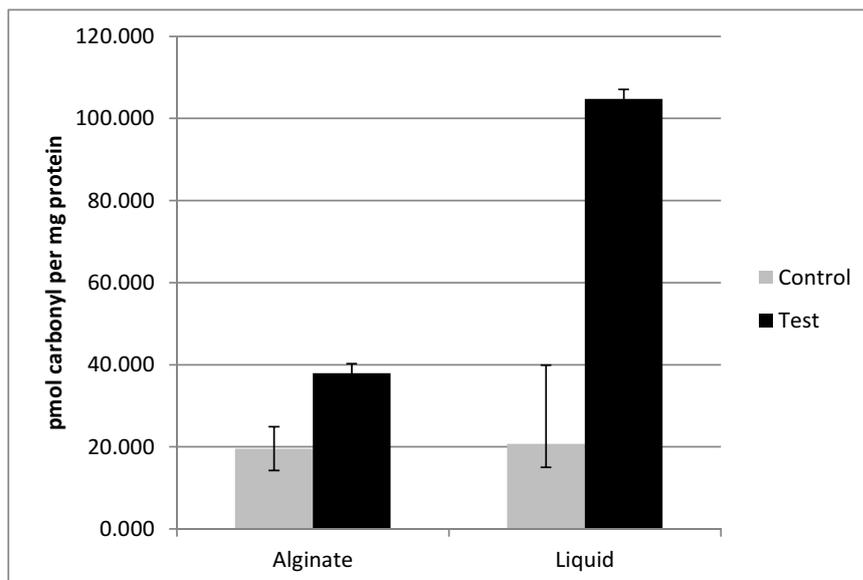
Figure 3.4: Mean nanomoles carbonyl per mg of protein as determined using colorimetric DNPH assay. Error bars show standard error of the mean.

These results suggest that a substantially greater amount of oxidation occurs in free BSA compared with BSA embedded in alginate, but it should be noted that results were very variable and so were not considered to be reliable. In particular, the amount of protein recovered was variable and the greatest amount of protein recovered was from the liquid test samples. It is possible that protein from these samples also retained a large amount of unincorporated DNPH, which would explain the far larger variance seen for these samples. Figure 3.5 shows the recovery of protein for each sample type.
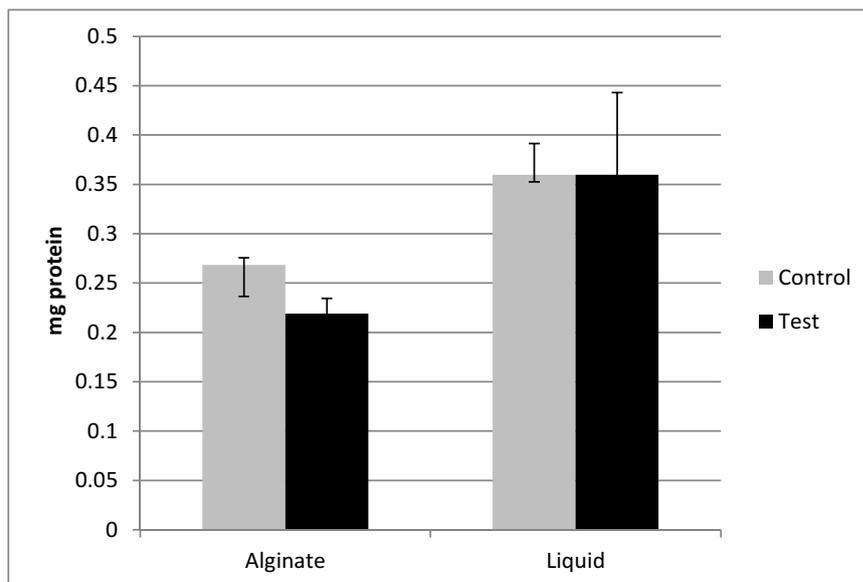
Figure 3.5: Mean recovery of protein from colorimetric DNPH assay. Error bars show standard error of the mean.

Measurement of *in vivo* oxidation of *E. coli* protein. Based on the results of the BSA oxidation measurements, it was decided to compare three conditions when treating bacterial samples – suspension, alginate gels treated with liquid and alginate gels treated with vapour in order that possible effects due to the presence of the alginate could be considered. It was also decided to modify that carbonyl assay procedure by addition of a sonication step when resuspending derivitised protein samples in order to reduce some of the variability seen.

Six 50 mL overnight cultures were resuspended in 5 mL deionised water as described in section 3.3.3.1. Two of the resuspended cultures were treated with 0.85% liquid H_2O_2 or deionised water (control) for 45 s as in section 3.3.3.1, two were embedded in alginate gel and treated with 0.85% liquid H_2O_2 or deionised water for 45 s as in section 3.3.3.2, and two were embedded in alginate gel and placed in vapourised H_2O_2 chamber at an injection rate of 3 grams per minute or a 37°C incubator (control) for 60 s as described in section 3.3.3.2. Crude cell extracts were made from the treated cell suspensions as described in section 3.3.3.3. The crude extracts were split into two equal volumes and half had outer membranes proteins extracted as in section 3.3.3.5 (except pellets were redissolved in 250 ţL collection buffer to give five aliquots) while half had cytoplasmic proteins extracted as in section 3.3.3.4 (except pellets were redissolved in 250 ţL collection buffer to give five aliquots), giving a total of 12 sets of extracts.

One aliquot of each protein type was defrosted and used to quantify protein content as described in section 3.3.3.6. One aliquot of each protein type was defrosted and used for a colourimetric assay of total protein carbonyl content. Protein yields from the extraction process were too low to allow more than 1 mg of protein to be used. This proved to be insufficient to allow retention of the protein pellet during the washing stages (results not shown), so the volume of overnight culture used to produce protein extracts was increased. However, it was not practical to produce sufficient outer-membrane protein to assay using the colourimetric method, so only whole cell extracts were assayed in the final study using this procedure.

3.2.2.4 Testing of colourimetric method for determination of protein thiol concentration.

Two hundred mL of *E. coli* cultured overnight in TSB was pelleted, washed and resuspended in 10 mL deionised water. The resuspended culture was divided into four 2.5 mL aliquots, and 2.5 mL of H_2O_2 dissolved in deionised water to give effective concentrations of 0, 0.85, 3 and 10 % H_2O_2 added. Samples were incubated with H_2O_2 for 45 s before the reaction was halted by addition of 40 mL neutraliser. Cells were recovered by centrifugation at 4000 x g for 15 min, the supernatant discarded and the pellet washed twice with deionised water. Washed pellets were resuspended in 5 mL deionised water and crude cell extracts prepared as before.

Protein thiol content was measured as follows: DTNB (5,5'-dithiobis-2-nitrobenzoic acid) stock was prepared by dissolving 0.3935 g DTNB in 10 mL of methanol and to give a final concentration of 10 mM. Protein thiol content was measured by addition of 50 ţL crude cell extract and 50 ţL 10 mM DTNB in methanol to 1 mL 100 mM tris, 10mM EDTA buffer, pH 8 in a cuvette. Thiol content of samples was measured in triplicate. A blank containing 50 ţL deionised water in place of sample was also prepared. Reaction mixtures were incubated at room temperature for 15 min, absorbance at 412 nm read in a spectrophotometer, and thiolate released calculated from the excitation coefficient for the thiolate ion at 412 nm of 14100 M^{-1}.

Protein content of the samples was measured using the BCA assay as described in section 3.3.3.6. Thiol content per mg of protein was then calculated to correct for any variation due to differences in protein recovery. As predicted, the method proved to be facile, with no further purification

of protein required. Figure 3.6 shows the thiol content per mg of protein for the samples treated with various H_2O_2 concentrations.
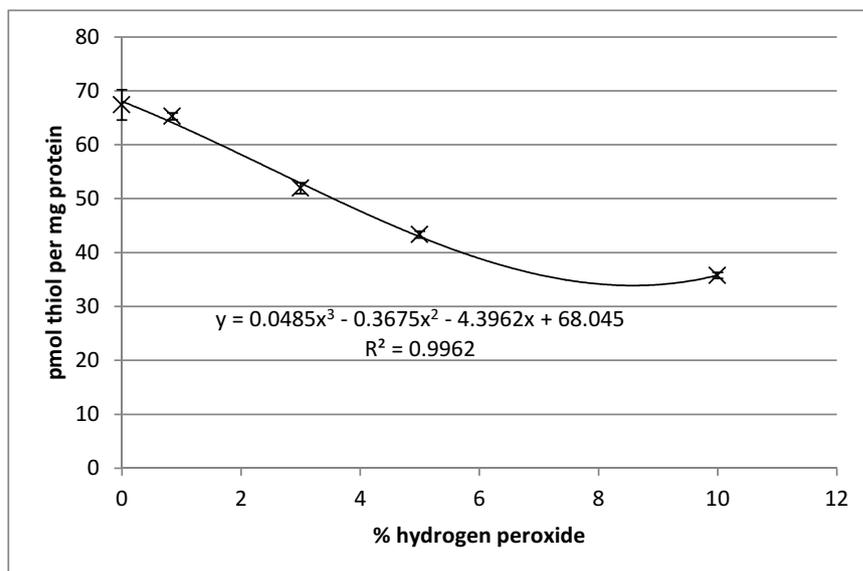
Figure 3.6: Protein thiol content per mg of protein for liquid hydrogen peroxide treated samples. Error bar show the standard error of the mean for three replicate measures.

3.2.2.5 Development of ELISA method for measurement of protein

oxidation.

Testing of ELISA assay.   Fully oxidised protein was produced by reacting 10 mL BSA at 10 mg/mL in PBS with hypochlorous acid overnight at 37řC. Protein in which all carbonyl groups had been reduced was produced by reacting 10 mL BSA at 1 mg/mL in PBS with 10 mg solid sodium borohydride for 1 h at room temperature. The reaction was quenched by addition of 5 N hydrochloric acid and the reduced protein was dialysed overnight against PBS. The total amount of carbonyl groups per microgram of the oxidised and reduced protein was measured using the colourimetric method described in section 3.2.3.

A set of protein carbonyl standards was produced by mixing varying proportions of the reduced and oxidised protein to give 0:1, 1:9, 2:8, 4:6, 6:4 and 8:2 oxidised:reduced standards. These were stored at -20řC until use.

One aliquot of each protein sample prepared in section was defrosted. Standards and samples were derivitised by adding 1 mg of protein to 7.5 mM DNPH in 6 M guanidine hydrochloride adjusted to pH 2.5 with 150

mM potassium phosphate to a total volume of 45 L followed by incubation at room temperature in the dark for 45 min. The reactions were quenched by adding 6 L of reaction mixture to 1200 L of PBS.

An ELISA plate was prepared by incubating 200 L of diluted derivitised sample or standard per well (5 wells per condition) overnight at 4řC. Blank wells were prepared by incubating 200 L coating buffer (10 mM sodium phosphate buffer pH 7.2 and 140 mM sodium chloride) per well. Following incubation, each well was washed 5 times with 300 L coating buffer using an OPSYS MW plate washer. Wells were blocked by incubating for 90 min at room temperature with 300 L blocking buffer (10 mM sodium phosphate buffer pH 7.2 and 140 mM sodium chloride + 0.1 % Tween-20), followed by washing as before. Wells were incubated with 200 L of a 1:1000 dilution of 2mg/mL rabbit anti-DNP-KLH fraction in blocking buffer at 37řC for 60 min, followed by washing as before. Finally, wells were incubated with 200 L of a 1:3000 dilution of goat anti-rabbit IgG HRP-conjugate for 60 min and room temperature and washed as before.

TMB substrate solution was prepared by dissolving 27 mg TMB in 4 mL methanol, adding 6 mL 0.16 M sodium acetate and 0.31 M citric acid solution and 4.3 L 35 % hydrogen peroxide stock. One hundred L TMB substrate was added to each well and allowed to react for 5 min at room temperature before stopping with 100 L 2 N hydrochloric acid. The absorbance of each well at 450 nm was read using an Opsys plate reader. All wells produced a signal too intense to be read.

Optimisation of antibody concentrations.   The first attempt at running the ELISA according to the method described in Buss *et al.* (1997) was not successful as all wells gave colour too intense to be read using the plate reader. Concentrations of antibodies were therefore adjusted as follows:

Fully oxidised protein standard was derivitised by adding 1 mg of protein to 7.5 mM DNPH in 6 M guanidine hydrochloride adjusted to pH 2.5 with 150 mM potassium phosphate to a total volume of 45 L followed by incubation at room temperature in the dark for 45 min. The reaction was quenched by adding 30 L of reaction mixture to 6 mL of PBS. An ELISA plate was prepared by incubating 200 L of diluted derivitised sample or standard per well in 27 wells overnight at 4řC. Five blank wells were prepared by incubating 200 L coating buffer (10 mM sodium phosphate buffer pH 7.2 and 140 mM sodium chloride) per well. Following incubation, each

well was washed 5 times with 300 L coating buffer using an OPSYS MW
plate washer. Wells were blocked by incubating for 90 min at room tem-
perature with 300 L blocking buffer (10 mM sodium phosphate buffer pH
7.2 and 140 mM sodium chloride + 0.1 % Tween-20), followed by washing
as before. Wells were incubated with 200 L of various dilutions of 2mg/mL
rabbit anti-DNP-KLH fraction in blocking buffer at 37řC for 60 min, fol-
lowed by washing as before. Finally, wells were incubated with 200 L of
various dilutions of goat anti-rabbit IgG HRP-conjugate for 60 min and
room temperature and washed as before. The combinations of primary and
secondary antibody dilutions used are shown in table 3.2, three replicates
were run for each combination.

Primary antibody dilution	Secondary antibody dilution
	1:3000
1:1000	1:4500
	1:6000
	1:3000
1:2000	1:4500
	1:6000
	1:3000
1:3000	1:4500
	1:6000

Table 3.2: Combinations of antibody dilutions tested during ELISA opti-
misation.

TMB substrate solution was prepared and the plate read as before.
ANOVA analysis was performed on the results of the factorial study of anti-
body concentration. There was no significant effect due to primary antibody
concentration (P = 0.299), but there was a large effect due to secondary an-
tibody concentration (P <0.0005). There was no significant effect due to
the interaction of the two concentrations (P = 0.091), so it was decided to
use a dilution of 1:2000 of primary antibody (purely for economy) and a
1:4500 dilution of secondary antibody.

An ELISA plate was prepared and run using the remaining bacterial
protein samples as described previously, with the modified antibody con-
centrations as determined.. The second attempt with modified antibody
concentrations gave absorbances too high to be read for the 60 and 80 %
oxidised standards, the response was linear only for the 0, 10 and 20% ox-
idised protein standards. This suggests that the range of concentrations of
oxidised protein was too wide, and that lower concentration standards only

should be used.

It was also found that the 0% standard gave a high reading, suggesting that the fully-reduced standard had not been sufficiently reduced. Following the method of Buss *et al.* (1997) resulted in a low concentration of fully-reduced protein which was insufficient to produce a usable result in the colourimeteric assay, so the amount of carbonyl groups present could not be checked.

Optimisation of reduced and oxidised standard production.   The original method for production of reduced BSA reacted 10 mL of a 1 mg/mL BSA solution with 10 mg sodium borohydride followed by quenching and overnight dialysis to give a final concentration of 2 mg/mL. This was insufficient to perform the colorimetric assay, so the reduction method was modified to use 10 mL of 10 mg/mL BSA and 100 mg sodium borohydride, followed by dialysis; or to use the original concentrations of reagents but to incorporate a desalting and concentration step using spin columns rather than dialysis. Using spin columns rather than dialysis was easier than dialysis and reduced the time during which reduced sample was exposed to air and could potentially be reoxidised.

Attempting to react 10 mg/mL BSA with 100 mg sodium borohydride produced heavy foaming and neutralisation produced further foaming making sample recovery difficult, so this method was abandoned.

Protein in which all carbonyl groups had been reduced was produced by reacting 30 mL BSA at 1 mg/mL in PBS with 10 mg solid sodium borohydride for 1 h at room temperature, followed by neutralisation with 5 N hydrochloric acid and desalting and 10 times concentration using a Pierce centrifuge column.

The concentration of the reduced BSA was estimated by measuring optical density at 280 nm in a spectrophotometer. Use of the centrifuge concentrator yielded 3 mL of sample at 13.8 mg/mL. This was sufficient to perform the colorimetric assay.

Fully oxidised protein was produced by reacting 10 mL BSA at 10 mg/mL in PBS with hypochlorous acid overnight at 37řC. The carbonyl content of the oxidised and reduced samples was measured using the standard colorimetric method as described previously. The fully reduced BSA was found to contain 0.29 nanomoles of carbonyl per mg of protein, whilst the fully-oxidised BSA was found to contain 12.06 nanomoles of carbonyl

per mg of protein. This suggests that either the improved method of buffer exchange prevents re-oxidation of the reduced BSA, or that there was some other reason for the high values for the negative sample in previous ELISA assays.

Optimisation of ELISA blocking. In order to reduce the high background signals, the method of blocking the ELISA plate was investigated. The original method of Buss *et al.* (1997) used reduced BSA as the blocking agent, however an erratum to the original paper suggested that Tween-20 was as effective (Buss *et al.*, 1997(2)), so initial studies were performed using the simpler Tween-20 blocking method. A study was therefore performed to compare the existing blocking method with an alternative method using reduced BSA as the blocking agent.

An ELISA plate was prepared with wells containing either no sample, or the DNPH-derivitised 0.5 nmol/mg carbonyl sample. The ELISA plate was processed as per the standard procedure, however 12 wells were blocked using the standard 1% Tween-20 in PBS and 12 wells were blocked using 0.1 mg/mL fully-reduced BSA. Following processing, the plate was read on the OPSYS plate reader as per the standard procedure. This experiment was performed once only.

For both blocking methods, the 0.5 nmol/mg carbonyl standard gave a signal too high to read. The wells without sample gave an average OD of 0.189 for the BSA blocking method, and 0.186 for the Tween-20 blocking method. This difference was not statistically significant ($p > 0.05$).

Addition of wash step to remove unincorporated DNPH. The previous results suggest that the blocking method is satisfactory, and that the high background and negative standard signals are given by unincorporated DNPH that has not been removed from the plate. Incorporating a washing step into the derivitisation stage to remove unincorporated DNPH before samples are loaded onto the plate would result in excessive sample loss, so it was decided instead to load samples onto the plate before derivitisation. In theory, this allows the plate to be washed thoroughly following the addition of DNPH with minimal sample loss as the sample will be bonded to the solid support.

Cytoplasmic and outer-membrane protein extracts were produced from liquid or vapourised H_2O_2-treated samples (and controls) as previously. One sample of each type was produced from a single overnight culture. A set of

0.29 to 9.71 nmol/mg carbonyl standards was produced from the previously prepared fully-reduced and fully-oxidised BSA standards. An ELISA plate was prepared by loading the underivitised samples and standards onto the plate at a concentration of 5 g/mL, and the plate was incubated overnight at 4 ℃. The plate wash washed five times with PBS using a plate washer and the standards and samples were derivitised by the addition of 200 ţL 0.05 mM DNPH in phosphate buffer, pH 6.2. The plate was incubated in the dark for 45 minutes, and unincorporated DNPH was removed by washing five times with 1:1::PBS:ethanol and once with PBS using a plate washer. The ELISA procedure was then followed as per the standard protocol. This experiment was performed once only.

The standard curve for the ELISA is shown in Figure 3.7.
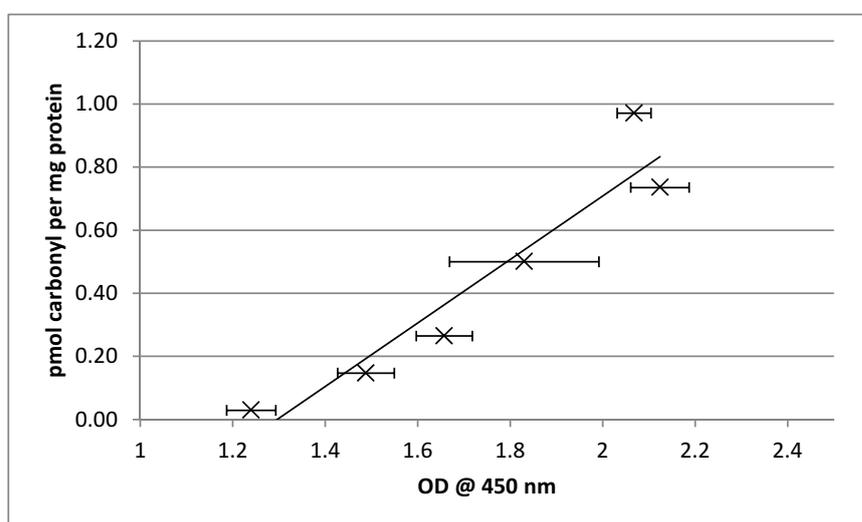
Figure 3.7: Standard curve for the improved ELISA – errors bars show standard error of the mean for five replicates.

This improved method gave a usable standard curve and so was used for further study.

3.2.2.6   Development of 2D gel electrophoresis and Western blotting method to determine carbonyl content of individual proteins.

Testing of 2D gel separation of outer-membrane proteins.   Outer-membrane proteins are often difficult to successfully resolve on a 2D SDS-PAGE gel due to their hydrophobic nature, therefore an initial gel was prepared using OMP extract in order to determine whether modifications needed to be made to the chosen method.

A 50 mL culture of *E. coli* was grown overnight in TSB, pelleted, washed and resuspended in 5 mL deionised water. This suspension was divided into two, half diluted 1:1 with deionised water and half mixed 1:1 with 4% sodium alginate in a 15 mL centrifuge tube. The alginate was cured by addition of 5 mL 2% $CaCl_2$ and rinsed twice with 5 mL deionised water. 10 mL 0.25 M sodium citrate was added to each tube and the tubes mixed until the alginate gel had dissolved. Cells were recovered by centrifugation at 4000 x g for 15 min, the supernatant discarded and the pellet washed twice with deionised water. Washed pellets were resuspended in 5 mL deionised water and crude cell extracts prepared as described previously. Outer membrane proteins were purified from the crude extracts as described in previously, except that 250 ţL of collection buffer was added to give a total of five aliquots of each sample type.

Two aliquots of the suspension protein were defrosted and one used to calculate the total protein content whilst the other was reduced and alkylated according to the protocol for the ProteoPrep Reduction & Alkylation kit (Sigma (Poole, UK)); briefly, 1.25 L tributyl phosphine (TBP) solution was added and the sample incubated for 30 min at room temperature, 1.54 L iodoacetamide (IAA) solution was added and the sample incubated at room temperature for 60 min, finally 1.32 L TBP solution was added and the sample incubated at room temperature for 15 min. Following reduction and alkylation, protein concentration was adjusted to give a total of 250 ng protein in 315 L buffer by addition of running buffer (7 M urea, 2 M thiourea, 1% ASB-14, 0.5% Ampholytes). The adjusted samples were used to rehydrate an 18 cm GE Healthcare IPG strip overnight at room temperature.

The rehydrated and loaded IPG strip was focused using an Amersham (GE Healthcare) IPGphor 2 at 50 mA peak current, 500 volts peak voltage for 500 volt-hours, 1000 volts peak voltage for 1000 volt-hours and finally 8000 peak voltage volts for 32000 volt-hours. The focused strip was equilibrated for 20 min in equilibration buffer (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 20% glycerol) with constant rocking. Following equilibration, the strip was transferred to a GE Healthcare pre-cast DALT 20 cm gel and fixed in place with agarose solution made up of 125 mg agarose in 25 mL running buffer (3.03 grams per litre Tris-HCl pH 8.3, 14.4 grams per litre glycine, 1 gram per litre SDS) and 5 L 10% bromophenol blue). The gel was run for 1 h at 80 volts, 10 mA and 1 watt per gel followed by 6 hours at

500 volts, 40 mA and 13 watts per gel in a GE Healthcare Ettan DALT six tank cooled to 25 řC powered by a GE Healthcare EPS-601 power supply with running buffer as described.

After running, the gel was fixed overnight by incubation in 5:4:1: deionised water:methanol:acetic acid at 4ºC and then stained for two hours at room temperature with colloidal Coomassie blue. The gel was destained overnight at 4ºC in 7:2:1: deionised water:methanol:acetic acid and imaged using a Biorad GS 700 densitometer. The image from the 2D gel is shown in Figure 3.8. Clearly resolved protein spots were obtained, comparable to those obtained by Molloy *et al.* (2000). This demonstrates that the extraction and separation methods were capable of purifying and resolving individual outer membrane proteins from *E. coli* cell suspensions, so no further modifications were made to these procedures.
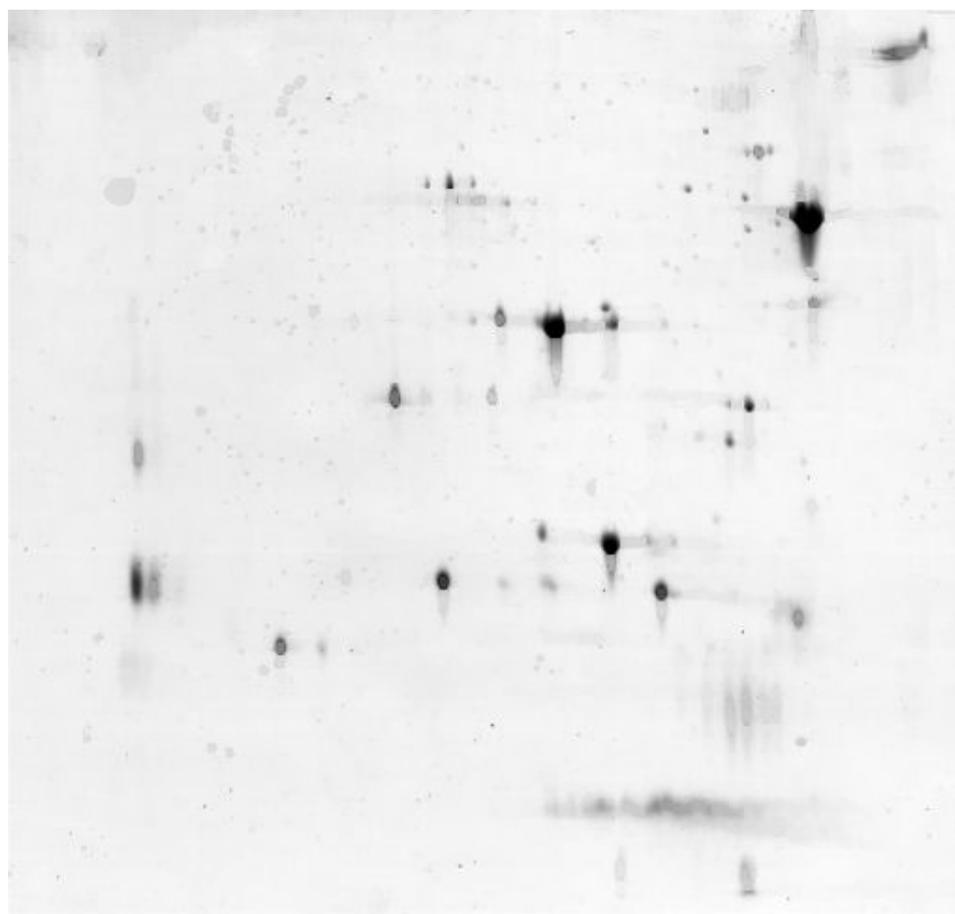
Figure 3.8: 2D gel of suspension outer-membrane protein extract. Gel was stained with Coomassie blue and imaged using GS 700 densitometer.

Dot-blot procedure to test detection of protein carbonyls.   A dot blot assay using reduced and oxidised BSA standards was performed in order to test the immunochemical procedure for detection of protein carbonyls.

Reduced and oxidised BSA standards produced for the ELISA carbonyl assay were dilute to 0.5 mg/mL in PBS, then serial dilutions to 50, 5, 0.5 and 0.05 ţg/mL in PBS made.

A PVDF membrane was wetted with methanol for 5 min, then equilibrated in PBS for 5 min.

A blotting stack was manufactured consisting of, top-to-bottom, one sheet of blotting paper wetted with PBS, one sheet of dry blotting paper, five sheets of dry tissue paper. The wet PVDF membrane was placed on the stack and left until all surface liquid had been absorbed. Once the surface of the membrane was dry, 10 ţL of each dilution was dotted onto membrane. The membrane was removed from the stack and allowed to dry for 10 minutes at room temperature.

Protein carbonyls were derivitised by wetting the membrane with methanol for 5 minutes, equilibrating the membrane in 2 N HCl for 5 minutes, and incubating the membrane for 5 minutes in 0.5 mM DNPH in 2 N HCl. Unincorporated DNPH was removed by washing three times in 2 N HCl, 5 mins per wash, followed by washing five times in methanol, 5 mins per wash.

The membrane was equilibrated for 5 minutes in PBS + 0.05% Tween-20 (PBST), then blocked by incubating for 1 hour at 25 řC in PBST + 5% milk.

The membrane was probed by incubating for 1 hour at 25ř C with a 1:16000 dilution of -KLH-DNP rabbit polyclonal antibody in PBST + 5 % milk, washed three times with PBST and incubated for 1 hour at RT with a 1:2500 dilution of goat anti-rabbit horse-radish peroxidase conjugated polyclonal antibody in PBST + 5 % milk.

The blot was developed by adding 5 mL of Immobilon Chemiluminescent Western HRP substrate and imaged in a UVIchemi cooled CCD camera system. Figure 3.9 shows the resulting dot-blot image, signals were produced for all oxidised BSA samples. These results show that the derivitisation and immunoblotting procedure works satisfactorily with the intended conditions.
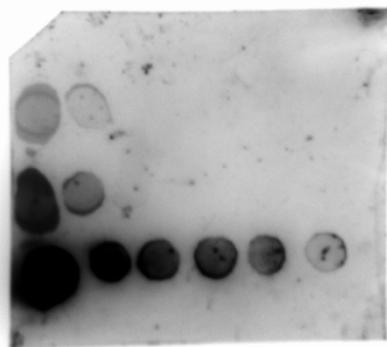
Figure 3.9: Protein carbonyl dot-blot using oxidised and reduced BSA standards to validate blotting method for carbonyl detection. Top row of dots is fully-reduced BSA, middle row is 1:1 mixture of oxidised and reduced BSA, and bottom row is fully-oxidised BSA. Samples were serially diluted 1:10 left-to-right.

Development of Western blot method. One aliquot of suspension cytoplasmic test protein, and one aliquot of suspension cytoplasmic control protein prepared previously were defrosted and electrophoretically separated on 10 cm minigel as follows:

Protein samples were reduced and alkylated according to the protocol for the ProteoPrep Reduction & Alkylation kit as before. Following reduction and alkylation, protein concentration was adjusted to give a total of 100 ţg protein in 125 L buffer (7 cm strips) by addition of running buffer (7 M urea, 2 M thiourea, 1% ASB-14, 0.5% Ampholytes). Adjusted samples were used to rehydrate IPG strips overnight at room temperature.

Whilst strips were focusing, 12% acrylamide resolving gel solutions were prepared using 4 mL acrylamide stock (29.2 grams acrylamide and 0.8 grams Bis per 100 mL), 2.5 mL 1.5 M Tris-HCl pH 8.8, 3.4 mL deionised water and 100 L 10% SDS per 10 mL. Four % acrylamide stacking gel solutions were prepared using 1.3 mL acrylamide stock, 2.5 mL 0.5 M Tris-HCl pH 6.8, 6.1 mL deionised water and 100 L 10% SDS per 10 mL. Gel solutions were degassed for 15 min and polymerisation initiated by addition of 50 L 10% APS solution and 5 L TEMED per 10 mL (resolving gel) or 50 L 10% APS solution and 10 L TEMED per 10 mL (stacking gel). A Biorad mini-Protean Tetra casting apparatus was used to cast 10 cm mini-gels.

Rehydrated and loaded IPG strips were focused using an Amersham (GE Healthcare) IPGphor 2 at 50 mA peak current, 4000 volts peak voltage for a total of 8000 volt hours. Focused strips were equilibrated for 20 min in equilibration buffer (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 20%

glycerol) with constant rocking. Following equilibration, strips were transferred to the cast gel cassettes and fixed in place with agarose solution made up of 125 mg agarose in 25 mL running buffer (3.03 g/L Tris-HCl pH 8.3, 14.g/L glycine, 1 g/L SDS and 5 L 10% bromophenol blue). Mini-gels were run for 45 min at 200 volts, 30 watts maximum power in a Biorad mini-Protean Tetra tank powered by a GE Healthcare EPS-601 power supply with running buffer as described earlier.

Following electrophoresis, the mini-gels were equilibrated for 20 min in blotting buffer (25 mM Tris pH 8.3, 192 mM glycine, 0.025% SDS) at room temperature. PVDF membrane was cut to size, prewet for 15 s in methanol and equilibrated for 20 min in blotting buffer at room temperature. Blots were transferred overnight at 30 volts, 40 watts maximum power in a Biorad mini-Transblot module powered by a GE Healthcare EPS-601 power supply with blotting buffer as described. Transferred blots were dried for 1 h at room temperature.

Dried blots were pre-wet with methanol for 15 s, then blocked by incubating for 20 min with 0.1% PVP in 2% acetic acid at room temperature with constant rocking. Blots were reversibly stained for total protein by incubating for 20 min with Ferrozine stain (0.75 mM Ferrozine, 30 mM iron (III) chloride, 5 mM thioglycolic acid in 2% acetic acid) at room temperature with constant rocking followed by three 1 minute rinses with 2% acetic acid. A second stain was performed by a 20 minute incubation with ferricyanide stain (100 mM potassium ferricyanide, 60mM iron (III) chloride, 100mM sodium acetate pH 4.0) followed by three 1 minute rinses with 100mM sodium acetate, pH 4.0.

The first attempt at blotting did not produce any visible proteins spots when stained with Ferrozine/ferrous stain or enhanced with ferricyanide/ferric stain, so it was decided to modify the blot transfer conditions.

A second aliquot of suspension cytoplasmic test protein, and one aliquot of suspension cytoplasmic control protein prepared previously were defrosted and electrophoretically separated on a 10 cm minigel as before. Mini-gels were equilibrated as before. Blots were transferred for a reduced time of 1 h at an increased voltage of 100 volts, 40 watts maximum power as described. Transferred blots were dried for 1 h at room temperature. Dried blots were stained with Ferrozine/ferrous and ferricyanide/ferric stains as before.

Changing the electroblotting conditions to 1 h at 100 volts produced a blot with faintly visible protein spots which were not able to be detected

using the GS 700 densitometer, so the process was repeated using 250 ţg of protein. The reversible total protein staining procedure was also modified by addition of a 15 min equilibration in 2% acetic acid following blocking before staining.

Repeating the procedure with a higher protein concentration, and modifying the staining procedure to ensure that membranes were first equilibrated before staining gave acceptable stains with the Ferrozine/ferrous complex. Enhancement with ferrocyanide/ferric complex gave high background with extensive streaking. The images obtained are shown in figures 3.10 and 3.11.

Figure 3.10: Suspension cytoplasmic test protein blot stained using Ferrozine/ferrous complex and imaged using GS 700 densitometer.

Figure 3.11: Suspension cytoplasmic control protein blot stained using Ferrozine/ferrous complex

It should be noted that stains looked considerably clearer to the naked

eye than images recorded on the densitometer. Outer-membrane protein blots were allowed to dry before imaging, as this appeared to improve contrast of densitometer images. Figures 3.12 and 3.13 show the images obtained from staining the outer membrane suspension protein blots.
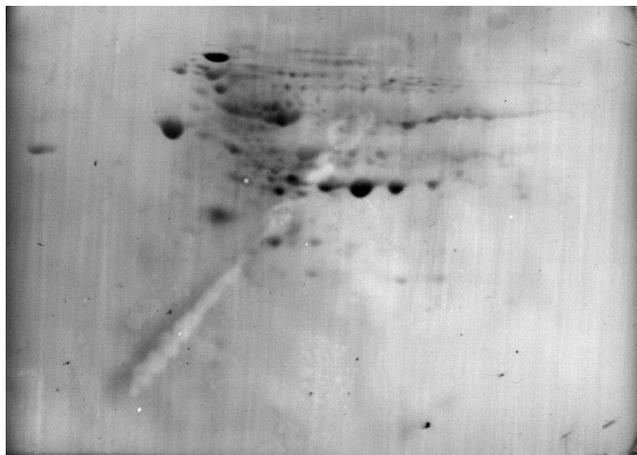
Figure 3.12: Suspension outer-membrane test protein blot stained with Ferrozine/ferrous complex and imaged using a GS 700 densitometer.
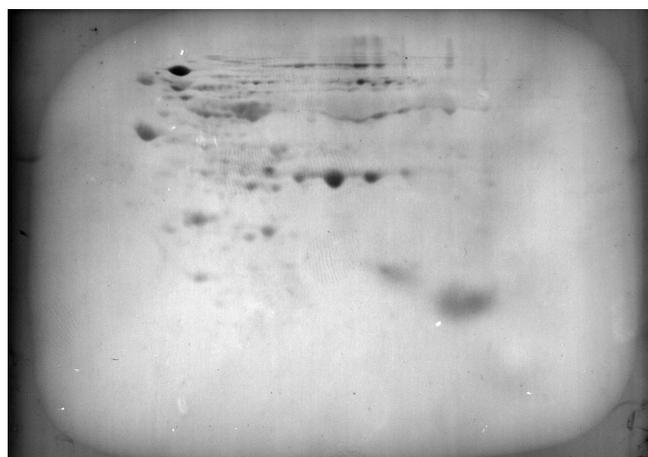
Figure 3.13: Suspension outer-membrane control protein blot stained with Ferrozine/ferrous complex and imaged using a GS 700 densitometer.

Immunoprobing was performed without blocking as described in Conrad *et al.* (2000). Membranes were incubated for 1 h at 25 řC with continuous shaking in rabbit anti-KLH-DNP antibody diluted 1:16000 with 0.1% Tween-20 and 5% non-fat milk in PBS, followed by three 5 min washes with 0.1% Tween-20 in PBS. Membranes were then incubated for 1 h at room temperature with continuous shaking in goat anti-rabbit IgG fraction

diluted 1:2500 with 0.1% Tween-20 and 5% non-fat milk in PBS, followed by three 5 min washes with 0.1% Tween-20 in PBS.

Blots were developed by incubating for 5 min in Immobilon Chemiluminescent Western HRP substrate (Millipore) and imaged in a UVIchemi cooled CCD camera system. The attempt to immunoprobe these membranes using the Western blotting procedure without blocking as described by Conrad *et al.* (2000) was not successful as very high levels of background were produced and individual spots could not be visualised, so a 1 hour blocking step in PBS + 5% milk was tested.

One aliquot of suspension outer-membrane test protein, and one aliquot of suspension outer-membrane control protein were defrosted and electrophoretically separated on 10 cm minigel as before. Gels were blotted, and the blots stained for total and oxidised protein as before. Immunoprobing with an overnight blocking step gave successful results for the control protein, but the test protein did not show any resolvable spots. Figure 3.14 shows the results for the control protein.
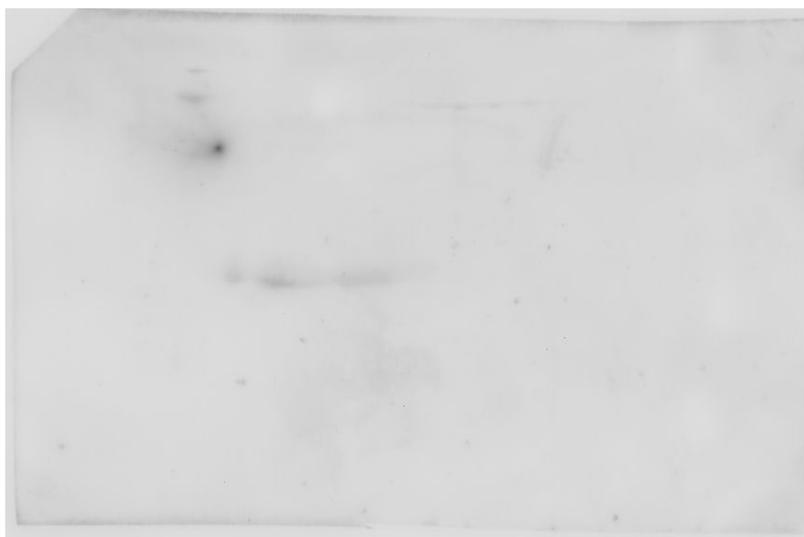
Figure 3.14: Western blot image of oxidised protein in suspension outer-membrane control sample.

It can be seen from figure 3.14 that the image produced for the control protein blot was very faint. A usable image was not obtained for the test protein. This suggested that there was insufficient protein present to give a signal with the chemiluminescent reagent used. Further blots were therefore produced using a larger, 14 cm gel with an 11 cm IEF strip that could be used with a greater amount of protein. A more sensitive chemiluminescent reagent was also used.

3.2.3 Methods.

3.2.3.1 Preparation of liquid hydrogen peroxide treated bacterial suspension.

Bulk *E. coli* cultures were produced by inoculating three 250 mL Erlenmeyer flasks each containing 100 mL TSB with culture from working slopes using a culture loop. These were then incubated overnight at 37°C with shaking (100 rpm) and used immediately. Cell suspensions were transferred into 50 mL centrifuge tubes, centrifuged at 4000 x g for 15 min, and the supernatant discarded. The pellets were washed three times with deionised water, and resuspended in 2.5 mL deionised water. All resuspended pellets were pooled, vortex mixed and five 2.5 mL subaliquots taken in 50 mL centrifuge tubes.

Stock 35 % H_2O_2 solution was diluted in deionised water in 5 mL centrifuge tubes to give 3 mL of 1.7, 3, 6 and 20 % H_2O_2 solutions. Cells were exposed to hydrogen peroxide by adding 2.5 mL of H_2O_2 solution to an aliquot of cell suspension to give the desired final concentrations of 0.85, 1.5, 3 and 10 %. A control was prepared by adding 2.5 mL of deionised water alone. The treatment mixtures were incubated for 45 s before the hydrogen peroxide was quenched by addition of 5 volumes of 5% sodium thiosulphate and 0.2 M sodium citrate solution. The mixture was then centrifuged at 4000 x g for 15 min, the supernatant discarded and the pellet washed three times with deionised water. The washed pellet was resuspended in 5 mL of deionised water for further treatment.

3.2.3.2 Preparation of alginate gel embedded bacterial sample and
vapourised hydrogen peroxide treatment.

The VHP 1000 instrument runs were started before gels were prepared in order to produce a constant hydrogen peroxide concentration in the treatment chamber. Treatments were timed such that gels were exposed immediately on production, following 20 min of decontamination cycle.

Bacterial cells suspended in deionised water were prepared as before. The resuspended cells were mixed with an equal volume of 4% sodium alginate solution and 1 mL of sample transferred to the lid of a miniature Petri dish. The gel was then spread thinly by gently lowering the base of the Petri dish into the upturned lid. The alginate was cured by addition of 2 mL of 2% calcium chloride solution with the Petri dish base still in position, the

base was then discarded and excess calcium chloride was removed by rinsing three times with deionised water. A total of five gels was produced for each exposure time tested, along with 5 gels to act as unexposed controls.

Dishes containing gels were exposed to the VHP treatment chamber by means of the sample tube for exposure times of 1, 2, 3 and 5 minutes. Dishes were removed from the treatment chamber, the gels were immediately scraped into centrifuge tubes containing 5% sodium thiosulphate and 0.2M sodium citrate solution and the alginate was completely re-dissolved by vortex mixing. The mixture was then centrifuged at 4000 x g for 15 min, the supernatant discarded and the pellet washed three times with deionised water. The washed pellet was resuspended in 5 mL of deionised water for further treatment.

3.2.3.3   Preparation of crude extracts.

PMSF dissolved in ethanol to a concentration of 100 mM was added to cell suspensions to give a resulting PMSF concentration of 0.4 mM. Cells were then broken apart by three cycles of 60 s sonication on ice separated by 30 s intervals. Intact cells and debris were removed by centrifugation at 4000 x g for 15 min at 4řC, the supernatant was retained and the pellet discarded.

3.2.3.4   Preparation of purified total protein.

Sucrose and 100 mM tris pH 8.8 solution was added to the crude cell extract to give final concentrations of 0.9 M sucrose and 100 mM tris. An equal volume of phenol was added and the mixture was incubated at room temperature for 1 h with agitation every 15 min. The mixture was then centrifuged at 5000 x g for 20 min to separate the organic (protein-containing) and aqueous (nucleic acid-containing) layers, the aqueous layer was removed and retained, and the organic layer discarded. Ice-cold methanol containing 50 mM ammonium sulphate was added to the retained organic layer, and the mixture was incubated at -20řC overnight to precipitate the protein. The protein was pelleted by centrifugation at 5000 x g for 20 min at room temperature, the supernatant was discarded, and the protein was washed by resuspending in 1.5 mL ice-cold 50 mM ammonium sulphate in methanol, followed by physical agitation of the pellet with a sterile disposable inoculating loop and sonication on ice for 5 s. The resuspended pellets were transferred into microcentrifuge tubes and incubated at -20řC for 20 min. The protein was re-pelleted by centrifugation at 22000 x g for 20 min,

the supernatant was discarded and the protein washed in 1 mL of 50 mM ammonium sulphate in methanol as before . This was repeated for a total of three washes in methanol containing 50 mM ammonium sulphate, two washes in ice-cold acetone and a single wash with ice-cold ethanol. Following the ethanol wash, the protein pellet was air-dried and redissolved depending on the downstream application. Samples were resuspended in 800 ţL 6 M guanidine by physical agitation and allowed to dissolve overnight at 4 řC before immediate assay.

3.2.3.5   Preparation of purified outer-membrane protein.

Outer membrane proteins were purified from the crude extracts by addition of Sarkosyl solution in deionised water to give a final volume of 10 mL and a final Sarkosyl concentration of , the mixture was then incubated at room temperature for 30 min with agitation every 10 min. Following incubation, the mixture was centrifuged at 26000 x g for 1 h at 4řC, the supernatant was discarded and the pellet washed three times with 1 mL of deionised water. The pellet was then dissolved overnight at 4řC in 150 L collection buffer containing 7 M urea, 1% ASB-14, 40 mM Tris, 0.5 % Ampholytes pH 3-10. Dissolved protein was divided into 50 L aliquots and stored at -80řC until use.

3.2.3.6   Protein quantification.

A 10 mg/mL BSA in PBS solution was prepared and then diluted in PBS to give a series of protein standards of concentration 0.1, 0.2, 0.4, 0.5, 0.75, and 1.0 mg/mL. Two aliquots of 190 L of each standard was taken and 10 L of 6 M guanidine added to each. Negative standards were prepared by adding 10 L of 6 M guanidine to 190 L PBS.

Three 10 L aliquots were taken from each sample including the control sample and the retained sample pool from the carbonyl assay and added to 190 L PBS to give a 1 in 20 dilution.

BCA working solution was prepared by adding 50 parts of the BCA solution to 1 part of 4% copper sulphate solution. A 1 mL volume of BCA working solution was added to each 200 L aliquot of standard or sample and mixed by vortexing. These solutions were then incubated at 37řC for 15 min and immediately transferred to ice before having absorbance at 462 nm read using a spectrophotometer blanked against the negative standards.

A standard curve was produced by plotting the mean absorbance at 462 nm of the duplicate standards against the concentration. A straight line was fitted to these points using least-squares regression and the equation of this line calculated. This equation was then used to convert the absorbance at 462 nm of the samples to a concentration in mg/mL. This was corrected for the dilution by multiplying by 20.

3.2.3.7 Quantification of protein carbonyls using colourimetric method.

Protein samples to be measured were divided into three 250 ţL subaliquots for derivitisation. Pooled samples were made by mixing 50 ţL of each sample and three 250 ţL subaliquots of this pooled sample were taken to act as controls, the remainder of the pooled sample was retained. Carbonyl groups were derivitised by addition of 1 mL of 10 mM DNPH in 2 N hydrochloric acid solution, the pellet was resuspended by sonication on ice for three cycles of 10 s with 10 s intervals, and the resuspended pellets were incubated at room temperature in the dark for 1 h. Control samples were treated identically, but 2 N hydrochloric acid with no DNPH was added.

Following derivitisation, unincorporated DNPH was removed by addition of 1.25 mL cold 20% trichloroacetic acid (TCA) in deionised water, followed by centrifuging at 5000 x g for 20 min. The supernatant was discarded and the pellet washed by resuspending in 1 mL 2 N hydrochloric acid with physical agitation using a sterile inoculating loop and sonication on ice for three cycles of 10 s with 10 s intervals. The resuspended protein was incubated at room temperature for 5 min, pelleted by centrifugation at 5000 x g for 20 min at 4řC and the supernatant discarded. This washing procedure was repeated for a total of three washes with 2 N hydrochloric acid and three washes with methanol. Following the final washing step, the pellet was air dried at room temperature for 15 min and the pellets dissolved in 1 mL 6 M guanidine hydrochloride, pH 2.5 with physical agitation and sonication on ice for three cycles of 10 s with 10 s intervals.

Incorporated DNPH was measured by taking the absorbance at 375 nm with a 6 M guanidine hydrochloride blank, and the amount of carbonyl groups present in each derivitised sample was calculated using the extinction coefficient of 22000 M^{-1}. The mean percentage of protein lost during the carbonyl assay was calculated by subtracting the control sample concentration from the retained pool sample and used to provide a correction

factor for the measured sample concentrations (for example, if it was found that 25% of protein was lost on average, then the concentrations of the samples were multiplied by 0.75 to correct for loss during the process).

The means of the calculated values of protein concentration and carbonyl content of the three replicates were used to calculate mean nanomoles of carbonyl groups present per microgram of protein. The whole process was repeated on three separate occasions, and mean values of nanomoles of carbonyl groups present per microgram of protein across the three occasions calculated.

3.2.3.8   ELISA determination of protein carbonyls.

Liquid and vapour treated cultures were prepared as described (sections 3.2.3.1 and 3.2.3.2) , except that only a single concentration (0.85 %) of liquid hydrogen peroxide and a single vapour exposure time (1 min) were tested.

Crude protein extracts were prepared as described (section 3.2.3.3). These extracts were divided into two aliquots and half used for a cytoplasmic protein extract, and half used for an outer-membrane protein extract.

Fully oxidised protein was produced by reacting 10 mL BSA at 10 mg/mL in PBS with hypochlorous acid overnight at 37 řC. Protein in which all carbonyl groups had been reduced was produced by reacting 30 mL BSA at 1 mg/mL in PBS with 10 mg solid sodium borohydride for 1 h at room temperature. The reaction was quenched by addition of 5 N hydrochloric acid and the reduced protein was desalted and 10 x concentrated using a Pierce centrifuge column.. The total amount of carbonyl groups per microgram of the oxidised and reduced protein was measured using the colourimetric method. A set of protein carbonyl standards was produced by mixing varying proportions of the reduced and oxidised protein to give 0%, 10%, 20%, 40%, 60% and 80% oxidised proteins standards. These were stored at -20řC until use.

ELISA plates were prepared by incubating 200 ţL sample or standard diluted to 5 ţg/mL in PBS per well (5 wells per condition) overnight at 4řC. Blank wells were prepared by incubating 200 L PBS per well. Following incubation, each well was washed 5 times with 300 L PBS using an OPSYS MW plate washer.

Standards and samples were derivitised by the addition of 200 ţL 0.05 mM DNPH in phosphate buffer, pH 6.2. The plate was incubated in the

dark for 45 minutes, and unincorporated DNPH was removed by washing five times with 1:1::PBS:ethanol and once with PBS using a plate washer. Wells were blocked by incubating for 90 min at room temperature with 300 L blocking buffer (PBS + 0.1 % Tween-20), followed by washing as before. Wells were incubated with 200 L of a 1:2000 dilution of 2mg/mL rabbit anti-DNP-KLH fraction in blocking buffer at 37řC for 60 min, followed by washing as before. Finally, wells were incubated with 200 L of a 1:4500 dilution of goat anti-rabbit IgG HRP-conjugate for 60 min and room temperature and washed as before.

TMB substrate solution was prepared by dissolving 27 mg TMB in 4 mL methanol, adding 6 mL 0.16 M sodium acetate and 0.31 M citric acid solution and 4.3 L 35 % hydrogen peroxide stock. Once completely dissolved, 100 L TMB substrate was added to each well and allowed to react for 5 min at room temperature before stopping with 100 L 2 N hydrochloric acid. The absorbance of each well at 450 nm was read using an Opsys plate reader. A standard curve was produced by plotting the mean absorbance against nanomoles carbonyl groups and a straight line fitted to these points. The equation of this line was calculated and used to convert the measured absorbance of the samples into values of nanomoles carbonyl groups present.

The whole procedure was repeated three times, and mean values for the three repeats calculated.

3.2.3.9   Western blot method for detection of specifically oxidised proteins.

Protein samples were reduced and alkylated according to the protocol for the ProteoPrep Reduction & Alkylation kit; briefly, 1.25 L tributyl phosphine (TBP) solution was added per 50 L sample and incubated for 30 min at room temperature, 1.54 L iodoacetamide (IAA) solution was added per 50 L sample and incubated at room temperature for 60 min, finally 1.32 L TBP solution was added per 50 L sample and incubated at room temperature for 15 min. Following reduction and alkylation, protein concentration was adjusted to give a total of 150 ţg in 200 L buffer (by addition of running buffer (7 M urea, 2 M thiourea, 1% ASB-14, 0.4% Ampholytes). Adjusted samples were used to rehydrate IPG strips overnight at room temperature.

Rehydrated and loaded IPG strips were focused using an Amersham (GE Healthcare) IPGphor at 50 mA peak current and 8000 volts peak voltage for 30000 volt-hours. Focused strips were equilibrated for 20 min in equili-

bration buffer (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 20% glycerol) with constant rocking. Following equilibration, strips were transferred to the gel cassettes and fixed in place with agarose solution made up of 125 mg agarose in 25 mL running buffer (3.03 g/L Tris-HCl pH 8.3, 14.g/L glycine, 1 g/L SDS and 5 L 10% bromophenol blue). Gels were run for 1 h at 200 volts (50 mA max current and 30 watts max power) in a Bio-Rad Criterion gel tank powered by a GE Healthcare EPS-601 power supply.

Second dimension gels were removed from the gel cassette and transferred into the Transblot PVDF membrane stack in a Transblot Turbo cassette according to the manufacturers instructions. The proteins were transferred at 25 volts constant voltage, 1 A maximum current for 30 minutes.

Dried blots were pre-wet with methanol for 15 s, equilibrated for 5 min in 20 % methanol, 80 % tris-buffered saline (TBS) solution (50 mM tris pH 8.4, 0.9% sodium chloride) at room temperature with continuous rocking, then equilibrated with 2 N hydrochloric acid for 5 min at room temperature with continuous rocking. Proteins were derivitised by incubating the membranes with 0.5 mM DNPH in 2 N hydrochloric acid for 5 min at room temperature with continuous rocking. Finally, unincorporated DNPH was removed by incubating the membranes three times in 2 N hydrochloric acid for 5 min at room temperature (19 to 25řC) with continuous rocking and five times in methanol for 5 min at room temperature with continuous rocking.

Derivitised blots were equilibrated for 5 min in 2% acetic acid solution at room temperature, then blocked by incubating for 20 min with 0.1% PVP in 2% acetic at room temperature with constant rocking, followed by rinsing for 5 min in 2% acetic acid. Blots were reversibly stained for total protein by incubating for 30 min with Ferrozine stain (0.75 mM Ferrozine, 30 mM iron (III) chloride, 5 mM thioglycolic acid in 2% acetic acid) at room temperature with constant rocking followed by three 1 min rinses with 2% acetic acid. Stain sensitivity was increased by a further 5 min equilibration in 100 mM sodium acetate, pH 4.0 followed by a 30 min incubation with ferricyanide stain (100 mM potassium ferricyanide, 60 mM iron (III) chloride, 100 mM sodium acetate pH 4.0) followed by three 1 minute rinses with 100 mM sodium acetate, pH 4.0. Blots were imaged using a Bio-rad ChemiDoc XRS+ cooled CCD system set to colorimetric gel mode, then stain was eluted by incubating for 15 min at room temperature with constant rocking in elution solution (200 mM sodium carbonate pH 9.6, 100 mM EDTA).

Once imaged for total protein, blots were blocked overnight by incubating at 4řC in PBS containing 0.1% Tween-20 and 5% non-fat dried milk, followed by a single 5 min wash with 0.1% Tween-20 in PBS at room temperature. Membranes were then incubated for 1 h at 25řC with continuous shaking in rabbit anti-KLH-DNP antibody diluted 1:16000 with 0.1% Tween-20 and 5% non-fat milk in PBS, followed by three 5 min washes with 0.1% Tween-20 in PBS. Membranes were then incubated for 1 h at room temperature with continuous shaking in goat anti-rabbit IgG fraction diluted 1:2500 with 0.1% Tween-20 + 5% non-fat milk in PBS, followed by three 5 min washes with 0.1% Tween-20 in PBS.

Blots were developed by incubating for 5 min in SuperSignal West Pico chemiluminescent reagent, then imaged using a Bio-rad ChemiDoc XRS+ cooled CCD system set to chemiluminescent blot mode.

3.2.3.10   Measurement of Protein Thiols.

DTNB (5,5'-dithiobis-2-nitrobenzoic acid) stock was prepared by dissolving 0.3935 g DTNB in 10 mL or methanol and to give a final concentration of 10 mM. Stock was stored at -20 řC until use. Protein thiol content was measured by addition of 50 ţL crude cell extract and 50 ţL 10 mM DTNB in methanol to 1 mL 100 mM tris, 10mM EDTA buffer, pH 8 in a cuvette. A blank containing 50 ţL deionised water in place of sample was also prepared.

Reaction mixtures were incubated at room temperature for 15 min, absorbance at 412 nm read in a spectrophotometer, and thiolate released calculated from the excitation coefficient for the thiolate ion at 412 nm of 14100 M^{-1}. Protein content was measured using the BCA assay as described in section 3.2.3.5, and thiol content per mg of protein calculated.

3.3   Results.

3.3.1   Quantification of protein carbonyls using colourimetric method.

Figures 3.15 and 3.16 show the results of the direct colorimetric assay of protein carbonyl content by derivitisation with dinitrophenyl hydrazine. Protein carbonyl content is measured in nanomoles and corrected for variations in protein content by dividing by the measured protein content in milligrams. Figure 3.15 shows the results obtained for *E. coli* suspensions

treated with increasing concentrations of liquid H_2O_2 for 45 seconds, and figure 3.16 shows the results obtained for *E. coli* cultures embedded in alginate gels exposed to vapourised H_2O_2 at an injection rate of 3.7 grams per hour for increasing exposure times. Similar results were seen with *E. coli* cultures embedded in alginate gels treated with liquid H_2O_2 as were seen with suspensions, and so these results are not shown. Both charts show the mean measurements from three repeats of the experiment performed on different occasions. Error bars show the standard error of this mean.
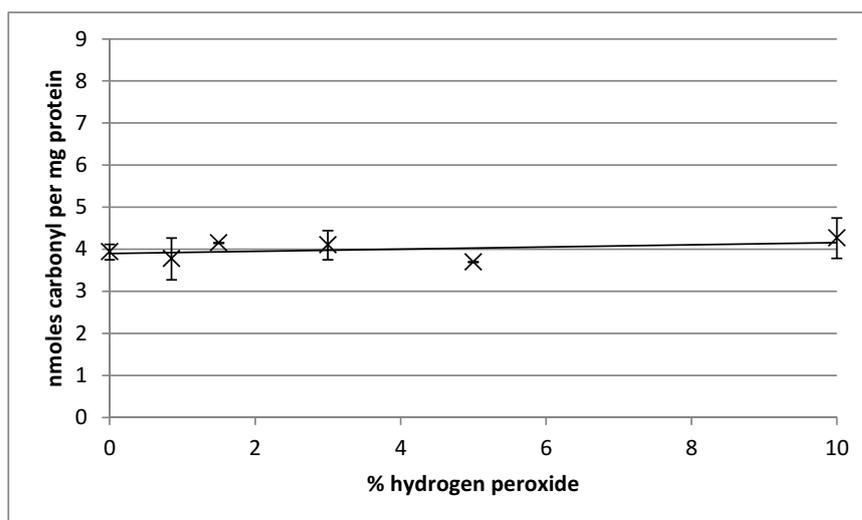
Figure 3.15: Nanomoles of protein carbonyl per milligram of protein for liquid hydrogen peroxide treated *E. coli* suspensions as measured using the colorimetric assay method. Error bars show the standard error of the mean of three repeated experiments.
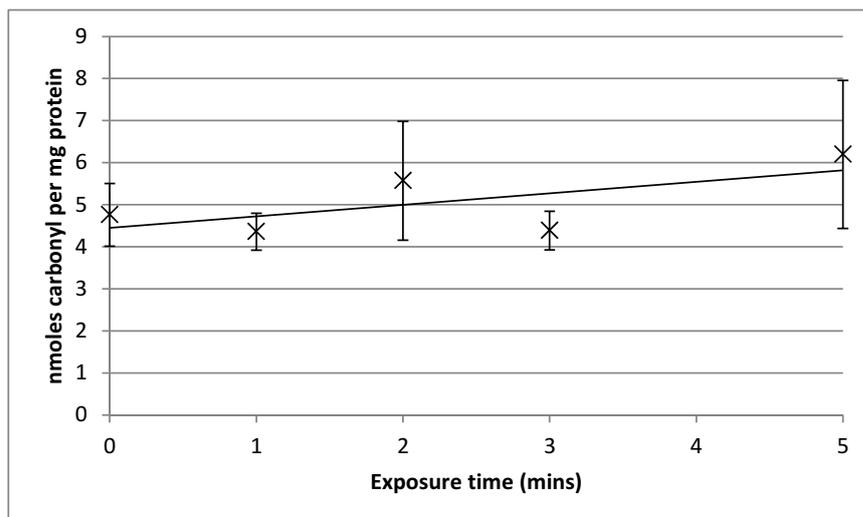
Figure 3.16: Nanomoles of protein carbonyl per milligram of protein for vapourised hydrogen peroxide treated _E. coli_ alginate gels as measured using the colorimetric assay method. Error bars show the standard error of the mean of three repeated experiments.

Analysis using ANOVA shows no significant effect of increasing liquid H_2O_2 concentration, or vapour exposure time on the protein carbonyl content, and nor did any of the treated samples contain significantly more protein carbonyl than untreated, control samples (p $>$0.05).

3.3.2 Protein carbonyl ELISA assays.

Figures 3.17 and 3.18 show the results of the ELISA measurement of protein carbonyl content of cytoplasmic and outer-membrane proteins respectively. Protein carbonyl content is measured by an increase in optical density at 450 nm. Both charts show the mean measurements from three repeats of the experiment performed on different occasions. Error bars show the standard error of this mean.
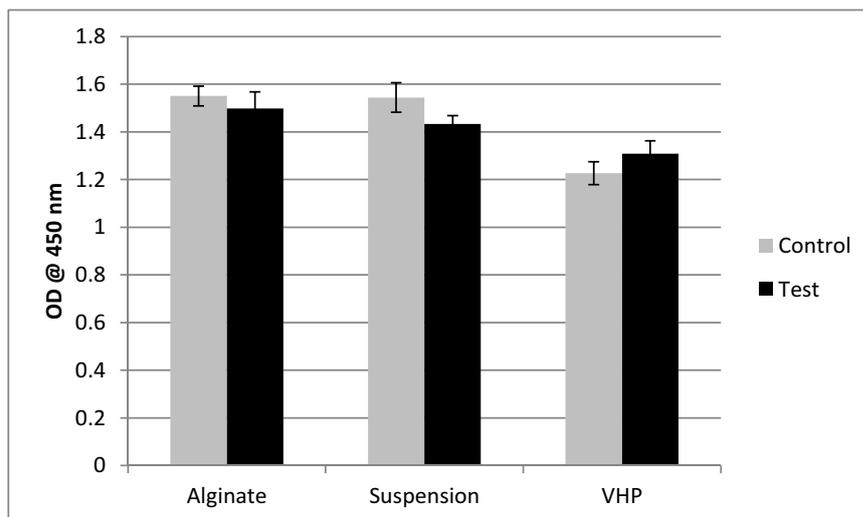
Figure 3.17: Carbonyl content for cytoplasmic *E. coli* protein samples from bacterial suspensions treated with 0.85 % liquid hydrogen peroxide for 45 seconds (suspension), bacteria embedded in alginate gels treated with 0.85 % liquid peroxide for 45 seconds (alginate), and bacteria embedded in alginate gels treated with vapourised hydrogen peroxide for 60 seconds (VHP). Test values are on the right in black, and control (untreated) values are on the left in grey. Error bars show standard error of the mean for three repeated experiments.
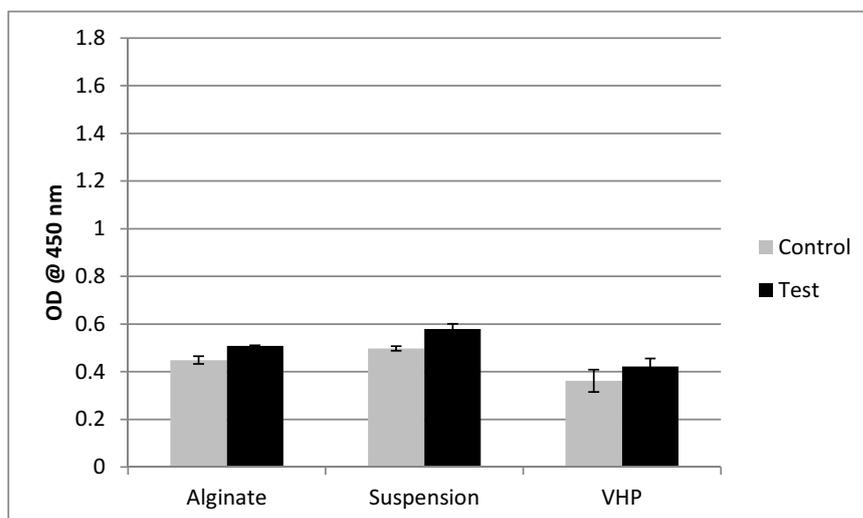
Figure 3.18: Carbonyl content for outer-membrane *E. coli* protein samples from bacterial suspensions treated with 0.85 % liquid hydrogen peroxide for 45 seconds (suspension), bacteria embedded in alginate gels treated with 0.85 % liquid hydrogen peroxide for 45 seconds (alginate), and bacteria embedded in alginate gels treated with vapourised hydrogen peroxide for 60 seconds (VHP)). Test values are on the right in black, and control (untreated) values are on the left in grey. Error bars show standard error of the mean for three repeated experiments.

There was no significant difference between the test and control values for any condition when analysed using ANOVA (p>0.05).

3.3.3   2D Western Blots for specific protein carbonyl formation.

Figures 3.19 and 3.20 show the total protein stains using the Ferrozine/ferrous and ferrocyanide/ferrous stain combination. It can be seen that there was substantial variation in the intensity of staining of these blots, particularly the outer-membrane protein samples. The amount of protein sample added to each IPG strip during first-dimension separation was adjusted to ensure the same amount of protein was added for each sample type, so these differences reflect variations in transfer of proteins into the IPG strips, from the strips into the second dimension gels and from the gels to the blotting membranes. They do not reflect variations in recovery of protein from the treated and untreated samples and so are not related to any effects due to H_2O_2 treatment. The purpose of the total protein stain of the blots is to control for these differences when examining the results of the Western blot for protein carbonyls.

Figures 3.21 and 3.22 show the protein carbonyl Western blot images. In most cases, there was no substantial difference between test and control blots for the various conditions, however the VHP-treated cytoplasmic protein samples appears to show a row of spots positive for carbonyl which did not appear on the corresponding control blot. However, reference to the total-protein stained images for these two blots shows that these spots were also not resolved on the control blot.

Outer-membrane carbonyl Western blot image from the *E. coli* suspensions treated with liquid H_2O_2 did appear to give a more intense image, and for more spots to be resolved than for the control image. However, reference to the total protein stains for these samples shows that the test sample showed more intense staining, and more resolved spots than the control sample.

3.3.4   Protein thiol assays.

Figure 3.23 shows the relationship between picomoles of protein thiol per milligram of protein measured using the DNTB assay for crude extracts from *E. coli* suspensions treated using 0, 0.85, 1.5, 5 and 10 % liquid H_2O_2 for
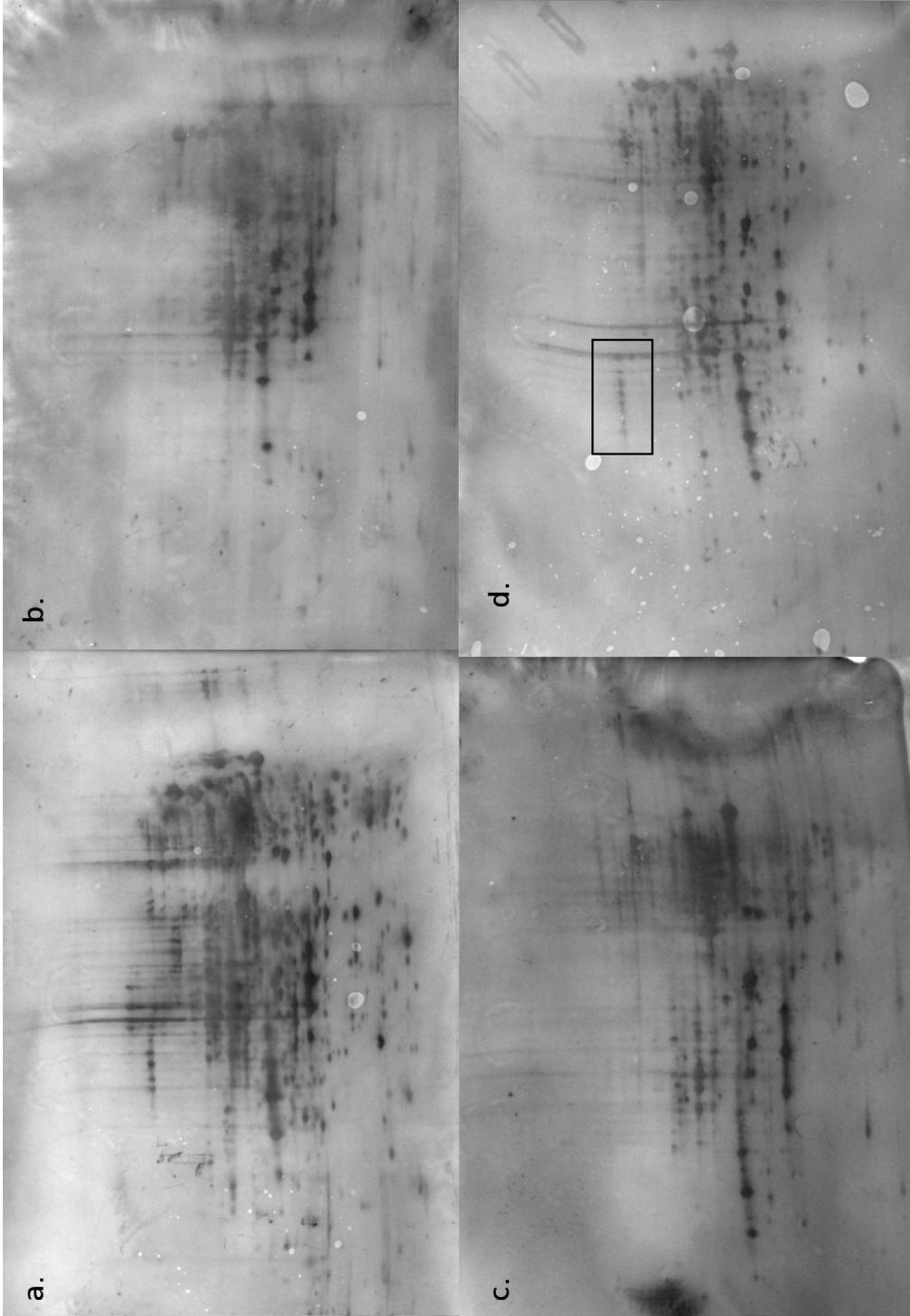
Figure 3.19: Total protein stain of 2D Western blot of: a. cytoplasmic protein from untreated *E. coli* suspensions, b. cytoplasmic protein from *E. coli* suspensions treated with 0.85 % liquid H_2O_2 for 45 seconds, c. cytoplasmic protein from untreated *E. coli* embedded in alginate gels, d. cytoplasmic protein from *E. coli* embedded in alginate gel treated with vapourised H_2O_2 for 60 seconds. Intensity of the stain is proportional to the amount of protein present. The highlighted region shows proteins present in extract treated with vapourised hydrogen peroxide that were not resolved in the control extract.
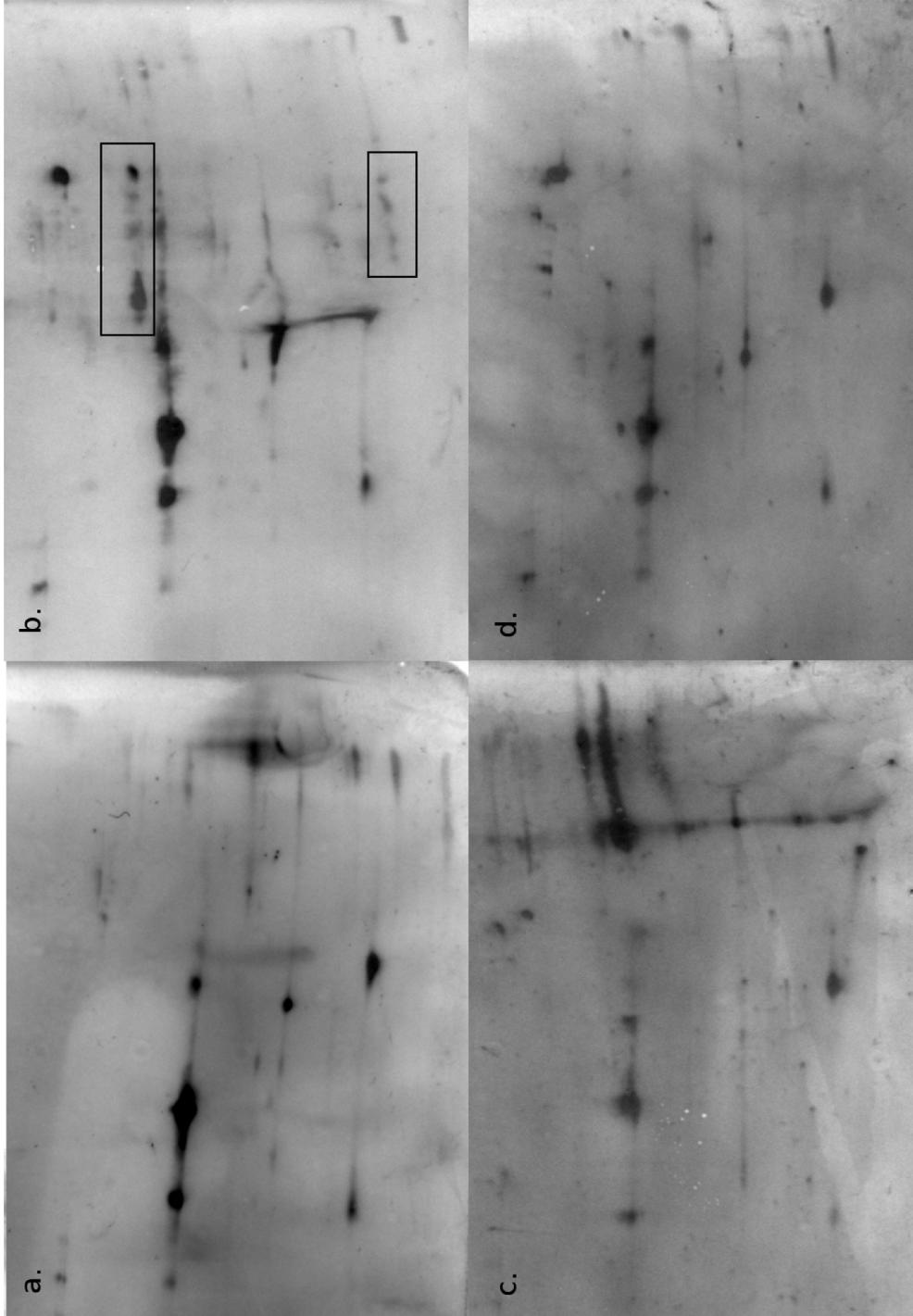
Figure 3.20: Total protein stain of 2D Western blot of: a. outer-membrane protein from untreated *E. coli* suspensions, b. outer-membrane protein from *E. coli* suspensions treated with 0.85 % liquid H_2O_2 for 45 seconds, c. outer-membrane protein from untreated *E. coli* embedded in alginate gels, and d. outer-membrane protein from *E. coli* embedded in alginate gel treated with vapourised H_2O_2 for 60 seconds. Intensity of the stain is proportional to the amount of protein present. The highlighted region shows proteins present in extract treated with liquid hydrogen peroxide that were not resolved in the control extract.
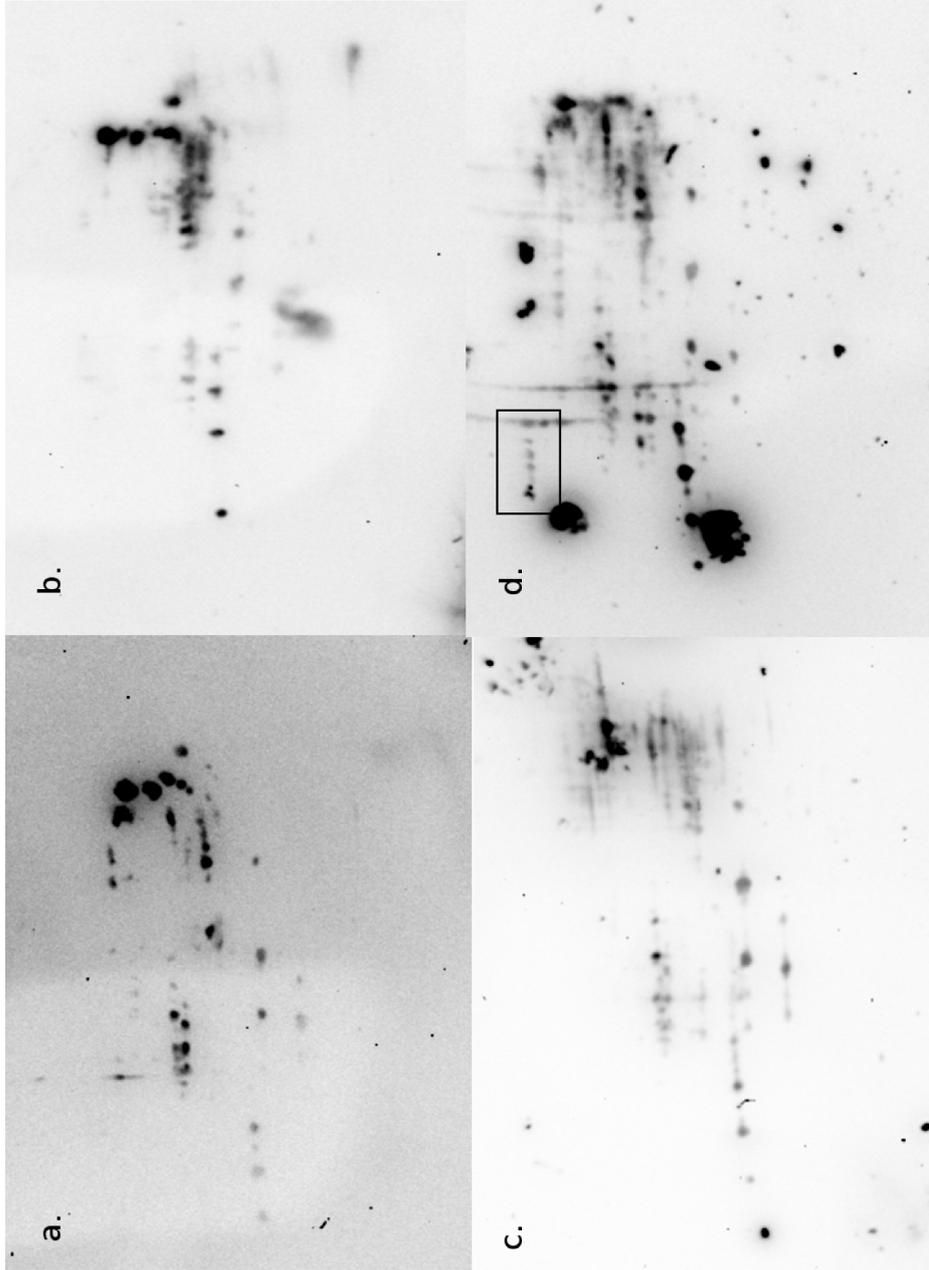
Figure 3.21: Western blot image showing carbonyl content of cytoplasmic protein from a. cytoplasmic protein from untreated *E. coli* suspensions, b. cytoplasmic protein from *E. coli* suspensions treated with 0.85 % liquid H$_2$O$_2$ for 45 seconds, c. cytoplasmic protein from untreated *E. coli* embedded in alginate gels, d. cytoplasmic protein from *E. coli* embedded in alginate gel treated with vapourised H$_2$O$_2$ for 60 seconds. Intensity of the blot image is proportional to the amount of protein carbonyls present for each dot. The highlighted region shows proteins stained in extract treated with vapourised hydrogen peroxide that were not stained in the control extract.
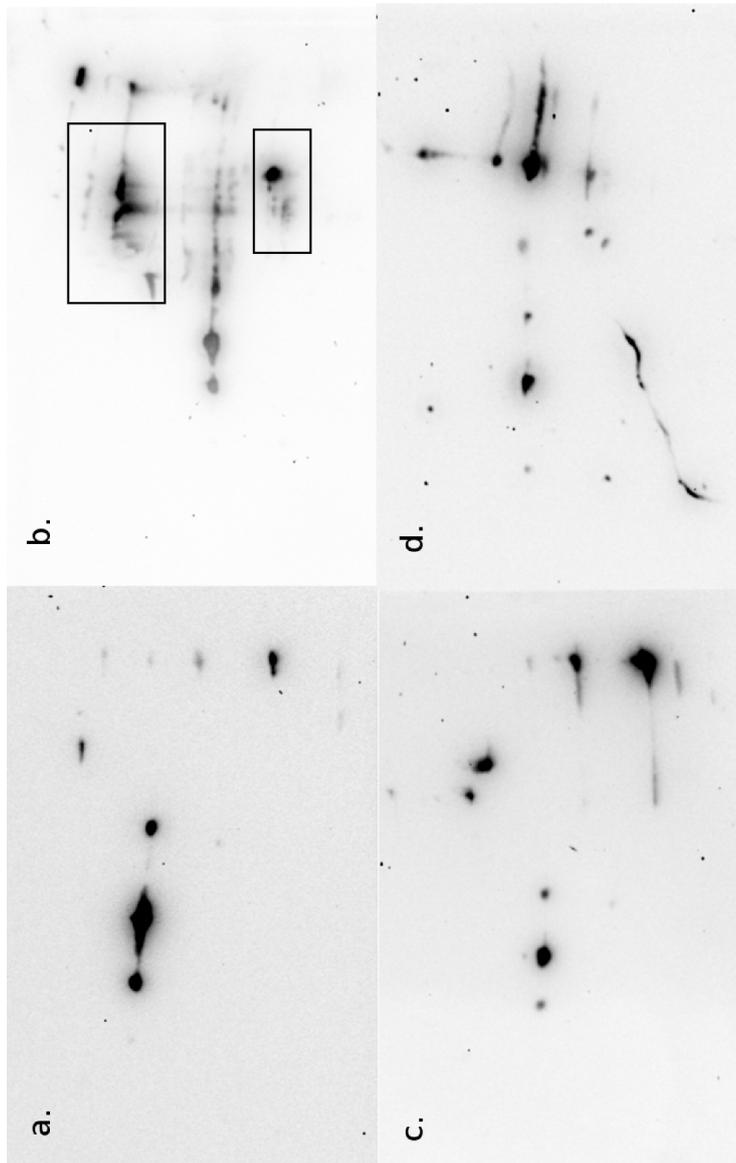
Figure 3.22: Western blot image showing carbonyl content of cytoplasmic protein from: a. outer-membrane protein from untreated *E. coli* suspensions, b. outer-membrane protein from *E. coli* suspensions treated with 0.85 % liquid H_2O_2 for 45 seconds, c. outer-membrane protein from untreated *E. coli* embedded in alginate gels, and d. outer-membrane protein from *E. coli* embedded in alginate gel treated with vapourised H_2O_2 for 60 seconds. Intensity of the blot image is proportional to the amount of protein carbonyls present for each dot. The highlighted region shows proteins stained in extract treated with liquid hydrogen peroxide that were not stained in the control extract.

45 seconds. The values shown are the mean of three experiments repeated on separate occasions, and error bars show the standard error of the mean.
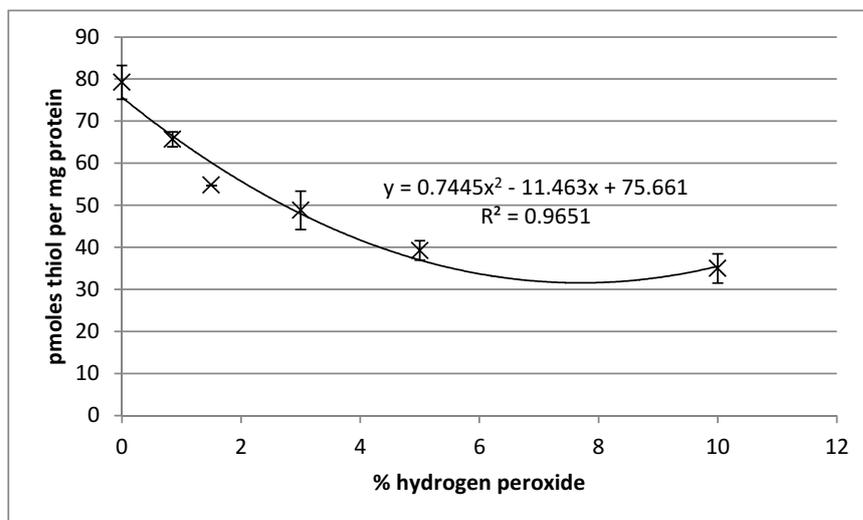
Figure 3.23: Picomoles thiol per milligram of protein as measured using the DTNB assay for *E. coli* suspension samples treated using increasing concentrations of liquid hydrogen peroxide for 45 seconds. Error bars show the standard error of the mean of three repeated experiments.

Figure 3.24 shows similar values for *E. coli* embedded in alginate gels and exposed to vapourised H_2O_2 for 0, 1, 2 and 5 minutes.
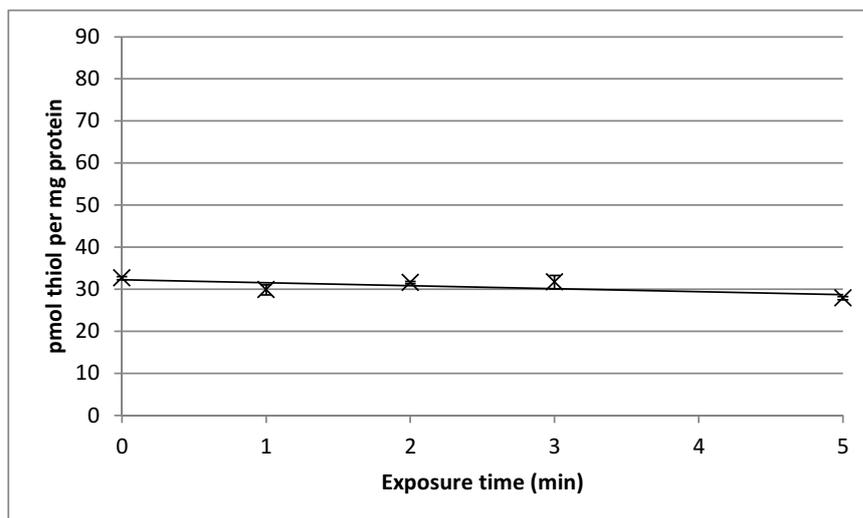
Figure 3.24: Picomoles thiol per milligram of protein as measured using the DTNB assay for *E. coli* embedded in alginate gels treated using increasing exposure times to hydrogen peroxide. Error bars show the standard error of the mean of three repeated experiments.

There was a substantial reduction in protein thiol content in samples

exposed to liquid H_2O_2. No such decrease was seen with samples exposed to vapourised H_2O_2.

Figure 3.25 shows the relationship between colony-forming unit counts (as measured in Chapter 2) and the corresponding protein thiol concentrations for samples treated with 0, 0.85, 1.5, 5 and 10 % hydrogen peroxide. A curve was fitted to these points using non-linear regression in Microsoft Excel.
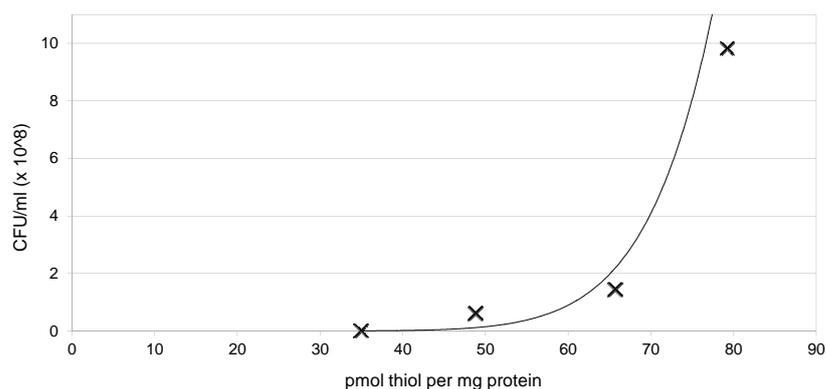
Figure 3.25: Relationship between picomoles thiol per milligram of protein and colony forming units per millilitre for *E. coli* suspension samples treated with increasing concentrations of liquid hydrogen peroxide for 45 seconds.

It can be seen that there is no simple linear relationship between the decrease in protein thiol content, and the decrease in colony-forming units. This suggests that whilst there is a correlation between these values, this decrease in protein thiol is not the sole cause of cell death.

3.4   Discussion.

3.4.1   Colourimetric carbonyl assays.

Of the assays used for protein carbonyl content, the colorimetric assay was the least sensitive but most direct measurement, allowing assays to be performed as quickly as possible following treatment of bacterial cultures. This assay was therefore the least likely to be affected by artefactual oxidation of protein during processing, which would increase the carbonyl content in both test and control samples, and thus reduce the sensitivity of the assay (although protein purification involved an overnight step, this was

performed at -20 řC and so oxidation would occur slowly if at all). However, with the equipment available, it was not possible to produce sufficient purified outer membrane protein to be used in the direct colorimetric assay, and so this method could only be used with total protein.

None of the conditions tested gave a measurable increase in protein carbonyl content, though these conditions resulted in reductions in viable colony forming units of between 1 and 5 $\log_{10}$ CFU/mL. As discussed previously, oxidation of a number of amino acids via Fenton-type hydroxyl radical reactions leads to the production of carbonyl groups, so carbonyl content can be used as an estimate of protein oxidation.

Previous work by Semchysyn *et al.* (2005) found that protein carbonyl levels, as measured using a similar colorimetric DNPH assay, did increase in *E. coli* strain KS400 on exposure to H_2O_2. They found an increase of up to 5 nanomoles of carbonyl per milligram of protein in cultures treated with H_2O_2, however, examination of the methods used provides reason why the results they obtained should not be considered to by contradictory to those obtained in the present study. Their study used low concentrations of H_2O_2 and long exposure times; a significant increase was seen with 100 țM (equivalent to 0.00034%) H_2O_2 exposure for 20 minutes or 20 țM (0.00007 %) H_2O_2 exposure for 1 hour. The study also used actively growing cells incubated at 37 řC. This type of low-level chronic stress from H_2O_2 at a concentration lower than the MIC should be expected to produce substantially different effects than acute exposure to potently biocidal concentrations, particularly at elevated temperatures that would be expected to increase the rate of reactions leading to the production of hydroxyl radicals and the oxidation of amino acids. They also used a simpler process to measure protein carbonyls – DNPH was reacted with crude cell extract with no attempt made to purify proteins before derivitisation, derivitised proteins were precipitated by addition of trichloroacetic acid before measurement. It has been demonstrated by Luo and Wehr (2009) that such a procedure is insufficient to remove contaminating DNA which will react with DNPH to give artefactually raised results. That this is the case in the study performed by Semchysyn *et al.* can easily be seen by the high carbonyl measurements obtained for their control values, they report values of approximately 7 nanomoles of carbonyl per milligram of protein. Luo and Wehr state that protein carbonyl content for a wide variety of organisms has been found usually to be below 5 nanomoles per milligram, a value in closer agreement to those obtained in

the current study. It should also be noted that oxidation of the DNA backbone can lead to the production of aldehydes and ketones within the DNA molecule, as DNPH reacts specifically with the carbonyl group present in aldehydes and ketones, but not specifically protein carbonyls, if contaminating DNA is not removed then it is not possible to determine whether an increase in carbonyl content in a sample is due to protein or DNA oxidation. Finally, Semchysyn *et al.* did not analyse their results appropriately – the results they obtained for the measured carbonyl values were very variable, and no clear trend could be seen either for increasing H_2O_2 concentration or exposure time. Statistical significance of the difference between the control values and those of the highest carbonyl content was determined using Student's t-test with a p-value < 0.05 being considered significant. This is not an appropriate test to use when multiple values are being compared, correction should have been made for multiple comparisons, or an alternative test such as ANOVA used.

3.4.2   ELISA measurement of protein carbonyls.

The ELISA method used was extremely sensitive and so could be used with much lower amounts of protein, allowing separate measurement of outer membrane proteins and total cell proteins. However, OMP samples gave consistently lower signals than would be expected from their protein content. This may mean that such proteins do not bind well to ELISA plates, or cannot be derivitised with DNPH when so bound as these samples gave a lower signal than the fully-reduced BSA standard used, which would not be expected to contain any carbonyl groups for derivitisation. A final possibility is that the results of the BCA assay to measure protein content are substantially different for outer-membrane proteins than for the BSA used a standard protein. This is perhaps the most likely explanation as outer-membrane protein samples also appeared to give substantially less intense stains with the total protein stain on 2D Western blots than the cytoplasmic protein samples though the same amount of protein was loaded as measured using the BCA assay for both sample types. The low signals given by the outer-membrane protein samples meant that the carbonyl content of these samples could not be calculated.

No significant difference was seen in carbonyl content between cytoplasmic protein samples treated with either 0.85 % liquid H_2O_2 for 45 seconds, or with vapourised H_2O_2 for 60 seconds and the corresponding untreated

controls. Calculating the carbonyl concentration by reference to the reduced/oxidised BSA standards gave values of between 3 to 6 nanomoles of carbonyl per milligram of protein, which was broadly in agreement with those obtained by the colorimetric assay. This validates the results of both assays, and provides strong evidence that oxidation of the total protein content within the cytosol of the bacterial cell does not occur on exposure to liquid or vapourised H_2O_2 in conditions that can cause a reduction of between 1 and 5 $\log_{10}$ CFU/mL. This shows that non-specific oxidation of cytosolic protein is not important to the bactericidal mechanism of H_2O_2.

3.4.3 2-dimensional Western blot identification of selectively oxidised proteins.

The 2-dimensional Western blots did not identify any specifically oxidised cytoplasmic proteins in samples treated with liquid H_2O_2, though it is a limitation of this method that reproducibility is poor, so it would require a large difference in carbonyl content to reliably identify an oxidised protein. Replicate runs of these blots would perhaps afford greater sensitivity as the intensity of spots could be averaged across runs; however the process is both expensive and time-consuming so it was not practical to perform repeats. The decision not to perform further repeats was reinforced by the results of the colorimetric and ELISA methods, which suggested that there was no overall difference in carbonyl content between the treated and untreated samples.

The cytoplasmic protein sample treated with vapourised H_2O_2 exhibited a series of spots positive for carbonyl that were not present in the control sample. However, as discussed, this same set of spots was also present in the test sample and absent from the control sample when stained for total protein, as illustrated in figure 3.19d. There are three possible explanations as to these observed results; these spots could be formed by fragments of proteins produced by the break-up of larger proteins by oxidation, they could be proteins joined by disulphide bridges formed by the oxidation of thiol groups, or they could be due to differences in transfer of proteins during IPG strip loading, running of the second dimension gel, or blotting. As these spots are towards the top of the gel, this shows that they are not smaller fragments, and the reduction and alkylation steps during sample preparation would break any disulphide bridge, so this suggests that these spots are due to the differences in protein transfer. As discussed, it was not

possible to perform repeats to confirm this.

The outer-membrane blots for the vapour-treated sample did not show any specifically oxidised proteins, confirming that treatment with H_2O_2 vapour sufficient to cause a 1 $\log_{10}$ drop in CFU/mL does not oxidise outer-membrane proteins.

The outer-membrane protein blot for the sample treated with 0.85 % liquid H_2O_2 gave more intense staining than for the control sample. However, as discussed, the total protein stain for this blot was substantially more intense than for the control, with several spot resolved on this blot that did not appear for the control. This suggests that the differences seen could be attributed to differences in protein transfer between these two blots.

The study mentioned in section 3.1.2.3 by Tamarit *et al.* (1998) detected several specifically oxidised proteins in both the cytoplasm and outer-membrane of cultures of *E. coli* K12 exposed to 2 mM (equivalent to 0.007 %) liquid H_2O_2 at 37 řC for10 minutes. Oxidised proteins were detected using a Western blotting procedure similar to the one used in this study, although they used 1-dimensional SDS-PAGE to separate proteins. The differences in results obtained in this study compared to those of Tamarit *et al.* are interesting, as it is possible that they indicate important differences between the effect of low concentrations of H_2O_2, which produce a stress effect (the conditions used by Tamarit *et al.* produced a 30 % reduction in cell viability after 1 hour in anaerobic cultures and 7 % reduction in cell viability in aerobic cultures) and high concentrations which produce a bactericidal effect (the conditions used for the Western blots in this study gave ~ 1 $\log_{10}$ reduction in colony forming units, equivalent to a 90 % reduction in viability after 45 seconds for liquid treatment). It appears that, whilst stress conditions do lead to the oxidation of protein both in the cytoplasm and the outer-membrane, bactericidal conditions do not. Whilst this discrepancy might be explained by the more than 10 times longer exposure time used by Tamarit *et al.*, the more than 100-fold greater concentration of H_2O_2 used in this study would be expected to more than compensate for this, assuming first-order kinetics for the reaction of H_2O_2 with bacterial proteins. Rather, it appears that the Fenton-type oxidation of proteins does not occur when high concentrations of H_2O_2 are present, which shows that such reactions are not involved in the bactericidal mechanism of H_2O_2 treatments.

3.4.4   Measurement of protein thiols.

Exposing *E. coli* suspensions to 0.85 % liquid H_2O_2 cause a drop of ˜15 picomoles of thiol per milligram of protein, or approximately 19 % of the total thiol content. The thiol content decreased approximately linearly in relation to the concentration of H_2O_2 up to 5 %, demonstrating that the addition of liquid H_2O_2 to *E. coli* suspensions is capable of oxidising protein thiol groups.

Comparison of the reduction in CFU/mL with the decrease in thiol content caused by addition of increasing amounts of H_2O_2 showed that there is no simple linear correlation between these two variables. Exposure to 0.85 % H_2O_2 for 45 seconds caused a decrease in CFU/mL of approximately 90 % for a corresponding decrease of thiol content of ˜19 %, and whilst was an approximately linear relationship was seen between H_2O_2 concentration and thiol content up to 5 % H_2O_2, the relationship between H_2O_2 concentration and CFU/mL was logarithmic.

This suggests two possibilities – the first is that a relatively small decrease in thiol content causes a large decrease in viable cells. This is unlikely because, as discussed in section 3.1.1.2, there is evidence that methionine residues in proteins act as a buffer against oxidative damage. That is to say, readily-oxidised, non-functional methionine residues in protein will react with H_2O_2 before cysteine residues, which are important for function and structure of proteins. This means that, if thiol oxidation is indeed the major cause of the bactericidal action of H_2O_2, the relationship between thiol content and CFU/mL should be the opposite of that seen: a very large initial decrease in thiol content would give rise to a small decrease in CFU/mL as the "sacrificial" methionine residues are oxidised, whilst increasing H_2O_2 concentration would give rise to a relatively smaller further decrease in thiol content as methionine residues are consumed, however this relatively small decrease in thiol would cause a large reduction in cell viability as the remaining, functionally-important cysteine residues are also oxidised. This hypothetical relationship is illustrated in figure 3.24.
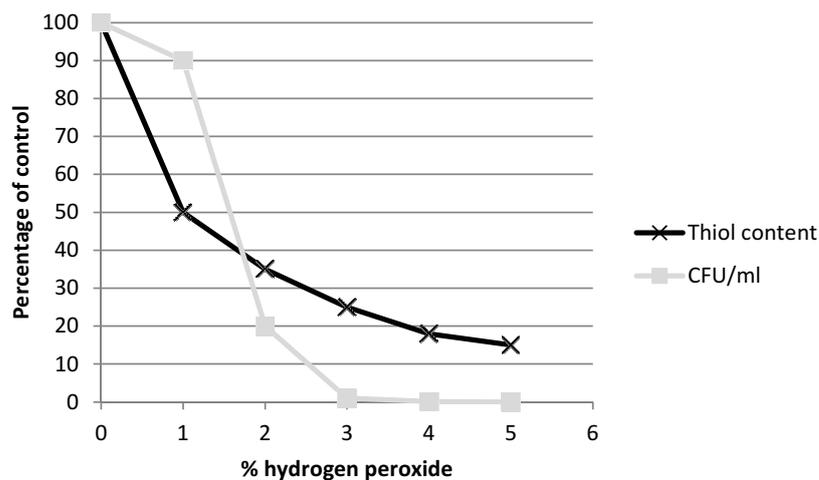
Figure 3.26: Expected relationship between hydrogen peroxide concentration and thiol content/ CFU/mL if thiol oxidation was major mechanism of bactericidal action of hydrogen peroxide.

No decrease in protein thiol content was seen with *E. coli* embedded in alginate gels. This suggests that the vapourised H_2O_2 does not build-up to give a concentration sufficient to measurably affect thiol content, and that the mechanism relies instead on the steady supply of more reactive H_2O_2 molecules to the system which are able to oxidise more important targets.

It is possible that the decrease in protein thiol content seen with liquid H_2O_2 treatment contributes in a secondary way to the bactericidal mechanism as it affects the cell's ability to repair oxidative damage and maintain the reducing environment within the cell. It is unlikely that such a phenomenon represents a major component of the mechanism however, as vapour treatment produced no such change and was yet still equally as effective at killing cells.

3.4.5   Conclusion.

These results suggest that the oxidation of proteins within the cytoplasm and outer-membrane is not important for the bactericidal effects of high (0.85 % and upwards) concentrations of liquid H_2O_2. Commercial disinfection solutions that use such concentrations of H_2O_2, such as contact lens solutions and dental disinfectants, therefore do not involve oxidative protein destruction as a mechanism of action. This is perhaps unsurprising, as discussed liquid H_2O_2 is not a potent oxidiser of protein, and very extensive protein damage would be required to render a cell non-culturable.

Such solutions can cause the specific oxidation of cysteine and methionine residues, though this is not a major component of their activity. However, as it has been shown that such reactions occur through non-radical mediated mechanisms, this shows that H_2O_2 at sufficiently high concentrations can have some deleterious effects without Fenton-type mechanisms being involved.

Vapourised H_2O_2 treatment of vegetative bacteria in moist conditions also does not appear to lead to the oxidative break-down of proteins. This is maybe more surprising as H_2O_2 vapour has been shown to be a more potent oxidiser of proteins, however studies showing this have still required longer exposure times than those used in this study, for example Finnegan *et al.* (2010) exposed BSA to VHP treatment for 10 minutes , compared to the maximum of five minutes used in this study. Such *in vivo* studies used protein dried on a surface and it is possible that conformational changes seen during drying render the protein more readily-accessible to reactive H_2O_2 or radicals. As unambiguous evidence of the potency of VHP as an oxidiser of proteins exists, this suggests that it is also a potent oxidiser of some other target within the vegetative bacterial cell and damage to this target occurs in sufficient quantity to cause cell death before measurable damage to proteins can occur.

Chapter 4

DNA.

4.1 Introduction.

4.1.1 DNA oxidation.

The DNA molecule is somewhat prone to oxidation, and the accumulation of such damage during normal respiration is implicated in both aging and disease. Consequently, the mechanisms and products of DNA oxidation have been extensively studied and a highly complex picture created of possible mechanisms and oxidation products that differ depending on the exact nature of the oxidant causing the damage and the environment in which the reaction occurs. DNA presents essentially two targets for oxidation – the sugar (deoxyribose) moiety, and the four base (adenosine, cytosine, guanine and thymine) moieties. The possible mechanisms and consequences of oxidation of these two types of moiety will be briefly outlined.

4.1.1.1 Oxidation of DNA sugar moiety.

Damage to the sugar moiety of DNA is initiated by abstraction of one of the seven carbon-bound hydrogens, forming a carbon-centred radical, followed by the addition of molecular oxygen at diffusion controlled rates, forming a peroxyl radical. This leads to the loss of the base, producing an abasic site, and in some cases the loss of part of the sugar, leading to the formation of a single-strand break (Dedon, 2008) .
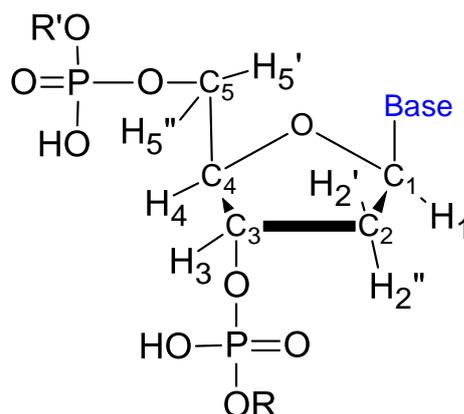
Figure 4.1: Diagram of the DNA sugar moiety enumerating the positions of the carbon and hydrogen atoms.

It has been shown that oxidation of the carbon positions occurs at a differential rate, in the order H5'&H5">H4>H3H2'&H2"H1 and that this order depends on the accessibility of the proton to solvent (Kuznetsova *et al.*, 2009).

H1 is buried in the minor groove of DNA, where it is inaccessible to solvent and can generally only be oxidized by certain minor groove binding molecules. Oxidation at the C1 position is therefore unlikely to occur on exposure to H_2O_2.

The H2' and H2" atoms are also relatively inaccessible, and in addition are unreactive, so oxidation at the C2 position is again unlikely.

The H3 atom is again inaccessible, and C3 oxidation believed to be of minor importance (Zhou, 2005).

The H4 atom is both accessible and has relatively low bond dissociation energy, hence the C4 position is considered to be a major target for oxidation. Oxidation here causes either the formation of a 2-deoxypentos-4-ulose abasic site, or a strand break with a 3'-phosphoglycolate residue, and the formation of a free base-product, either base propenal or malonaldehyde and free nucleobase. Henle and Linn suggest that such damage is a frequent outcome of deoxyribose oxidation (Henle and Linn, 1997) and it has been demonstrated that treatment with 1mM H_2O_2 produces strand-breaks of this nature in mammalian cells (Bertonici and Menegheni, 1995).

The H5' and H5" atoms are believed to be the most accessible, and oxidation at the C5 position has been observed with a number of oxidation agents. Oxidation at this position has been shown to cause strand breaks with either a formylphosphate at the 3' end and a 2-phosphoryl-1,4-dioxo-

2-butane at the 5' end or a 3'-phosphate and a 5'-nucloeside-5'-aldehyde (Dedon, 2008).

4.1.1.2 Consequences of deoxyribose oxidation.

The most obvious consequence of deoxyribose oxidation is the cleavage of the sugar-phosphate backbone of the DNA molecule, and the generation of single-strand breaks. Although these are repairable by the action of exonuclease III, endonuclease IV and DNA polymerase I (Demple and Harrison, 1994), accumulation of such breaks can be fatal.

Abasic sites generated by deoxyribose oxidation have been shown to be mutagenic, despite the existence of repair mechanisms to remove them. For example, Kroeger *et al.* have shown that the formation of 2-deoxypentos-4-ulose abasic site in Escherichia coli results in the production of three-nucleotide deletions (Kroeger *et al.*, 2004).

Some of the end products of deoxyribose oxidation can further react with DNA to form mutagenic adducts. For example, Zhou *et al.* have shown that base propenals formed during 4'-oxidation are the major source of the exocyclic pyrimido[1,2-]purin-10-(3H)-one (M1dG) adduct, which can cause base pair substitutions and frame-shift mutations, and also arrest transcription (Zhou *et al.*, 2005). The 2-phosphoryl-1,4-dioxobutane residue formed during 5' oxidation can also form damaging adducts with cytosine and adenine – Chen *et al.* demonstrated that specific 5' oxidation of deoxyribose led to the formation of oxadiazabicyclo(3.3.0)octaimine adducts of dC and dA, while the trans-1,4-dioxo-2-butene elimination product has been shown to form adducts with dA and dC with a variety of oxidizing agents (Chen *et al.*, 2006) .

4.1.1.3 Oxidation of base residues.

Oxidation of the base residues of DNA leads to base alterations, primarily through OH addition to electron rich double bonds, though hydrogen abstraction from thymine-methyl groups can also occur (Henle and Linn, 1997). A wide variety of such base alterations has been reported and it is beyond the scope of this introduction to consider the nature and mechanisms of formation of these, Cooke *et al.* have given a thorough overview of the products formed under various conditions (Cooke *et al.*, 2003).

Of the four bases, guanine is the most easily oxidized, having the lowest oxidation potential, reported by Kawanisih *et al.* as 1.29 V versus normal

hydrogen electrode (NHE) compared with 1.42 V for adenine, 1.6 V for cytosine, and 1.7 V for thymine (Kawanishi *et al.*, 2001) . The product 7,8-dihydro-8-oxoguanine (8-oxo-Gua) is probably the most widely-studied of the base-oxidation products, largely because enzymes for the excision of this product are ubiquitous across organisms, and because it is highly mutagenic, pairing with adenine and causing GT substitutions during replication (Michaels *et al.*, 1992).

4.1.1.4   Consequences of base oxidation.

Unlike sugar-phosphate backbone oxidation, damage to the base residues does not cause direct damage to the integrity of the DNA molecule, resulting instead in mutagenic effects. Base oxidation products were therefore considered to be of lesser importance to the acute bactericidal effects of exposure to high concentrations of H_2O_2.

4.1.2   Methods to measure DNA oxidation.

There exist two main strategies for the measurement of oxidative DNA damage – the detection of DNA oxidation products, or the measurement of strand breaks produced either directly through the action of the oxidizing agent or indirectly as part of the repair response of the cell.

4.1.2.1   Detection of oxidized DNA products.

The detection of oxidized DNA products typically involves the isolation and hydrolysis of DNA, followed by some system of chromatography and/or mass spectroscopy, for instance, Nunoshiba *et al.* (1999) investigated the effect of iron and superoxide on DNA oxidation in *E. coli*. Bacterial samples were treated, and DNA extracted using a commercially available kit and removing RNA using RNAse. The purified DNA was lyophilised, hydrolysed with formic acid in evacuated tubes, lyophilised again and dissolved in ethanol. DNA bases were collected by passage through a semi-preparative HPLC column, evaporated, lyophilised and trimethylsilylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide/acetonitrile mixture under argon. The concentration of oxidatively modified bases was measured using gas chromatography/mass spectroscopy-selected ion monitoring (GC/MS-SIM).

The use of GC/MS methods has been criticized for introducing artefactual DNA oxidization into samples during isolation (by ambient oxygen and metal ions introduced by contamination of reagents and liberated during cell lysis) and hydrolysis, and for the simultaneous destruction of modified bases (especially 8-oxo-Gua) during hydrolysis. Halliwell (2000) discusses such potential problems associated with MS methods in detail. Methods have been developed and validated with the aim of reducing such problems, based on the use of tandem MS techniques, and the earlier GC/MS techniques have now been rejected; Cadet *et al.* (2003) discuss these improvements in detail, but such methods rely on the availability of specialized equipment and generally involve time-consuming sample preparation which has to be performed with great care in order to minimize introduction of artifacts.

The two major advantages offered by MS based techniques are the very high sensitivity and the wide-range of base modifications that can be detected (Halliwell, ibid.); however, as this study is investigating bacterial cultures great sensitivity is not required as the supply of bacterial cells is not limited. Whilst the ability to detect a range of modifications is possibly useful, it is beyond the scope of this study to identify the individual modifications occurring. Due to the potential difficulties involved in MS-based methods, it was decided not to investigate these further. A simpler method of measurement of oxidized bases relies on high-performance liquid chromatography (HPLC) combined with electrochemical detection of oxidized products. For instance, Mandsberg *et al.* (2009) measured the levels of 8-oxo-Gua in *Pseudomonas aeruginosa* by isolating DNA using a commercial system, removing RNA using RNAse and precipitating DNA using ethanol. Purified DNA samples were hydrolysed with nuclease P1 and digested nucleotides separated using HPLC. Unmodified dG was detected using UV absorbance whilst 8-oxo-Gua was detected electrochemically. Becerra *et al.* (2006) investigated DNA oxidation in *Staphylococcus aureus* as a consequence of exposure to ciproflaxin using a similar method. Whilst such methods require less specialized equipment, they also rely on quite extensive sample preparation and hydrolysis steps with the potential to introduce errors. The range of products that can be detected is also narrower, with the 8-oxo-Gua product the most frequently used.

4.1.2.2 Detection of strand breaks.

As well as the chemical detection of DNA oxidation products, there exists several methods to directly measure the production of both double- and single-strand breaks in DNA. One of the earliest methods described is size separation of extracted DNA on a sucrose density gradient using ultracentrifugation. Use of neutral and alkaline gradients allows on to distinguish double-strand breaks (which cause fragments to separate under neutral conditions) from single-strand breaks (which only cause separation of fragments under denaturing, alkaline conditions). Mitra and Bernstein (1978) describe a method whereby single-stand and double strand breaks were measured in *E. coli* exposed to Cd2+by first incubating the cultures with radioactively labeled thymidine, treating with Cdň2+ and lysing the cells using sodium lauryl sulphate. The lysates were layered onto sucrose gradients, centrifuged, and fractions collected by piercing the bottom of the tube. Measurement of radioactivity allowed the determination of DNA concentrations in each fraction, and the size of the fragments present could thus be calculated.

An alternative method of measuring DNA fragmentation is the TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling) assay, whereby the enzyme terminal deoxynucleotidyl transferase is used to catalyze the addition of labeled dUTP to the ends of DNA strands. The assay was originally described by Gavrieli *et al.* (1992), who used the method to label DNA nicks in histological sections and cell suspensions from various rat tissues with biotinylated dUTP which could then be detected by avidin-peroxidase. The method has been further refined, and is commonly used in with fluorescently labeled dUTP for the flow-cytometric detection of DNA fragmentation brought about by apoptosis. The method has been adapted to for use with prokaryotic cells by Rohwer and Azam (2000) who used the method to measure DNA damage in *E. coli* strain K37 and the archeon *Haloferax volcanii* exposed to 0.2 % (86 mM) H_2O_2. The method is limited however in that it can only detect strand-breaks with 3'-OH ends. These are more often brought about by the action of DNA repair enzymes (as mentioned above, the chemical oxidation of the DNA backbone gives rise to other groups present on the 3'-end of a strand break), so the method will not detect DNA fragmentation with a purely chemical cause that has brought about necrosis. The method is also fairly complex to carry out and requires analysis using a flow cytometer or fluorescence microscope. For

these reason, this method was not considered in this study.

A simpler method for the detection of strand breaks is the alkaline unwinding assay. This method, originally described by Birnboim & Jevcak (1981) relies on the denaturation of DNA into single strands under alkaline conditions, the rate of which is increased by breaks within the strand (such breaks allowing additional sites for initiation of unwinding). Cell lysates can therefore by incubated for some set time in alkaline solution before the addition of neutralizing solution to reduce the pH and terminate unwinding. Addition of a fluorescent dye that preferentially binds double-stranded DNA (such as ethidium bromide) allows the amount of double-stranded DNA present in the sample to be measured. By reference to two controls – a negative control whereby neutralizer is added prior to the alkaline solution such that no unwinding occurs, allowing the total amount of DNA in the sample to be measured; and a positive control whereby DNA is massively fragmented by sonication or shearing, allowing background fluorescence to be measured – the degree to which strand breaks are present can be estimated.

Georgiou and Papapostolou (2006) describe a method of quantifying fragmentation of DNA using the fluorescent dyes Hoechst 33258 and PicoGreen. They found that the fluorescence of these dyes is reduced by approximately 70% when bound to fragmented double-stranded DNA (< 23 kbp) compared to larger DNA molecules. Thus DNA fragmentation can be assessed by purifying DNA from cells under investigation, dividing the sample into two and fragmenting one aliquot by sonication or nuclease treatment, and measuring the difference in fluorescence between the two aliquots.

A very simple method of quantifying DNA fragmentation during apoptosis was described by Chen (1996). Chen lysed human promyelocytic leukemia cells using Triton-X100 and precipitated DNA from the crude lysate using polyethylene glycol 8000. By pulsed-field agarose gel analysis of the supernatant following precipitation, they found that fragments of 300 kilo base pairs (kbp) and below were not precipitated, thus by measuring the concentration of DNA remaining in the supernatant using a fluorescent dye such as Hoechst 33258, the amount of DNA fragmentation produced by some treatment can be measured.

The methods described above have the advantage of producing fully quantitative data, they allow either the direct measurement of fragment production or a measurement that by calibration can be related to the number

of breaks produced. However, all rely on the extraction and (to a greater or lesser degree) purification of DNA from cells. The large chromosomal DNA molecule is very susceptible to breaking once liberated from the cell due to shearing forces during processing, thus artefactual strand breaks can be introduced, the likelihood and frequency of which increases as purification steps are added. Such risks can be avoided by making use of agarose gel electrophoresis methods that have been developed that allow cells to be embedded in agarose before lysis, thus providing a support matrix for the DNA, minimising the effects of shear forces.

One commonly used method for detection of strand breaks electrophoretically is the single cell electrophoresis or Comet assay. This was originally described by Ostling and Johnason (1984) and refined by Singh *et al.* (1988) and involves embedding cells of interest in a miniature low-melting point agarose gel cast on a microscope slide. The cells are then lysed and subjected to electrophoresis, during which fragmented DNA migrates out of the cell forming a 'tail' in the direction of the current. Staining of the gels with fluorescent dye and visualisation under fluorescence or confocal microscopes allows the counting and measurement of these tails, such that the number of cells containing fragmented DNA, and the number of strand breaks occurring in each cell can be estimated. The assay can be run under neutral or alkaline conditions to allow detection of double-strand breaks only, or both double-strand and single strand breaks respectively. The gels can also be preincubated with certain bacterial repair enzymes which cleave at oxidatively damaged sites, allowing the detection and identification of such lesions. Although generally performed using eukaryotic cells, the large size and relatively high DNA content of which aid visualisation, the assay has been performed with bacterial cells; Singh *et al.* (1999) used the assay to measure double-strand breaks formed in *E. coli* strain JM 101 on exposure to x-rays.

An alternative method is the agarose gel smearing assay. As described by Wlodek *et al.* (1991) , this involves embedding cells of interest in a 'plug' of low melting point agarose, lysing the cells and then running the plug in the well of an agarose gel. Fragmented DNA can migrate out of the well and into the gel lane, producing a 'smear' of DNA in the lane when the gel is stained. As for the Comet assay, the gel can be run under neutral or alkaline conditions to detect double-strand breaks only or both single and double-strand breaks. Zirkle and Krieg (1996) used an alkaline gel method

to investigate strand breaks and subsequent repair in *E. coli* cells treated with H_2O_2.

4.2 Materials and methods.

4.2.1 Materials.

Sodium chloride, low melting point agarose, agarose, RNAse A, 30% Sarkosyl solution, lysozyme, ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane (tris), tris(hydroxymethyl)aminomethane hydrochloride (tris-HCl), Triton X-100, sodium acetate, ammonium acetate, sodium dodecyl sulphate (SDS), sodium hydroxide, proteinase K, bromocresol green, sucrose, unsheared *E. coli* DNA, phenol/chloroform/isoamyl alcohol solution were purchased from Sigma (Poole, UK).
Absolute ethanol, 1% ethidium bromide solution, microscope slide, cover slips and fluorescence microplates were purchased from Fisher (Loughborough, UK).
Acridine orange and Hoechst 33258 were purchased from Invitrogen (Paisley, UK).

4.2.2 Method development.

4.2.2.1 Comet assay.

Comet assays were performed according to a modified version of the method of Singh *et al.* (1999). A low melting point agarose solution (0.5 % agarose + 0.89 % NaCl solution in deionised water) was prepared, heated to 90 řC until molten, and used to precoat microscope slides with 100 ţL agarose solution. The coated slides were air-dried in a laminar flow hood and the molten agarose solution kept at 37 řC for further use.

Bulk *E. coli* cultures were produced by inoculating a 250 mL Erlenmeyer flask containing 100 mL TSB with culture from a working slope using a culture loop. This was incubated overnight at 37°C with shaking (100 rpm) and used immediately. Cell suspensions were transferred into 50 mL centrifuge tubes, centrifuged at 4000 x g for 15 min, and the supernatant discarded. The pellets were washed three times with deionised water, and resuspended in 2.5 mL deionised water. The resuspended pellets were pooled, vortex mixed and 500 ţL subaliquots taken in 15 mL centrifuge tubes.

Cells were exposed to hydrogen peroxide by adding 25 ţL of H_2O_2 solution to an aliquot of cell suspension to give the desired final concentrations of 3.5 %. A control was prepared by adding 25 ţL of deionised water alone. The treatment mixtures were incubated for 45 s before the hydrogen peroxide was quenched by addition of 5 volumes of 5% sodium thiosulphate and 0.2 M sodium citrate solution. The mixture was then centrifuged at 4000 x g for 15 min, the supernatant discarded and the pellet washed three times with deionised water. The washed pellets were resuspended in 5 mL of deionised water, and 1 ţL of each cell suspension was mixed with 50 ţL agarose solution, carefully pipette onto separate dried precoated slides and cover slips applied.

The slides were placed onto an ice pack, and left for 10 minutes until the agarose mixture set. The cover slips were removed and 200 ţL of lysis layer consisting of 5 ţg/mL RNAse A, 0.25 % Sarkosyl and 0.5 mg/mL lysozyme in LMP agarose was pipetted over the original gels. The cover slips were replaced and the slides incubated at 4 řC for 10 minutes. The cover slips were then removed and the slides incubated at 37 řC for 1 hour in a humid chamber.

Following incubation, the slides were placed into lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM tris, 1% Sarkosyl, 1% Triton X-100, pH 10) and incubated at room temperature for 1 hour. Following lysis, the slides were placed into enzyme digest solution (2.5 M NaCl, 10 mM EDTA, 1 mg/mL proteinase K, 10 mM tris pH 7.4) and incubated at 37 řC for 2 hours. Following digestion, the slides were electrophoresed in neutral conditions for 1 hour at 12 volts (running buffer 300 mM sodium acetate, 100 mM tris, pH 9), then fixed by immersing in ethanol containing 1 M ammonium acetate for 20 minutes, then absolute ethanol and allowed to air dry overnight. Finally, the gels were rehydrated with deionised water for 10 minutes, and stained with ethidium bromide for 5 minutes, followed by destaining with deionised water. The slides were then photographed using the 100x objective on a confocal microscope with emission and excitation. The image from the control gel is shown in figure 4.2.

Figure 4.2: Initial Comet assay gel image (original image converted to grey scale and inverted).

Initial results showed no clearly resolvable bacteria, so the method was modified in several ways. During the initial run it was noticed that the gels cast on microscope slides tended to float away from the slide and disintegrate during electrophoresis. Removing the cover slip during the casting process was also difficult, often resulting in tearing of the gels. A novel method of casting the Comet gels was developed in order to avoid loss of the gel during running. Microscope slides were coated by dipping into molten 1 % high melting point (HMP) agarose and allowed to dry overnight. Three cover slips were placed on the slides, and a mixture consisting of 200 ţL 1% low melting point (LMP) agarose containing 3 ţL treated cells was pipetted onto each slide. A cover slip and a layer of parafilm was then placed on each gel and the whole slide incubated at 4 ºC for 10 minutes. The cover slip and parafilm was removed and 200 ţL of lysis layer was pipetted over the original gel. The cover slip and parafilm was replaced and the slide once again incubated at 4 řC for 10 minutes. The cover slip and parafilm

was then removed and the slide incubated at 37 řC for 1 hour in a humid chamber. Following incubation, the gels were lysed and digested as before. Following digestion, the slides were electrophoresed , fixed, dried rehydrated and stained as before. Finally, the cover slip with a gel layer was cut off the slide and imaged face-down. The staining method was also modified to use acridine orange instead of ethidium bromide as this appeared to give superior results. Images of the control and test (3.5 % hydrogen peroxide) samples are shown in figures 4.3 and 4.4.
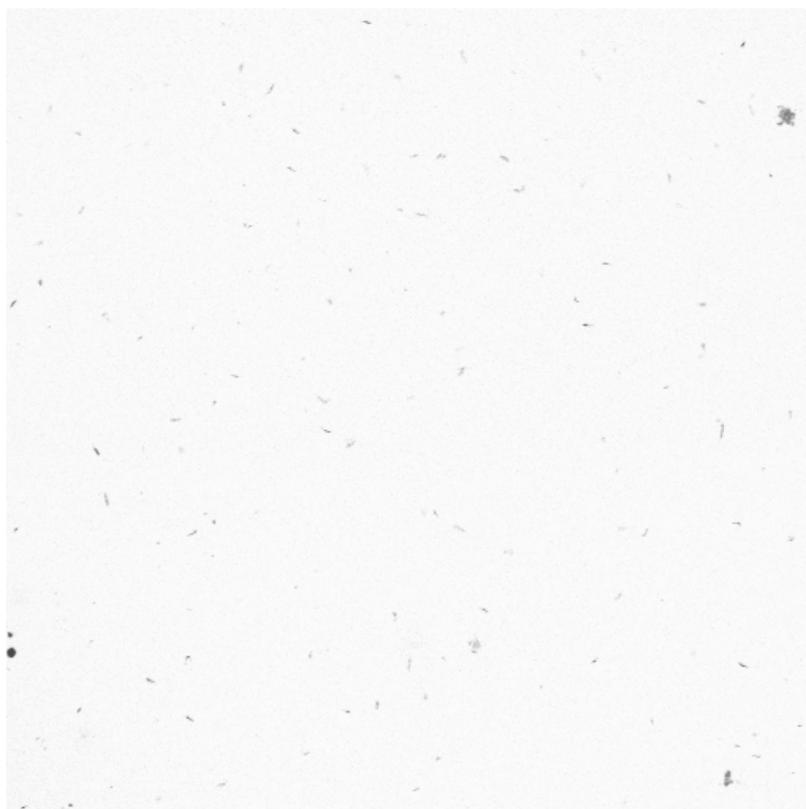
Figure 4.3: Acridine orange stained Comet assay image from control sample (original image converted to grey scale and inverted).

Figure 4.4: Acridine orange stained Comet assay image from test sample (original image converted to grey scale and inverted).

Whilst these modifications appeared to give much improved results, the images of individual bacterial cells were ambiguous and it was not clear whether DNA migration was occurring. Although the test samples appeared to give elongated 'comets' compared with the control, these did not point in the same direction as would be expected if DNA migration had occurred during electrophoresis. Higher magnification images of some of the test sample cells are shown in figure 4.5.
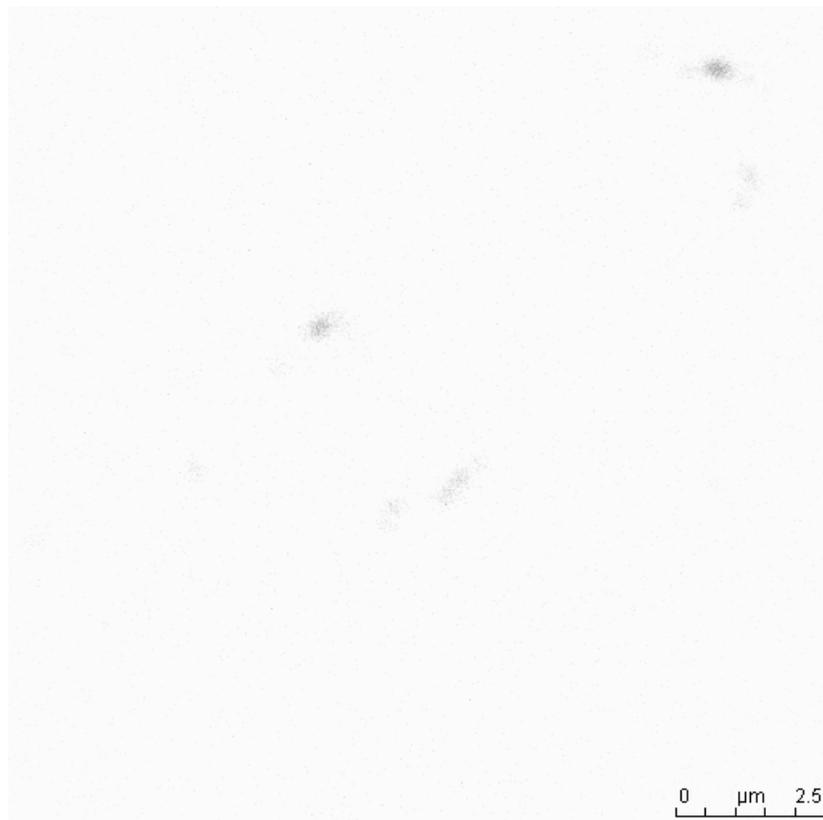
Figure 4.5: High magnification detail of the test sample cells on the Comet assay.

Again, it was impossible to determine whether DNA migration had occurred, or if massively fragmented DNA had formed a 'cloud' around the cell.

In the original paper, *Singh et al.* (1999) used the YOYO-1 dye, which gives much higher sensitivity than acridine orange and a fluorescent microscope with a 1000 x magnification. However, the YOYO dye is extremely expensive, and as there was no similar microscope available it was decided that no further work would be performed using this method.

4.2.2.2 Alkaline gel electrophoresis.

Alkaline gel electrophoresis was performed according to a modification of the method of Zirkle and Krieg (1996). A 1.5 % low melting point (LMP) agarose in TE buffer (50 mM tris-HCl, 5 mM EDTA, pH8) solution was prepared, the agarose was melted by heating to ˜60 řC and the solution kept at 37 řC until use. Treated cell suspensions were prepared as for the Comet assay, 15 ţL resuspended cells were mixed with 1 mL molten LMP

agarose, and 200 ţL of this mixture pipetted onto parafilm to form a gel 'block'. Three blocks were made for each condition.

The gel blocks were incubated at 4 řC for 10 minutes until set, and then transferred into lysis solution (0.1 mM EDTA, 0.5 NaOH, 0.05 % SDS) and incubated at 37 řC overnight.

An alkaline gel solution (0.5 % agarose in deionised water) was prepared and the agarose melted by heating to boiling point. The molten agarose was cooled to 60 řC, 0.1 volumes of 10 x alkaline electrophoresis (AE) buffer (10 mM EDTA, 500 mM NaOH) was added and the solution was mixed thoroughly. A 20 cm gel was cast using a Bio-Rad gel cassette and the gel allowed to set for 1 hour. The lysed gel blocks transferred to the wells of the gel, and the gel was electrophoresed in 1 x alkaline buffer at 30 volts for 6 hours. The gel was then removed from the electrophoresis apparatus and neutralised by immersing in neutralising solution (30 mM NaCl, 50 mM tris-HCl, pH 6) for 1 hour. The gel was stained with 0.5 ţg/mL ethidium bromide in TE buffer for 45 minutes and imaged with a gel camera. The resulting gel image is shown in figure 4.6.
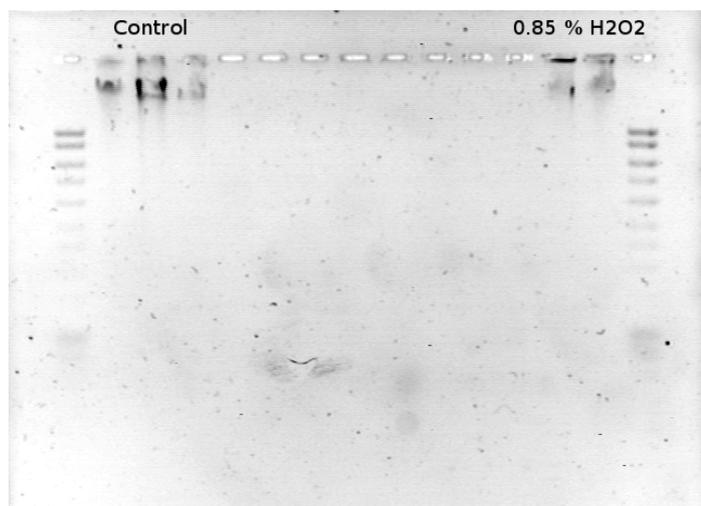
Figure 4.6: Image of alkaline gel electrophoresis gel. Untreated control samples are one the left and samples treated with 0.85 % hydrogen peroxide for 45 seconds are on the right.

It can be seen that only faint bands and 'smears' were produced using these conditions, suggesting that the amount of bacteria in the gel blocks was insufficient to give good quality results. The volume of bacteria added to the LMP agarose to prepare the gel blocks was therefore titrated so that more intense bands could be obtained.

Treated and untreated bacterial suspensions were prepared as before and gel blocks containing 30, 60, 90 or 120 ţL bacterial suspension per 1 mL LMP agarose produced. These were electrophoresed, stained and imaged as before, and the resulting gel is shown in figure 4.7.
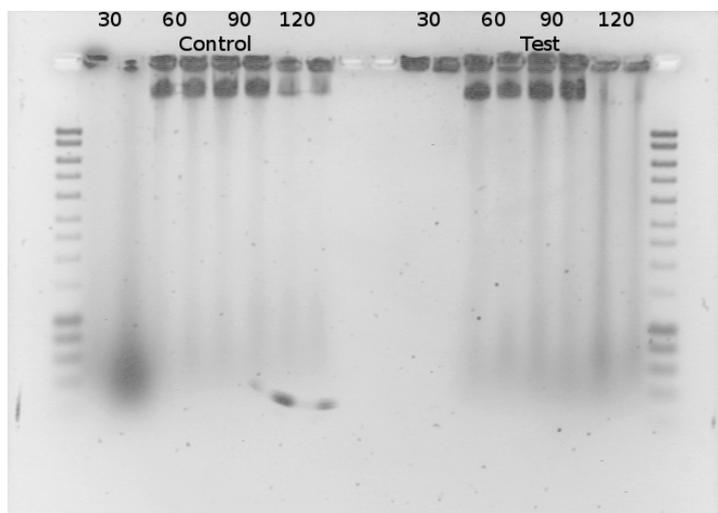
Figure 4.7: Image of alkaline gel electrophoresis gel. Untreated control samples are one the left and samples treated with 0.85 % hydrogen peroxide for 45 seconds are on the right. The amount of bacterial suspension added to the LMP agarose plugs increases from right to left.

It can be seen that 60 ţL bacterial suspension per mL of agarose and higher gave intense bands. It can also be seen that both treated, and untreated conditions gave long, intense smears suggesting that DNA fragmentation was occurring during the preparation process. It was also noticed during trial runs that the alkaline agarose gel was very difficult to handle with a tendency to disintegrate during running, and that the gel blocks containing bacteria were very difficult to insert into the wells of the gel, resulting in the loss of several blocks and poor results for others (this is particularly clear in figure where the higher amount of cells gave weak bands due to problems inserting the gel blocks). Several modifications were therefore made to the procedure in order to optimize results.

All solutions were autoclaved before use to avoid any possible nuclease contamination.

A mold to produce the LMP agarose/bacteria gel blocks was made by pressing the gel comb from the casting equipment into softened paraffin wax. The wax was allowed to harden and the comb removed. The resulting mold produced gel blocks of the exact size of the cast alkaline gel, allowing for

easier insertion, whilst the hydrophobic nature of the paraffin wax prevented sticking during setting.

Bacterial suspensions treated with 0, 0.85, 3, and 10 % H_2O_2 were prepared as before, however cell pellets were resuspended to OD 2.0, 4.5 mL aliquots of these suspensions removed, pelleted by centrifugation at 5000 x g for 10 minutes and then resuspended in 30 ţL TE buffer. The resuspended cells were mixed with 150 ţL molten LMP agarose solution and gel blocks cast by pipetting 60 ţL of this mixture into each position on the mold, three blocks were prepared for each condition. The mold was incubated at 4 řC for 10 minutes to allow the blocks to set. The blocks were then transferred into lysis solution and incubated at room temperature for 2 hours instead of overnight as before.

An alkaline gel was prepared as before, however the concentration of agarose used was increased to 1.5 %, which produced a stable gel. Lysed and washed LMP agarose blocks were inserted into the wells and sealed in place with molten LMP agarose containing bromocresol green. The dye was added to give an indication of running time. In order to reduce heating during running and thus prevent artefactual degradation of DNA, the electrophoresis voltage was reduced to 7 v and running time increased to 14 hours. The gel was stained and imaged as before. The gel image thus produced is shown in figure 4.8.
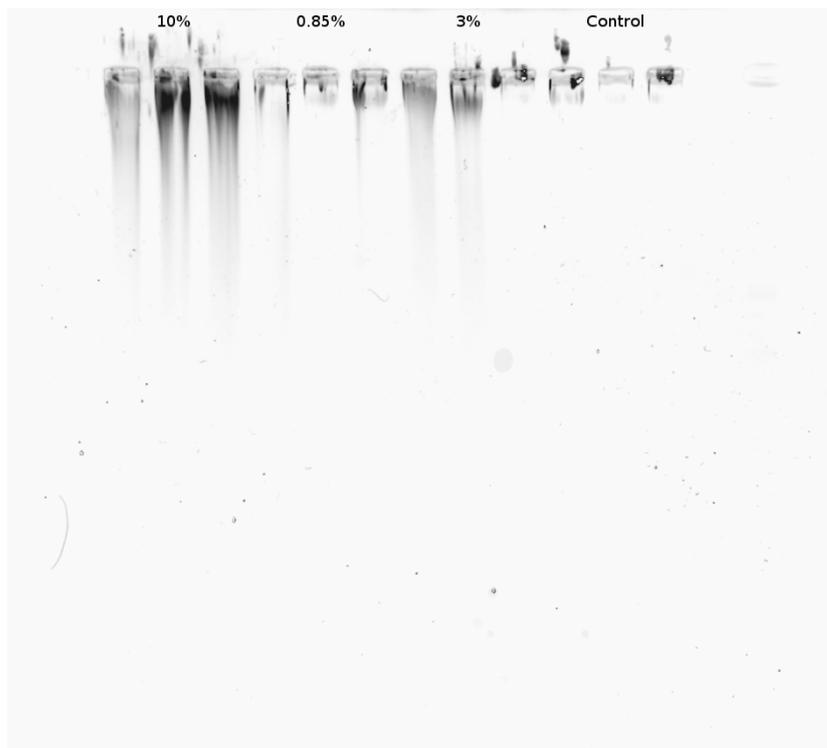
Figure 4.8: Alkaline gel electrophoresis image with improved conditions.

It can be seen that these conditions gave clearly resolvable 'smears' for hydrogen peroxide treated samples and clean bands without broad smears for the control sample. These conditions were therefore used for all further work.

4.2.2.3   DNA fragmentation assay – Hoechst method.

Whilst the alkaline gel electrophoresis (AGE) method used gave a good qualitative indication of DNA fragmentation, quantification was limited to estimating the length of the 'smears' produced. Inherently poor reproducibility of gel electrophoresis methods means that such measurements contain a large error, making statistical analysis difficult. There is also no means of calibrating these measurements – the relationship between the length of the 'smear' and the degree of DNA fragmentation is uncertain, so a direct correlation between DNA fragmentation and killing cannot be established using this method.

A relatively facile method of quantifying DNA fragmentation which would not introduce artefactual fragmentation in treated bacterial samples was therefore sought. As previously mentioned, Georgiou and Papapostolou (2006) have shown that the fluorescent DNA dye Hoechst 33258 gives an

approximately 70 % reduction in fluorescence when bound to fragmented DNA compared to high-molecular weight DNA. It was therefore hypothesized that a gentle DNA extraction method would allow the quantification of fragmented DNA present in treated bacterial samples.

A 0.25 mg/mL unfragmented DNA stock was prepared by resuspending 1 unit of unfragmented *E. coli* DNA in 200 ţL TE buffer. A fragmented DNA stock was prepared from this by addition of 80 ţL unfragmented DNA stock to 10 ţL of DNAse I solution (9 mM MnCl2, 0.9 mg/mL BSA, 2.5 mg/mL DNAse I in TE buffer) followed by incubation for 3 hours at 25 řC. The reaction was stopped by the addition of 10 ţL 50 mM EDTA. Fragmented and unfragmented DNA standards were prepared by serially diluting the stock solutions 1:2 in TE buffer to give concentrations of 50, 25, 12.5, 6.25, 3.125 and 1.5625 ţg/mL. A 1 mg/mL Hoechst stock was prepared in deionized water and stored in the dark at 2-8 řC until use. A working strength Hoechst solution was made by diluting the stock 1:1000 in TNE buffer (0.1 M NaCl, 10 mM EDTA, 10 mM tris-HCl, pH7) and 270 ţL Hoechst working solution placed in each well of a black fluorescence microplate. In sextuplate, 30 ţL of each DNA standard was placed in a well and fluorescence read at excitation 365 nm, emission 465 nm using a Fluorstar plate reader. The two standard curves are shown in figure 4.9.
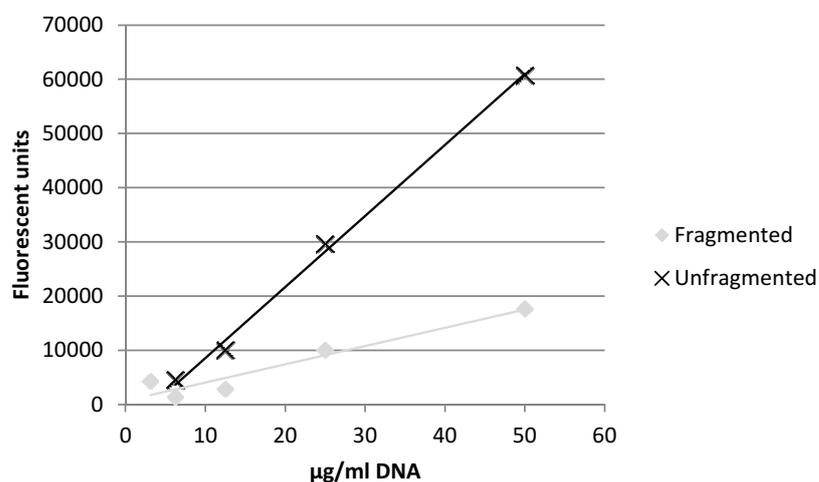
Figure 4.9: Standard curves for intact and fragmented *E. coli* DNA in Hoechst assay.

It can be seen that the fragmented DNA standards gave signals ˜30 % of those for unfragmented samples at the same concentration.

A bacterial suspension treated with 3% liquid hydrogen peroxide and an

untreated control sample were prepared as before. Cells were gently lysed by incubating 100 ţL of bacterial suspension in 700 ţL of sucrose lysis buffer (50 mM tri-HCl, 40 mM EDTA, 0.75 M sucrose, 1 mg/mL lysozyme, pH8) at 37 řC for 1 hour, followed by addition of 200 ţL 5x proteinase K/SDS solution (2.5 mg/mL proteinase K, 5% SDS) and incubation at 55 řC for 2 hours.

DNA was extracted from the lysed cells by addition of 1 mL of phenol/chloroform/isoamyl alcohol solution, the samples were mixed by gently inverting and then centrifuged at 5000 x g at room temperature for 10 mins. The aqueous phase was collected and the extraction repeated by addition of an equal volume of phenol/chloroform/isoamyl alcohol solution, followed by mixing and centrifugation as before. The aqueous phase was again collected, and dissolved phenol removed by addition of an equal volume of chloroform followed by mixing and centrifugation as before. The aqueous phase was again collected.

DNA was precipitated by addition of 0.1 volumes of 3 M sodium acetate and 3 volumes of ice cold ethanol. Samples were incubated at -20 řC overnight and centrifuged at 14000 g for 10 minutes. The supernatant was removed and the pellet washed with 1 mL of ice cold 70 % ethanol. The samples were centrifuged as before, the supernatant removed and the samples air dried at room temperature. DNA was resuspended in 1 mL of TE buffer (10 mM tris-HCl, 1 mM EDTA, pH 7.5).

It was noted during the DNA purification process that collecting the aqueous phases and pelleting the DNA was very difficult due to the extremely viscous lysate that was produced, and it proved impossible to obtain a satisfactory DNA pellet. The process was therefore repeated using a 1 in 10 dilution of treated bacterial suspension – 10 ţL of treated suspension was added to 90 ţL deionized water before addition of 700 ţL lysis buffer as before.

This reduced amount of bacterial suspension gave improved results, however the DNA pellet was still difficult to collect and yield proved to be very variable. An alternative method was therefore tested.

4.2.2.4   PEG – 8000 DNA fragmentation assay.

An overnight culture of *E. coli* was produced and treated with liquid hydrogen peroxide as previously described. Following treatment, the cell suspension was diluted 1 part of suspension in 9 parts deionised water to give

approximately 10^6 cells per mL.

Cells were gently lysed by incubating 70 ţL of bacterial suspension in 630 ţL of sucrose lysis buffer (50 mM tri-HCl, 40 mM EDTA, 0.75 M sucrose, 1 mg/mL lysozyme, pH8) at 37 řC for 1 hour, followed by addition of 200 ţL 5x proteinase K/SDS solution (2.5 mg/mL proteinase K, 5% SDS) and incubation at 55 řC for 2 hours.

Following incubation, an equal volume of 5 % PEG-8000 in 2 M NaCl solution was added to give a final concentration of 2.5 % PEG-8000 and 1 M NaCl. The samples were incubated on ice for 10 minutes, followed by centrifugation at 16,000 x g for 10 minutes at 4 řC.

Following centrifugation, 500 ţL of supernatant was removed, with care being taken not to disturb the DNA pellet. DNA concentration of the supernatant was measured by adding 100 ţL of supernatant to 100 ţL of 0.2 ţg/mL Hoechst 33258 in PBS, pH 7.4 in duplicate, followed by incubation at room temperature for 10 minutes. Fluorescence was read using Fluorstar plate reader, excitation 365 nm, emission 465 nm. Concentration was then calculated by reference to an *E. coli* DNA standard. The measured fluorescence, and hence the amount of DNA retained in the supernatant, increased following treatment with increasing concentrations of H_2O_2. The PEG-8000 assay was facile and rapid to perform, and so was used for the final study.

4.2.3 Methods.

4.2.3.1 Preparation of liquid hydrogen peroxide treated bacterial

suspension.

Bulk *E. coli* cultures were produced by inoculating three 250 mL Erlenmeyer flasks each containing 100 mL TSB with culture from working slopes using a culture loop. These were then incubated overnight at 37°C with shaking (100 rpm) and used immediately. Cell suspensions were transferred into 50 mL centrifuge tubes, centrifuged at 4000 x g for 15 min, and the supernatant discarded. The pellets were washed three times with deionised water, and resuspended in 2.5 mL deionised water.

All resuspended pellets were pooled, vortex mixed and five 2.5 mL sub-aliquots taken in 50 mL centrifuge tubes.

Stock 35 % H_2O_2 solution was diluted in deionised water in 5 mL centrifuge tubes to give 3 mL of 1.7, 3, 6 and 20 % H_2O_2 solutions. Cells were

exposed to H_2O_2 by adding 2.5 mL of H_2O_2 solution to an aliquot of cell suspension to give the desired final concentrations of 0.85, 1.5, 3 and 10 %. A control was prepared by adding 2.5 mL of deionised water alone. The treatment mixtures were incubated for 45 s before the H_2O_2 was quenched by addition of 5 volumes of 5% sodium thiosulphate and 0.2 M sodium citrate solution. The mixture was then centrifuged at 4000 x g for 15 min, the supernatant discarded and the pellet washed three times with deionised water. The washed pellet was resuspended to OD 2 in deionised water for further treatment.

4.2.3.2   Preparation of alginate gel embedded bacterial sample and <br> vapourised hydrogen peroxide treatment.

The VHP 1000 instrument runs were started before gels were prepared in order to produce a constant H_2O_2 concentration in the treatment chamber. Treatments were timed such that gels were exposed immediately on production, following 20 min of decontamination cycle.

Bacterial cells suspended in deionised water were prepared as before. The resuspended cells were mixed with an equal volume of 4% sodium alginate solution and 1 mL of sample transferred to the lid of a miniature Petri dish. The gel was then spread thinly by gently lowering the base of the Petri dish into the upturned lid. The alginate was cured by addition of 2 mL of 2% calcium chloride solution with the Petri dish base still in position, the base was then discarded and excess calcium chloride was removed by rinsing three times with deionised water. A single gel was produced for each exposure time tested, along with a gel to act as an unexposed control.

Dishes containing gels were exposed to the VHP treatment chamber by means of the sample tube for exposure times of 1, 2, 3, 5 and 10 minutes. Dishes were removed from the treatment chamber, the gels were immediately scraped into centrifuge tubes containing 5% sodium thiosulphate and 0.2M sodium citrate solution and the alginate was completely re-dissolved by vortex mixing. The mixture was then centrifuged at 4000 x g for 15 min, the supernatant discarded and the pellet washed three times with deionised water. The washed pellet was resuspended to OD 2 at 500 nm in deionised water for further treatment.

4.2.3.3   Alkaline gel electrophoresis.

A 1.5 % low melting point (LMP) agarose in TE buffer (50 mM tris-HCl, 5 mM EDTA, pH8) solution was prepared, the agarose was melted by heating to ~60 řC and the solution kept at 37 řC until use. Treated cell suspensions were prepared as in sections 4.2.3.1 and 4.2.3.2, 4.5 mL aliquots of these suspensions removed, pelleted by centrifugation at 5000 x g for 10 minutes and then resuspended in 30 ţL TE buffer. The resuspended cells were mixed with 150 ţL molten LMP agarose solution and gel blocks cast by pipetting 60 ţL of this mixture into each position on a paraffin wax mold, two blocks were prepared for each condition.

The gel blocks were incubated at 4 řC for 10 minutes until set, and then transferred into lysis solution (0.1 mM EDTA, 0.5 NaOH, 0.05 % SDS) and incubated at 37 řC overnight.

An alkaline gel solution (1.5 % agarose in deionised water) was prepared and the agarose melted by heating to boiling point. The molten agarose was cooled to 60 řC, 0.1 volumes of 10 x alkaline electrophoresis (AE) buffer (10 mM EDTA, 500 mM NaOH) was added and the solution was mixed thoroughly. A 20 cm gel was cast using a Bio-Rad gel cassette and the gel allowed to set for 1 hour. The lysed gel blocks transferred to the wells of the gel and sealed in place with molten LMP agarose containing bromocresol green. The gel blocks were placed in random order to avoid any bias in results due to the position on the gel.

The gel was electrophoresed in 1 x alkaline buffer at 21 volts for 7 hours using a Bio-rad tank and power supply. The gel was then removed from the electrophoresis apparatus and neutralised by immersing in neutralising solution (30 mM NaCl, 50 mM tris-HCl, pH 6) for 1 hour. The gel was stained with 0.5 ţg/mL ethidium bromide in TE buffer for 45 minutes and imaged with a gel camera. The images thus produced were analysed by measuring the length of the 'smear' for each sample using ImageJ software (National Institutes of Health).

The whole process was repeated on three occasions for liquid-treated samples and three occasions for vapour treated samples.

4.2.3.4   Effects of hydroxyl radical scavenger on DNA fragmentation.

An overnight culture of *E. coli* was produced as in section 4.2.3.1. The cell suspension was transferred into two 50 mL centrifuge tubes, centrifuged at 4000 x g for 15 min, and the supernatant discarded. The pellets were washed

three times with deionised water, and resuspended in 2.5 mL deionised water or 2.5 mL 1 M Tris-HCl, pH 7.4.

Stock 35 % H_2O_2 solution was diluted in deionised water or 1 M Tris-HCl pH 7.4 in microcentrifuge tubes to give 1 mL of 1.7, 3, 6 and 20 % H_2O_2 solutions. Cells were exposed to H_2O_2 by adding 50 ţL of H_2O_2 solution to a 50 ţL aliquot of cell suspension to give the desired final concentrations of 0.85, 1.5, 3 and 10 %. H_2O_2 diluted in water was added to cells suspended in water, and H_2O_2 diluted in Tris-HCl was added to cells suspended in Tris-HCl. Controls were prepared by adding 50 ţL of deionised water or Tris-HCl alone. The treatment mixtures were incubated for 45 s before the H_2O_2 was quenched by addition of 5 volumes of 10 g/l sodium thiosulphate and 0.2 M sodium citrate solution. The mixtures were then centrifuged at 5000 x g for 15 min, the supernatant discarded and the pellets washed three times with deionised water. The washed pellets were resuspended to OD 2 at 500 nm in deionised water for further treatment.

An alkaline gel was run and imaged as in section 4.2.3.3.

4.2.3.5 Effects of inhibition of protein synthesis on DNA fragmentation.

An overnight culture of *E. coli* was produced as in section 4.2.3.1. The cell suspension was transferred into two 50 mL centrifuge tubes, centrifuged at 4000 x g for 15 min, and the supernatant discarded. The pellets were washed three times with deionised water, resuspended in 2.5 mL deionised water, pooled and two 2.5 mL aliquots taken.

One aliquot was treated with the addition of 100 ţg/mL chloramphenicol for 10 minutes at 37 řC, whilst the other was incubated alongside without chloramphenicol. Following incubation, both samples were further divided into two aliquots and one aliquot from each sample were treated with 3 % H_2O_2 as in section 4.2.3.1, and one aliquot from each sample left untreated as control. Alkaline gel electrophoresis performed as previously described, each sample was run in triplicate.

4.2.3.6 PEG – 8000 DNA fragmentation assay.

An overnight culture of *E. coli* was produced and treated with liquid H_2O_2 as in section 4.2.3.1. Following treatment, the cell suspension was diluted 1 part of suspension in 9 parts deionised water to give approximately 106 cells per mL.

Cells were gently lysed by incubating 70 ţL of bacterial suspension in 630 ţL of sucrose lysis buffer (50 mM tri-HCl, 40 mM EDTA, 0.75 M sucrose, 1 mg/mL lysozyme, pH8) at 37 řC for 1 hour, followed by addition of 200 ţL 5x proteinase K/SDS solution (2.5 mg/mL proteinase K, 5% SDS) and incubation at 55 řC for 2 hours.

Following incubation, an equal volume of 5 % PEG-8000 in 2 M NaCl solution was added to give a final concentration of 2.5 % PEG-8000 and 1 M NaCl. The samples were incubated on ice for 10 minutes, followed by centrifugation at 16,000 x g for 10 minutes at 4 řC.

Following centrifugation, 500 ţL of supernatant was removed, with care being taken not to disturb the DNA pellet. DNA concentration of the supernatant was measured by adding 100 ţL of supernatant to 100 ţL of 0.2 ţg/mL Hoechst 33258 in PBS, pH 7.4 in duplicate, followed by incubation at room temperature for 10 minutes. Fluorescence was read using Fluorstar plate reader, excitation 365 nm, emission 465 nm. Concentration was then calculated by reference to an *E. coli* DNA standard.

The assay was repeated three times.

4.3   Results.

4.3.1   Alkaline gel electrophoresis.

An example of an alkaline gel image produced for *E. coli* suspensions treated with liquid H_2O_2 is shown in figure 4.10.
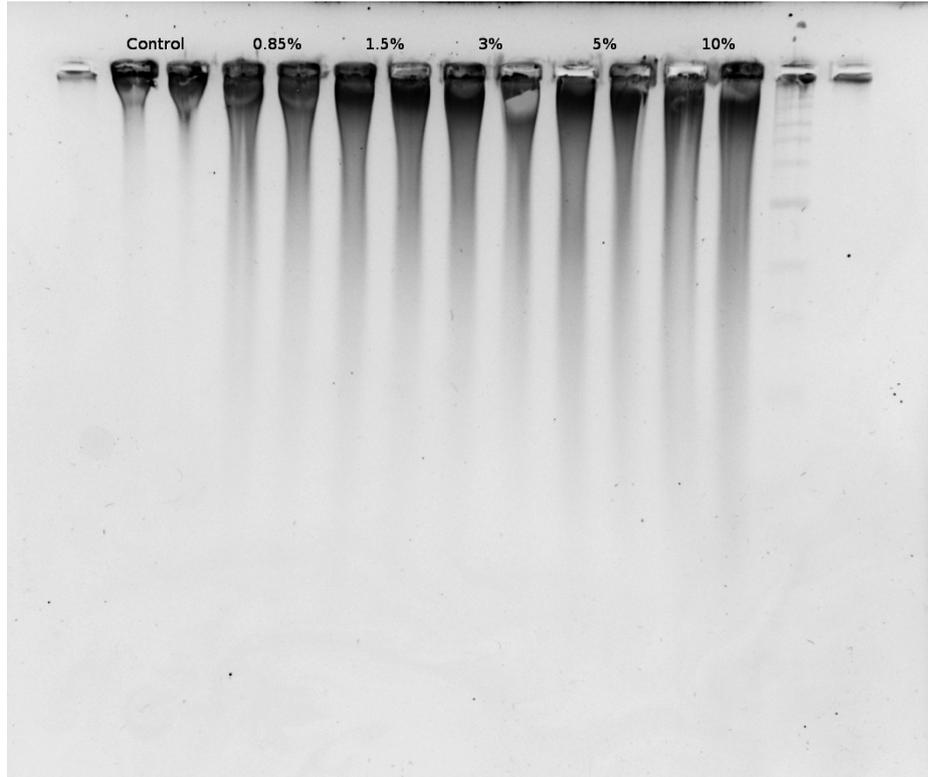
Figure 4.10: Example of alkaline gel run with liquid hydrogen peroxide treated samples

Figure 4.11 shows a chart of the average measured smear lengths for the three repeated experiments.
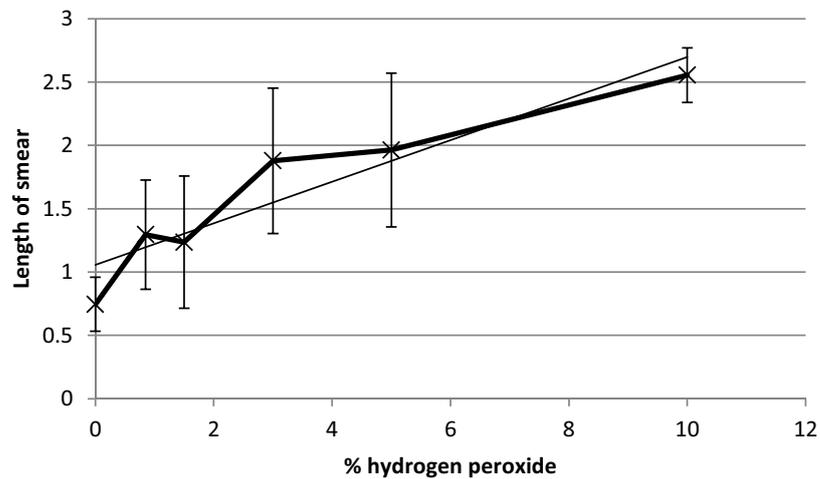
Figure 4.11: Mean measured smear lengths for liquid hydrogen peroxide treated samples. Error bars show the standard error of the mean of three repeated experiments.

Exposure to liquid H_2O_2 at any of the concentrations tested produced a

longer "smear" than that seen for the untreated, control samples. This shows that exposure to liquid H_2O_2 caused the formation of strand breaks. On average, the length of the smear increased with H_2O_2 concentration, showing that higher H_2O_2 concentration caused a greater incidence of strand break formation.

Figure 4.12 shows an example image produced from an alkaline electrophoresis gel run with *E. coli* embedded in alginate gels and treated with vapourised H_2O_2,

Figure 4.12: Example of alkaline gel run with vapourised hydrogen peroxide treated samples.

Figure 4.13 shows a chart of the average measured smear lengths for the three repeated experiments.
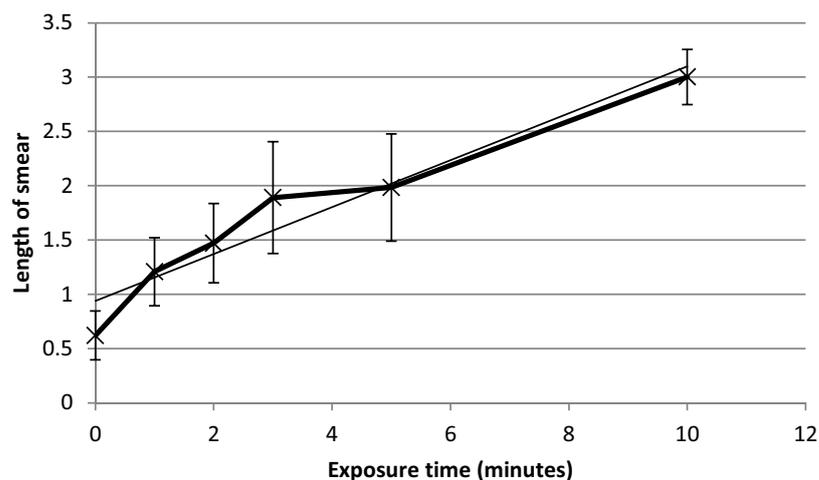
Figure 4.13: Mean measured smear lengths for liquid hydrogen peroxide treated samples. Error bars show the standard error of the mean of three repeated experiments.

Similarly as for liquid treatment, exposure to vapourised H_2O_2 for any of the times tested produced a longer "smear" than that seen for the untreated, control samples. This shows that exposure to vapourised H_2O_2 caused the formation of strand breaks. Again on average, the length of the smear increased with exposure time, showing that longer exposure times caused a greater incidence of strand break formation.

4.3.2 Effect of hydroxyl radical scavenger on strand break formation.

Figure 4.14 shows the alkaline electrophoresis gel image produced for *E. coli* suspensions treated with 3 % liquid H_2O_2 in the presence or absence of tris as a hydroxyl scavenger.
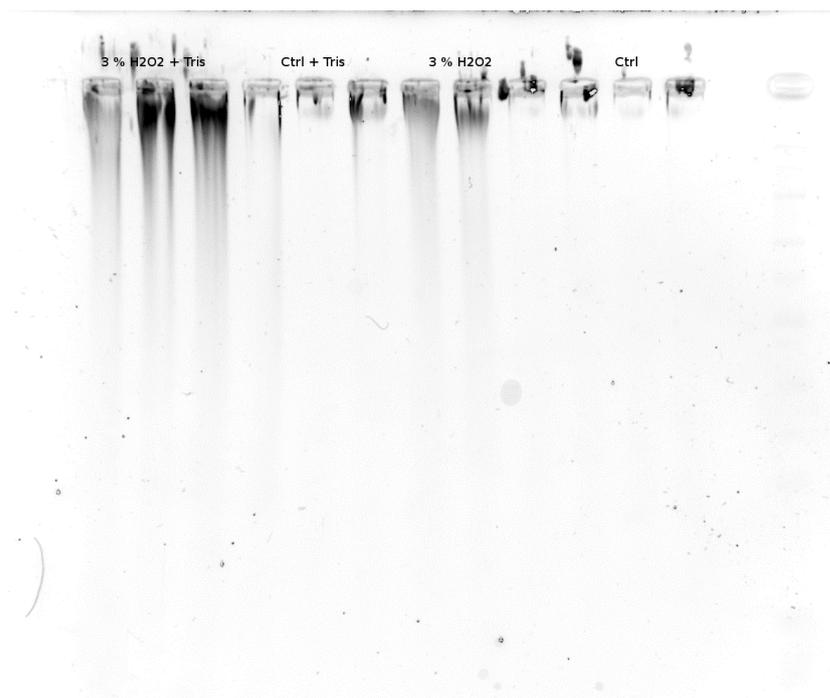
Figure 4.14: Alkaline gel image of *E. coli* suspensions treated with 3 % hydrogen peroxide or untreated (Ctrl) in 1 M Tris-HCl (+ Tris) or deionised water.

Samples treated with 3 % H_2O_2 in 1 M Tris-HCl gave clear smears at least as long as those produced by the samples treated in deionised water alone. Neither of the control samples gave resolvable smears, showing that the Tris-HCl alone did not produce strand breaks. This shows that the presence of Tris-HCl did not prevent formation of strand breaks during H_2O_2 treatment.

4.3.3   Effect of protein synthesis inhibition on strand break formation.

Figure 4.15 shows the alkaline electrophoresis gel image produced for *E. coli* suspensions treated with 3 % liquid H_2O_2 with and without prior treatment with chloramphenicol.
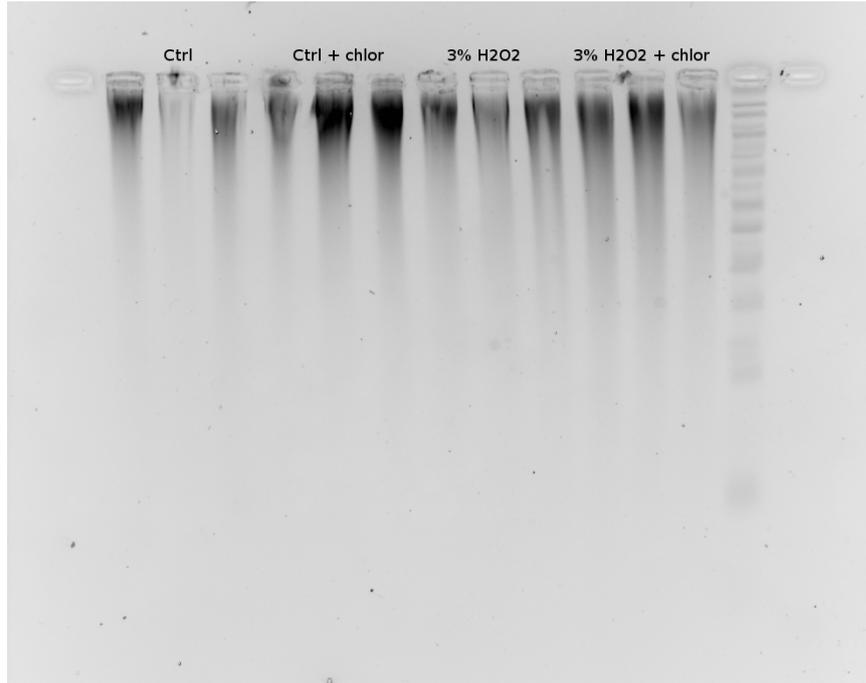
Figure 4.15: Alkaline gel image of *E. coli* suspensions treated with 3 % hydrogen peroxide or untreated (Ctrl) following exposure to chloramphenicol (+ chlor) or otherwise.

Figure 4.16 shows a chart of the average measured smear lengths for the three replicate samples.
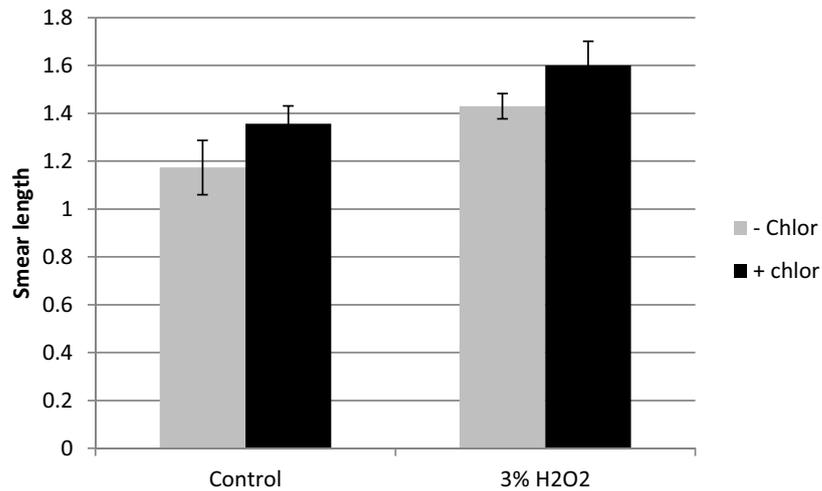
Figure 4.16: Mean measured smear length of *E. coli* suspensions treated with 3 % hydrogen peroxide or untreated (Control) following exposure to chloramphenicol (+ chlor) or otherwise (- chlor). Error bars show standard error of the mean of three replicate samples.

Samples exposed to 3 % H_2O_2 following chloramphenicol treatment gave

smears at least as long as those produced by the samples without chloramphenicol treatment. Both control samples gave smears longer than those normally seen, suggesting that incubation in deionised water at 37 řC for 10 minutes produced some cell necrosis or direct damage leading to the formation of DNA strand breaks. Inhibition of protein synthesis by treatment with chloramphenicol did not appear to reduce the formation of DNA strand breaks following H_2O_2 treatment.

4.3.4   PEG – 8000 DNA fragmentation assay.

Figure 4.17 shows a chart of the measured fluorescence of the supernatants for cell lysates from suspensions treated with increasing concentrations of liquid H_2O_2 following PEG-8000 precipitation.
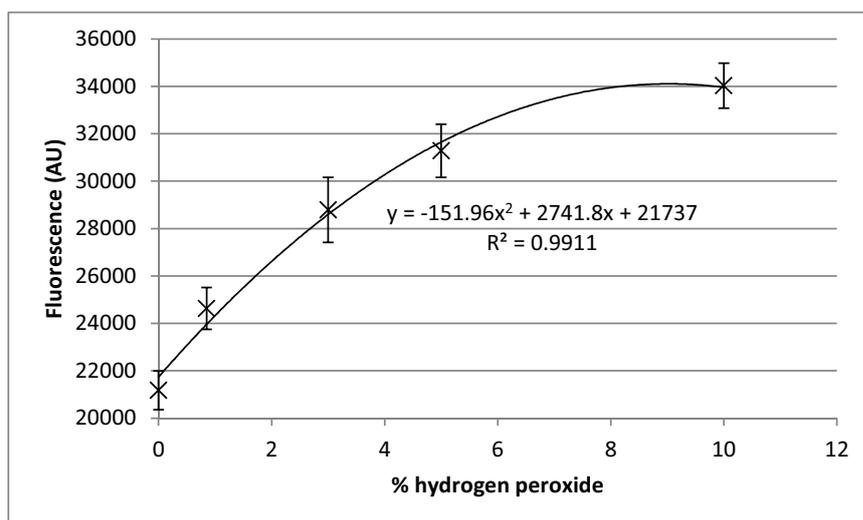
Figure 4.17: Fluorescence of PEG-8000 precipitated supernatants labeled with Hoechst dye. Error bars show the standard error of the mean of three repeated experiments.

The fluorescence of the supernatants on reaction with Hoechst dye is proportional to the amount of DNA retained in those supernatants, and thus provides a measure of the amount of DNA fragments present in a sample. It can be seen that treatment with increasing concentrations of liquid H_2O_2 gave a corresponding increase in the fluorescence of the supernatants, and therefore an increase in DNA fragmentation.

4.4 Discussion.

4.4.1 DNA damage by hydrogen peroxide treatment.

The results obtained clearly show an increase in DNA strand break formation on treatment with increasing concentrations of liquid H_2O_2 or with vapourised H_2O_2 for increasing exposure times.

Measuring the length of the 'smear' produced on the gel image provides a crudely quantitative estimate of the amount of strand breaks formed. The values given here show a roughly linear relationship between the concentration of liquid H_2O_2 used, and the length of the smear produced. A similar relationship was seen between exposure time to VHP and length of smear.

Assuming a linear relationship between smear length and number of strand breaks, this would suggest that there is not a linear correlation between number of strand breaks (which have a linear relationship with H_2O_2 concentration) and reduction in CFU/ml ($\log_{10}$ reduction in CFU/ml is linearly correlated with H_2O_2 concentration). However, there is no proof that the smear length is linearly related to number of strand breaks, so no conclusion can be drawn as to the mathematical nature of the relationship between number of strand breaks and reduction in CFU/ml.

This study shows for the first time that DNA damage is correlated with bactericidal activity for higher concentrations of liquid H_2O_2, and that an increase in H_2O_2 concentration causes an increase in strand break formation. It also shows for the first time that short exposures to vapourised H_2O_2 causes a similar increase in strand break formation.

4.4.2 Evidence for direct damage to DNA.

Treatment with chloramphenicol prior to exposure to liquid H_2O_2 did not cause an decrease in measured number of strand breaks in DNA. Chloramphenicol inhibits synthesis of proteins, and so prevent the DNA repair response which is generally brought about by the *de novo* synthesis of repair enzymes in response to damage (Rohwer and Azam, 2000). These repair enzymes remove oxidised DNA bases and so their action results in the formation of single strand breaks in response to base oxidation. This study found no effect on the number of strand breaks when protein synthesis was inhibited, suggesting that the majority of breaks are formed by the direct action of H_2O_2, not the action of repair enzymes. This is in contrast to the

study by Rohwer and Azam (2000) who state that chloramphenicol treatment prevents formation of strand breaks in *E. coli* during treatment with 0.2 % H_2O_2 for 30 minutes. However, their conclusion is greatly flawed as they measured strand breaks using TUNEL, a technique that will only label 3-OH' ends produced by the repair process, not the other breaks formed by direct chemical action.

4.4.3 <u>The mechanism of DNA damage by hydrogen peroxide.</u>

4.4.3.1 <u>Indirect measurements of DNA damage.</u>

Imlay and Linn (1986) exposed *E. coli* K12 to varying concentrations of H_2O_2 for 15 min at 37řC in K medium. They observed that cells were more sensitive to low (<3 mM) concentrations of H_2O_2 than to intermediate (5-20 mM) concentrations. At greater than 20 mM H_2O_2 survival was inversely proportional to concentration. This response is shown in Figure 4.18.
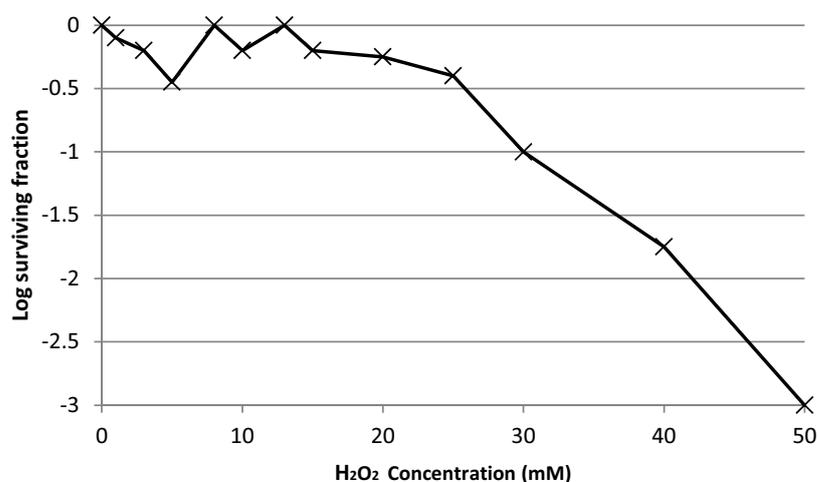
Figure 4.18: Log_{10} surviving fraction of wild-type *E. coli* K12 culture after 15 min exposure to various hydrogen peroxide concentrations. . Adapted from Imlay & Linn (1986).

A slight dip in the surviving fraction of culture can be seen at concentrations <3 mM. This effect was found to be reproducible, and was greatly magnified in DNA-repair deficient and anoxically-grown strains; these were particularly sensitive to H_2O_2 at low concentrations but not especially sensitive to higher concentrations when compared to aerobically grown wild-type

cells. Cells starved by incubation in M90 salts for 80 min before H_2O_2 were not killed by low concentrations of H_2O_2.

As part of their study, comparisons of the kinetics of killing of an exonuclease-II deficient strain at various H_2O_2 concentrations were made. Total kill by both lower and higher H_2O_2 concentrations was found to be time dependent (Figure 2). They postulated that killing of *E. coli* cells by H_2O_2 occurs according to two distinct modes: mode-1 killing occurring at low concentrations due to DNA damage and mode-2 killing occurring at higher concentrations due to damage to other target(s).

Similar results were obtained by Brandi *et al.* (1987) who also produced the bimodal killing pattern seen by Imlay & Linn (1986) after challenging *E. coli* in M9 salts with various H_2O_2 concentrations for 15 min. As part of this study, the effect of anoxia during H_2O_2 exposure was also studied. It was found that mode-2 killing was markedly reduced in anoxic conditions, whereas no effect was seen on mode-1 killing.

Brandi *et al.* (1987) also compared the effect of the hydroxyl scavenger thiourea on killing by 2.5 mM or 25 mM H_2O_2. 35 mM thiourea was found to markedly reduce killing by 25 mM H_2O_2 (hypothesised to be due to the mode-2 mechanism) whilst the same concentration had no effect on killing by 2.5 mM H_2O_2 (hypothesised to be due to the mode-1mechanism). An obvious criticism of this work is that thiourea, a potent reducing agent, is capable of reacting directly with H_2O_2. This reaction is first-order with respect to H_2O_2 concentration, so it is possible that the thiourea simply reduces the higher concentration of H_2O_2 without scavenging hydroxyl radicals. Brandi *et al.* (1987) concluded that mode-2 killing was dependent on the presence of oxygen and hydroxyl radicals, and suggested that this mechanism is indeed due to the Fenton chemistry previously outlined, whilst the mode-1 killing was not dependent on oxygen and hydroxyl radicals.

A study by Macomber *et al.* (2007) using copper-export deficient strains of *E. coli* grown in a copper-supplemented medium showed that these strains accumulated copper within the cell, but that this increase actually inhibited both killing and mutagenesis in a DNA repair deficient strain by millimolar concentrations of H_2O_2. Though they could find no definitive explanation for the inhibitory effect of the copper, their work shows that mode-1 and mode-2 killing due to DNA damage is mediated only by iron.

4.4.3.2 *In vitro* investigations of DNA damage.

Investigation of the mode-1 killing mechanism by Imlay *et al.* (1988) lead
to the creation of an *in vitro* model capable of producing the same pattern
of damage to purified DNA as that observed in the process of killing of
bacteria. This model consisted of phage PM2 DNA incubated with ferrous
sulphate and various concentrations of ethanol and H_2O_2. This system pro-
duced single strand breaks in the purified DNA. The production of breaks
was reduced by approximately half by the addition of micromolar concen-
trations of ethanol, but was not further decreased by addition of up to 10
mM ethanol. Addition of a range of H_2O_2 concentrations to the system con-
taining 10 mM ethanol produced a DNA nick dose response similar to that
seen in mode-1 killing of *E. coli*; the highest amount of nicks was produced
by 50 ţM H_2O_2, and this was reduced by half and remained approximately
constant on addition of 1 to 10 mM H_2O_2. As ethanol is a potent scavenger
of hydroxyl radicals, it was concluded that the mode-1 killing of bacterial
cells due to DNA damage by H_2O_2 is not dependent on production of free
hydroxyl radicals, and is more likely to be due to production of ferryl radical
intermediates from DNA-complexation.

Further work on Imlay and Linn's *in vitro* model by Luo *et al.* (1994)
found that DNA nicking was maximal at 50 ţM H_2O_2, dropping to one third
maximum at 3 mM, and remaining roughly constant between 3 and 50 mM.
Addition of 17 ţM ethanol to the model reduced nicking by 30 to 50 % at all
H_2O_2 concentrations. Increasing ethanol concentration to 10 mM reduced
nicking by a further 50 % at H_2O_2 concentrations below 100 ţM, but had no
effect at higher H_2O_2 concentrations. Increasing the ethanol concentration
to 100 mM caused further inhibition at H_2O_2 concentrations less than 3
mM but again had no effect at higher concentrations. These findings are
summarised in Table 4.1.

	<100 ţM H$_2$O$_2$	0.1 - 3 mM H$_2$O$_2$	3 -50 mM H$_2$O$_2$
No ethanol	Maximal nicking	1/3 x maximal	1/3 x maximal
17 ţM ethanol	Reduced nicking	Reduced nicking	Reduced nicking
10 mM ethanol	Further reduced nicking	Further reduced nicking	No further reduction in nicking
100 mM ethanol	Further reduced nicking	No further reduction in nicking	No further reduction in nicking

Table 4.1: Effects of increasing hydrogen peroxide and ethanol concentration on DNA nicking *in vitro*.

Luo *et al.* (1994) therefore concluded that there are at least three chemically distinct classes of oxidant species produced by Fenton-type reactions in the presence of iron; type I are sensitive to H$_2$O$_2$ but moderately resistant to ethanol, type II are resistant to both H$_2$O$_2$ and ethanol, and type III are sensitive to H$_2$O$_2$ and ethanol.

4.4.3.3   Evidence for different types of DNA oxidation reactions.

Luo *et al.* (1994) went on to investigate the effects of the iron chelators 1,10-phenanthroline (1,10-phen) and 2,2'-dipyridyl (2,2'-dipy) both on E. coli killing and on DNA nicking in the *in vitro* model. Both chelators blocked mode-1 killing, whilst 2,2'-dipy had no effect on mode-2 killing, and 1,10-phen substantially enhanced it. Both chelators also blocked DNA nicking at low H$_2$O$_2$ concentrations but not at higher concentrations; the peak of DNA nicking activity at 50 ţM H$_2$O$_2$ was completely eliminated by the addition of either chelating agent plus 100 mM ethanol, nicking was constant at around 50 % of the maximum between 50 ţM and 50 mM H$_2$O$_2$ with 2,2'-dipy and 100 mM ethanol, and was enhanced 6-fold by the addition of 1,10-phen between 50 ţM and 50 mM H$_2$O$_2$. As 2,2'-dipy chelates unbound Fe^{2+} and remains in solution, whilst 1,10-phen chelates unbound Fe^{2+} and then intercalates into the DNA backbone, they proposed the following model of oxidant damage. Type I oxidants are formed by Fe^{2+} ions associated but not bound to DNA - suggesting that these could exist in a "cationic cloud surrounding the polyanionic DNA helix" - such oxidants are accessible to H$_2$O$_2$ quenching, but would require higher concentrations of ethanol

to effectively quench them due to high localized concentrations within the "cationic cloud". These oxidants are responsible for mode-1 killing. Type II oxidants are formed by Fe2+more tightly associated with DNA molecule, once formed they are not accessible to H_2O_2 or ethanol quenching, and are responsible for mode-2 killing. Type III oxidants are produced by free Fe^{2+} ions in solution, they are easily available to H_2O_2 and ethanol to quench, and due to their short half-life are unlikely to be involved in killing due to DNA damage _in vivo_. The action of the chelating agents can be explained thus: 2,2'-dipy removes the "cationic cloud" and causes the formation of type III rather than type I oxidants, inhibiting mode-1 killing; 1,10-phen removes the "cationic cloud" and possibly free Fe^{2+} ions and intercalates them into the DNA backbone and causes the formation of type II rather than type I or type II oxidants, enhancing mode-2 killing and inhibiting mode-1 killing. These findings are summarised in Table 4.2.

	Type I	Type II	Type III
Resistant to H_2O_2?	No	Yes	No
Resistant to ethanol?	Moderately	Yes	No
Effect of 1, 10 phen	Decreased	Increased	Possibly decreased
Effect of 2,2'-dipy	Decreased	None	Increased
Position	Cationic cloud	DNA backbone	Free in solution
Killing mode	Mode 1	Mode 2	None

Table 4.2: Properties of three types of DNA oxidant formed by H_2O_2 and Fe^{2+}.

Studies by Henle and Linn (1997) suggest that the type I and II oxidants have different preferred cleavage sequences on the DNA molecule, with type I preferentially cleaving the sequences RTGR, TATTY, and CTTR and type II preferentially cleaving the sequence NGGG (where the highlighted base shows the cleavage site). They suggested that this selectivity could be due to sites of iron localisation, or that such sequences act as sinks for radical electrons formed elsewhere on the DNA chain.

4.4.4   Conclusion.

Treatment with H_2O_2, either in vapour or liquid phase, leads to the formation of DNA strand breaks through direct chemical action of the H_2O_2.

The formation of these lesions is not prevented by the inhibition of protein synthesis, suggesting that these breaks are not caused by the action of DNA repair enzymes, and so are likely to be caused by oxidation of the DNA backbone. Nor does addition of a hydroxyl radical scavenger prevent the formation of these breaks.

The extensive work performed investigating the genotoxic action of H_2O_2 shows that formation of strand breaks by oxidative attack of the DNA backbone is not caused by the production of free hydroxyl radicals reacting with free ferrous ions, but rather by the formation of ferryl radicals on reaction of H_2O_2 with iron closely associated with the DNA molecule. This study provides strong evidence that this ferryl radical mechanism is the one by which DNA damage by bactericidal concentrations of liquid H_2O_2 proceeds, and that this mechanism is a major contributor to the bactericidal action of H_2O_2.

Chapter 5

Lipids & Membranes.

5.1 Introduction.

5.1.1 Oxidation of lipids.

The oxidative degradation of membrane lipids proceeds by a free radical-initiated chain-reaction. A free radical abstracts a hydrogen atom from a fatty acid chain, most commonly an allylic hydrogen residing between two carbon-carbon double bonds on a polyunsaturated fatty acid, forming a lipid radical. This lipid radical then undergoes molecular rearrangement forming a resonance structure by delocalisation of the free electron, stabilising the structure. In aerobic cells, the stabilised peroxide is then most likely to react with molecular oxygen to form a peroxyl radical. This radical can then abstract a hydrogen atom from a neighbouring lipid molecule, with the original lipid forming a lipid hydroperoxide whilst the neighbouring lipid forms a lipid radical similarly to the initiating reaction, propagating the chain reaction through the membrane. A single initiating event is therefore able to lead to the conversion of many lipid molecules within a membrane into lipid peroxides, until some chain-terminating reaction can occur. These chain-terminating reactions include reaction of two peroxyl radicals, reaction of the peroxyl radical with a membrane protein, or the reaction of the peroxyl radical with a chain-breaking antioxidant such as tocopherol (Gutteridge and Halliwell, 1990). The series of reactions producing lipid peroxides from membrane lipids are summarized in figure 5.1.
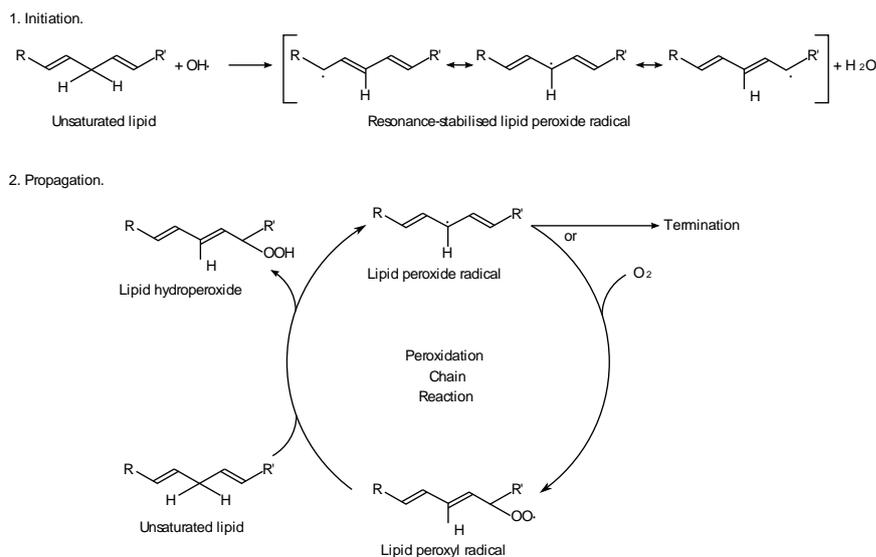
Figure 5.1: Summary of lipid peroxidation chain reaction.

The lipid hydroperoxides produced by the peroxidation chain reaction breakdown in the presence of reduced metal ions by a wide variety of mechanisms, leading to the formation of a number of classes of end-products, including saturated aldehydes such as ethanal and hexanal, alkenals such as acrolein and 2-butenal, dicarbonyls such as malondialdehyde, and 4-hydroxy-2-alkenals such as 4-hydroxy-2-nonenal (Negre-Salvayre *et al.* 2008, Burcham, 1998).

5.1.2 Lipid peroxidation *in vivo.*

Lipid peroxidation has been shown to occur in many different organisms, though due to the importance of this process to aging and disease, the majority of studies investigate peroxidation in eukaryote tissues. For instance, Williams *et al.*(2006) found elevated levels of lipid peroxidation endproducts in vulnerable brain regions of human subjects with Mild Cognitive Impairment and early stage Alzheimer's disease; Kumar (2001) estimated lipid peroxidation by measurement of the lipid peroxidation end-product malondialdehyde in human subjects between the ages of 20 and 65 and found levels increased with age; reperfusion injury has been shown to elevate levels of lipid peroxidation in various organs in rats (e.g. Yun *et al.* 2009; Hosseinzadah *et al.* 2007); and the levels of the lipid peroxidation end-product 4-hydroxynonenal were found to increase in a murine model of traumatic brain injury (Hall *et al.* 2004).

Peroxidation of lipids is also an important process in food spoilage, and

many studies have been performed measuring rates of peroxidation to investigate methods of preservation. Once again, these studies investigate eukaryotic tissues and products, for instance the effects of various preservation methods on lipid peroxidation in salmon (Tuckey *et al.* 2009); chicken (Narciso-Gaytán *et al.* 2010) and pork (Cheng *et al.* 2011) have been investigated.

Studies have been performed that investigate lipid peroxidation in prokaryotes, for instance Maness *et al.* (1999) investigated lipid peroxidation as a mechanism of the bactericidal activity of the photocatalytic titanium dioxide reaction. However, it is likely that membrane damage by lipid peroxidation occurs less readily in bacteria, as (apart from certain classes of marine psychrophilic bacteria) they do not produce polyunsaturated fatty acids (Russell and Nichols, 1999) . Saturated and monounsaturated fatty acids are more resistant to peroxidation as they do not contain allylic hydrogens flanked by two carbon-carbon double bonds. Considering the specific case of *E. coli*, investigations of the total lipid content of *E. coli* K12 report that the relative amounts of saturated and monounsaturated lipids present change as a function of growth temperature. Morein *et al.* (1996) found that, at 37 řC, the membrane lipid content is 44.8 % monounsaturated and 55.2 % saturated.

5.1.3   Consequences of lipid peroxidation.

Apart from the expected loss of membrane integrity associated with extensive oxidative damage to the lipids therein, the products of lipid peroxidation can have damaging effects on other cellular components. For instance, the lipid peroxides and hydroperoxides are themselves oxidising reagents and are able to react directly with protein thiols (Little and O'Brien, 1968), leading to protein aggregation (Richter, 1987). Incubation of plasmid DNA with oxidised PUFAs has been shown to result in extensive DNA single- and double-stranded nick formation, base modification and DNA adduct formation, though it is not certain that this is through the direct reactivity of the lipid peroxide (Burcham, 1998).

More reactive still are the advanced end-products of lipid peroxidation; the 4-hydroxy-2-alkenal 4-hydroxynonenal (4-HNE) can react with histidine (His), cysteine (Cys) or lysine (Lys) residues of proteins; the dicarbonyl malondialdehyde (MDA) can react with Lys residues, and lead to protein cross-linking; the unsaturated aldehyde acrolein reacts with Cys, His and

Lys residues; and the -oxoaldehydes methylglyoxal and glyoxal react with Lys and arginine residues (Negre-Salvayre *et al.* 2008). These advanced end-products can also react with DNA, MDA has been shown to form adducts with guanine, cytosine and adenine, and to have mutagenic, carcinogenic and genotoxic effects , whilst similar effects are seen with 4-HNE and enal-derived end-products (Burcham, 1998).

5.1.4   Methods for the measurement of lipid peroxidation and membrane damage.

There exists two strategies for the consideration of membrane damage – the first is the physical measurement of damage to the membrane itself, and the second is the assay of chemical damage to the lipid and protein components of the membrane. These two main strategies lead to a multitude of methods by which membrane damage can be assessed, and use of several different methods will allow a greater degree of confidence in the conclusions of this study.

5.1.5   Assessment of physical damage to the membrane.

This strategy can again be divided into two main classes – the direct visualisation through some microscopic method of the membrane *in situ*, or the assay of egress or ingress of certain agents as a measure of membrane integrity.

5.1.5.1   Visualisation of cell membrane damage.

Direct visualisation of the membrane can give clear evidence of membrane damage, but it is by its nature a qualitative measure – a correlation between number of cells showing signs of damage and reduction in viable cell count can be obtained, but overall extent of damage cannot be easily quantified and must be to some degree subjectively interpreted.

Typically, studies investigating membrane damage use some form of electron microscopy to visualise the cell surface and/or membranes. A recent study by Zhou *et al.* (2010) investigating the biocidal mechanism of polyhex-amethylene guanidine hydrochloride measured the extent of membrane damage in *E. coli* strain 8099 following treatment using field emission scanning electron microscopy (SEM) and transmission electron microscopy (TEM). They found a dose-dependent formation of local pores in the cytoplasmic

and outer-membranes. Similarly, DeQuieroz & Day (2007) investigated the effect of treatment with a combination of sodium hypochlorite and H_2O_2 on the cell membrane of *Pseudomonas aeruginosa* in biofilms using a combination of SEM and TEM. They found evidence of increasing distortion of cells and formation of perforations in the cell wall and cytoplasmic membrane as exposure time to the mixture was increased.

An alternative to electron microscopy is atomic force microscopy (AFM). This has an advantage over EM in that cells do not require any fixing, so investigations can be performed on live cells *in situ*. Meincken *et al.* (2005) used AFM to investigate membrane damage caused to *E. coli* strain HB101 by the antimicrobial peptides magainin 2, PGLa and melittin. They found evidence of morphological changes following treatment with any of the three peptides.

5.1.5.2   Measure of egress of cell components.

The first indication of a change to membrane permeability is potassium leakage (Lambert & Hammond, 1973) which can be measured using a variety of methods. For instance, Marshall & Reiter (1980) measured potassium leakage from *Strepococcus lactis* and *E. coli* caused by the hypothiocyanite ion by growing cultures in the presence of $^{42}K^+$ and then measuring loss of radioactivity in filtered cultures following treatment with hypothiocyanite using a gamma spectrometer. Newer methods involve treating cell suspensions with some biocidal agent, centrifuging to remove cells and then measuring the potassium ion concentration in the supernatant. Using variations on this method, Cox *et al.* (1998) measured potassium leakage caused in exponential and stationary phase *E. coli* strain AG100 cultures following treatment with tea-tree oil using a selective potassium ion electrode; whilst Suller & Russell (2000) measured potassium leakage in *Staphylococcus aureus* following Triclosan (2,4,4-trichloro-2-hydroxydiphenyl ether) treatment using an atomic absorption spectrophotometer.

More extensive membrane leakage can be assessed by measuring the concentrations of cell components and metabolites released. These can often by measured quite simply using spectrophotometric or biochemical methods. For example, Aronsson *et al.* (2005) investigated membrane permeabilisation by pulsed electric field processing by measuring ATP release from *E. coli, Listeria innocua* and *Saccharomyces cerevisiae* following treatment using the luciferase assay in a luminometer. In addition to measuring potas-

sium leakage, Marshall & Reiter (1980) also measured the concentration of
amino acids liberated from *E. coli* and *S. lactis* after hypothiocyanite treat-
ment by growing cultures in the presence of [14C] amino acids and then
measuring loss of a radioactivity in filtered cultures similarly to the potas-
sium study. Amino acid leakage into suspension fluid can also be measured
by more direct biochemical methods, such as the ninhydrin assay (Allwood
& Russell, 1968), or estimated by measuring the change in UV absorbance
at 260 and/or 280 nm using a spectrophotometer. Whilst this latter method
will only detect aromatic amino acids, it provides a useful measure of leak-
age of metabolites from the cell as nucleotides, nucleic acids and proteins
all absorb in this range, and it is commonly used in investigating potential
membrane damage by various biocidal agents. For example, Zeng *et al.*
(2010) used this method whilst investigating the disinfection mechanism of
electrolyzed oxidising water using *E. coli* and *S. aureus* as model organisms,
Kim *et al.* (2007) used it in investigations of the inactivation of *E. coli* by
super critical carbon dioxide, and Ukuku *et al.*(2009) used it in investigating
the behaviour of high hydrostatic pressure injured *E. coli.*

Finally, as an indicator of very extensive membrane damage, the re-
lease of protein into the suspension fluid can be measured. This is typically
done using standard biochemical assays for protein concentration such as
the Lowry, Bradford or BCA assay. For example, Klotz *et al.* (2010) mea-
sured protein leakage from three strains of *E. col*i exposed to high hydro-
static pressure treatment using the Bradford assay. Alternatively, leaking
proteins can be analysed using SDS-PAGE. Whilst this method is at best
semi-quantitative, it does allow the size of proteins so released to be mea-
sured, offering an indication of the extent of membrane damage (the more
damaged the membrane, the more large proteins that are released). Liou *et
al.* (2011) used this method when investigating damage to *E. coli* caused
by visible light responsive photocatalysts.

Another approach to the measurement of protein leakage is to assay for
specific enzyme activity, and methods exist to assay for activity of several
endogenous enzymes. For example, Chung and Chen (2008) investigated
the effect of acid-soluble chitosan treatment on membrane permeability of
E. coli and *S. aureus* by treating suspensions with chitosan and then as-
saying for alkaline phosphatase activity by measuring the evolution of the
coloured p-nitrophenol product from p-nitrophenylphosphate substrate; and
for glucose-6-phosphate dehydrogenase activity by measuring the evolution

of reduced triphosphopyridine nucleotide (TPN), both using a spectrophotometer. This approach is potentially useful in that, be selecting enzymes known to be localised in various regions of the cell, the location of the damage can be inferred. For example, Sahalan and Dixon (2008) investigated membrane damage by polymyxin B by treating *E. coli* K12 suspensions with polymyxin B, centrifuging the reaction mixture to remove cells and assaying the supernatant for -lactamase (a periplasmic enzyme) activity using the chromogenic cephalosporin assay and -galactosidase (a cytoplasmic enzyme) activity using the evolution of *O*-nitrophenol from *O*-nitrophenol--D-galactopyranoside.

Methods based on measuring the release of cell components require the retention of the reaction mixture in which cell suspensions are treated. In this study, this will contain high concentrations of H_2O_2. As H_2O_2 absorbs in the 260-280 nm region, this means that a simple spectrophotometric measure of 260/280 nm absorbants will not be possible. It is also possible that the H_2O_2 present will react with and destroy these cellular components as they are released, causing an underestimate of the extent of leakage. Finally, it is possible that the presence of high concentrations of H_2O_2remaining in the reaction mixture could cause interference with any biochemical assays by oxidising the substrates or products. In particular, the Lowry and BCA protein assays are not compatible with H_2O_2. The effect of H_2O_2 on enzymes, substrates and products had therefore to be assessed before any such assay could be used.

5.1.5.3 Measure of ingress of indicators into cells.

As an alternative (or compliment) to the measurement of leakage of components out of the cell, it is also possible to measure the ingress of substances not normally membrane-permeant in to the cell as an indicator of changes to membrane integrity. Typically, these take the form of fluorescent, coloured or luminescent lipophobic substances the uptake of which can be measured using colorimetric, fluorometric, luminescence or microscopic techniques.

The most commonly used of these is the dye propidium iodide. This is a DNA intercalating dye which, when bound to DNA exhibits a 20 to 30 fold increase in fluorescence, but which cannot pass through the membrane of healthy cells. When cells with compromised membranes are exposed to the dye, it thus passes into cell and stains the DNA therein whilst healthy cells remain unstained (Jones & Senft, 1985) . Detection of the fluorescence

in a population of cells treated with propidium iodide therefore allows one to estimate the degree of membrane damage within that population.

Propidium iodide staining as a measure of membrane damage has been used by Pagán & Mackey (2000) in a study of the relationship between membrane damage and cell death in pressure-treated *E. coli*. Treated cells were stained with propidium iodide and fluorescence measured using a spectrofluorometer. Similar assays can be performed using other dyes including SYTOX green (Roth *et al.* 1997), and ethidium bromide and ethidium homodimer (Calvello *et al.* 2011) . These are again impermeant to healthy membranes, and fluoresce on binding to DNA. The choice of dye used for this assay is generally determined by the excitation and emission wavelengths of the dyes, and the intensity of fluorescence, with high intensity dyes useful in situations were high sensitivity is required, though comparison of results with differently sized dyes (in particular, ethidium bromide and ethidium homodimer) can allow some measure of the degree of membrane damage.

Studying the influx of fluorescent dyes into treated bacterial suspensions affords a major advantage over the measure of metabolite efflux – cells can be treated, removed from the treatment, and then assayed for damage. This allows removal of the biocide used to treat the cells before assay, so that it will not interfere. It also allows the effects of dry treatments to be investigated more easily, whereas measuring efflux from cells requires them to be in suspension during treatment. This method, using the commercially available LIVE/DEAD stain was therefore selected for testing.

5.1.6   Assay of chemical damage to membrane components.

Damage to the membrane can occur due to oxidation of membrane proteins and to the lipids making up the membrane. Measurement of oxidation of membrane proteins is treated in chapter 3 previously.

5.1.6.1   Assay of lipid peroxidation products.

As discussed previously, lipid peroxidation leads to the production of a wide variety of breakdown products, and several methods have been used to measure the formation of these as an indicator of membrane damage. The most commonly reported method for the measurement of lipid peroxidation is the Thiobarbituric Acid Reactive Substances (TBARS) assay. This assay relies on the reaction of thiobarbituric acid (TBA) with malonaldehyde formed as

the end-product of lipid peroxidation to give a coloured product which can be measured colourimetrically.

The TBARS assay has been criticised as a means of measuring absolute lipid peroxidation as malonaldehyde is not the only aldehyde produced by lipid peroxidation, nor is malonaldehyde the only aldehyde capable of reacting with TBA, many others can do so, and will produce differently coloured products. Lipid peroxidation is also not the only means by which MDA can be produced, as previously noted that oxidation of the sugar backbone of DNA can also produce MDA. In addition, it has been suggested that the harsh reaction conditions required (high temperature and pH) can generate malonaldehyde from any lipid peroxides present in the sample, indeed it has been suggested that the majority of malonaldehyde thus measured is produced during, rather than prior, to the reaction. However, for the purposes of this project, an absolute measurement of complete lipid peroxidation is not required, rather the relative degree of lipid peroxidation in a H_2O_2 treated sample compared to an untreated control is sought. Due to the wide-spread acceptance of the TBARS assay as an index of lipid peroxidation, this was the preferred method.

5.2 Materials and methods.

5.2.1 Materials.

Trichloroacetic acid, thiobarbituric acid, tetramethoxypropane, butylated hydroxytoluene, tert-butyl hydroperoxide, triolein, vanillin, sodium cacodylate, 25 % glutaraldehyde solution, osmium tetroxide, carboxyfluorescein, Bio-beads, sodium cholate, phosphatidyl ethanolamine from *E. coli*, cytochrome C oxidase from bovine heart, sodium chloride, ferric chloride, sodium sulphate, potassium thiocyanate, tris-HCl, tryptone, sodium chloride, sodium thiosulphate, sodium citrate and calcium chloride and Triton X-100 were purchased from Sigma (Poole, UK).

Ethanol, chloroform, 85 % phosphoric acid solution, asolectin, phosphate-buffered saline tablets, methanol, glacial acetic acid, 2 N hydrochloric acid, sodium alginate, 5 ml syringes and glass fibre discs were purchased from Fisher (Loughborough, UK).

Sephadex G25 was purchased from GE Healthcare (Little Chalfont, UK).

Float-a-lyzer G2 devices were purchased from Spectrum Europe BV (Breda, Netherlands).

LIVE/DEAD Baclight bacterial viablity kits were purchased from Invitrogen (Paisley, UK)

35% hydrogen peroxide stock was provided by Steris.

5.2.2   Method development.

5.2.2.1   TBARS assay for measurement of lipid peroxidation.

Similarly to the methods developed to investigate protein and DNA oxidation, methods were also required to be developed to measure and compare the degree to which lipids are oxidised by H_2O_2 treatment. As previously stated, the TBARS assay was the preferred method of measuring lipid peroxidation, so initial studies were performed to validate and optimise the method used, which was based on that described by Semchysyn *et al.* (2005).

A total of 50 mL of *E. coli* cultured overnight in TSB was pelleted, washed and resuspended in 10 mL deionised water. The resuspended culture was divided into two 5 mL aliquots, and 5 mL of deionised water added to one aliquot to give the control sample whilst 5 mL of 1.7 % H_2O_2 solution was added to the second aliquot to give an effective H_2O_2 concentration of 0.85 %. Samples were incubated for 45 s before the reaction was halted by addition of 40 mL neutraliser. Cells were recovered by centrifugation at 4000 x g for 15 min, the supernatant discarded and the pellet washed twice with deionised water. Washed pellets were resuspended in 10 mL deionised water and crude cell extracts prepared as described in section 3.3.3.3.

Protein was removed from the samples by adding an equal volume of 20 % trichloroacetic acid (TCA). The samples were incubated for 15 minutes on ice, centrifuged at 2200 g for 15 min at 4řC. The supernatants were retained and the pelleted protein discarded.

A 500 ţM malonaldehyde stock solution was prepared by dissolving 4.17 ţL 1,1,3,3-tetramethoxypropane (TMP) in 1 mL ethanol before making up to 50 mL with deionised water. This stock was diluted in deionised water to give 0, 0.75, 1.5, 3 and 6 ţM standards.

In triplicate, 1.5 mL of each retained supernatant or standard was mixed with 2 mL of a saturated solution of thiobarbituric acid (TBA) containing the antioxidant butylated hydroxytoluene. The mixtures were then incubated at 100 řC for 60 min, followed by immediate chilling on ice. The optical density at 535 nm of a 1.5 mL aliquot of each sample or standard

was read using a spectrophotometer.

The standard curve for the MDA standards is shown in figure 5.2.
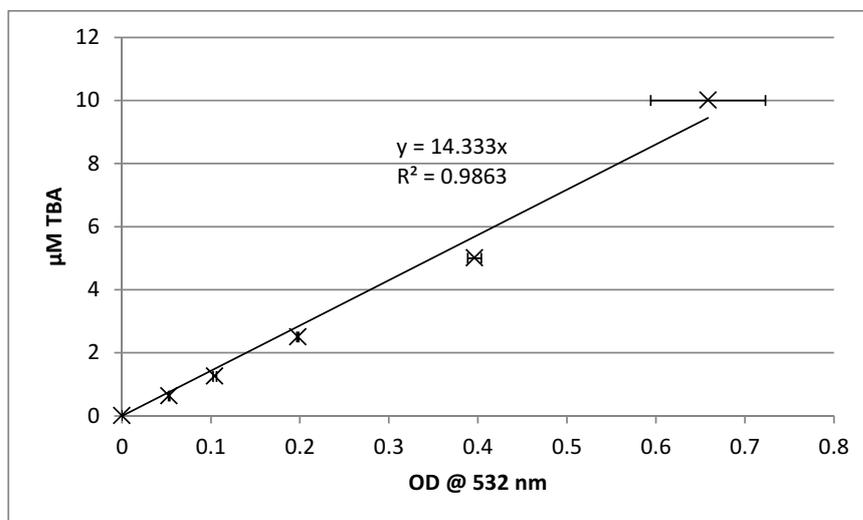
Figure 5.2: Standard curve for TBARS assay. Error bars show standard error of the mean of three replicate measurements.

The initial attempt at the TBARS assay gave no measurable MDA in either the control or test samples. The assay was therefore repeated using a long incubation with the potent oxidiser tBHP in order to completely oxidise any oxidisable lipids.

A total of 50 mL of *E. coli* cultured overnight in TSB was pelleted, washed and resuspended in 10 mL deionised water. The resuspended culture was divided into two 5 mL aliquots, and 5 mL of deionised water added to one aliquot to give a negative control sample whilst 5 mL of 60 mM tBHP solution was added to the second aliquot to give a positive control. Samples were incubated for 1 hour at 37řC before the reaction was halted by addition of 40 mL neutraliser. Cells were recovered by centrifugation at 4000 x g for 15 min, the supernatant discarded and the pellet washed twice with deionised water. Washed pellets were resuspended in 10 mL deionised water and crude cell extracts prepared and assayed for TBARS as before.

No measurable MDA was obtained using this positive control, so the test was repeated using double the quantity of culture resuspended in half the volume of deionised water before sonication to give a four-fold increase in cell concentration.

A total of 100 mL overnight culture was resuspended in 5 mL deionised water to give twice the amount of *E. coli* cells. Following treatment and neutralisation, washed cells were resuspended in 5 mL deionised water (com-

pared with 10 mL used originally) to give a total of four times the concentration of cells originally used.

This gave a value of 0.466 ţM TBARS for the negative control, and 1.533 ţM TBARS for the positive control. This demonstrated that the TBARS assay protocol used was capable of detecting TBARS if the amount of culture used in the assay was increased, however it can be concluded that it is only possible to oxidise a very small fraction of the lipid content. The assay was therefore used with the increased amount of *E. coli* culture.

5.2.2.2 Lipid hydroperoxide assay - two-phase TBARS assay.

In order to determine whether partial lipid peroxidation occurs during H_2O_2 treatment (that is, the lipid peroxidation chain reaction begins, but insufficient to generate measurable quantities of malonaldehyde) two alternative methods of estimating the peroxide number of the lipid were investigated. The first of these is a modification of the standard TBARS assay whereby a sample of crude cell extract is taken and assayed as normal for TBARS in order to determine the amount of complete lipid peroxidation. The remainder of the crude extract has the lipids purified by the Bligh Dyer method and the breakdown of any lipid peroxides catalysed by the addition of metal ions. The malonaldehyde thus produced is then assayed using TBA as usual.

This method was performed as described by Schmedes and Hølmer (1989). A total of 100 mL of *E. coli* cultured overnight in TSB was pelleted, washed and resuspended in 10 mL deionised water. The resuspended culture was divided into two 5 mL aliquots, and 5 mL of deionised water added to one aliquot to give a negative control sample whilst 5 mL of 1.7 % H_2O_2 solution was added to the second aliquot. Samples were incubated for 45 s before the reaction was halted by addition of 40 mL neutraliser. Cells were recovered by centrifugation at 4000 x g for 15 min, the supernatant discarded and the pellet washed twice with deionised water. Washed pellets were resuspended in 5 mL deionised water and crude cell extracts prepared as for the TBARS assay.

Lipids were extracted using the Blythe Dyer method. Briefly, methanol and chloroform were added to give the ratio methanol:chloroform:sample::2:1:0.8 and this mixture was incubated on ice for 10 min. One volume of chloroform and one volume of water was added, and the mixture was centrifuged at 3000 g for 15 min. A total of 9 mL of organic layer and 18 mL of aqueous layer were removed and retained from each sample.

An MDA stock was made by dissolving 6.6 ţL TMP in 10 mL of ethanol, then diluting 2.4 mL of the solution with 97.6 mL of deionised water. Aqueous standards of 0, 3.75, 7.5, 11.25 and 15 ţM were made by diluting the stock in deionised water. Organic standards of 0, 5, 10, 15 and 20 ţM were made by diluting the stock in chloroform.

An organic layer reagent was prepared by dissolving 0.875 grams of TBA in 15 mL of deionised water and adding 135 mL glacial acetic acid. This was mixed immediately before use with 0.475 grams of sodium sulphate and 11 mg ferric chloride.

In triplicate, 2.5 mL organic sample or standard was pipetted into glass vials and 4 mL of TBA reagent added. Samples were heated for 30 min in a boiling water bath then cooled for 5 min in a room temperature water bath. Samples were transferred to centrifuge tubes with 3.5 mL 5% TCA and centrifuged at 1500 g for 10 min. The aqueous layer was removed and the optical density at 532 nm measured using a spectrophotometer with the 0 ţM MDA standard used as a blank.

An aqueous reagent was prepared by dissolving 0.875 g TBA in 15 mL diH$_2$O and adding 135 mL glacial acetic acid, then 2.5 mL of TBA reagent was added in triplicate to 2.5 mL aqueous sample or standard in glass vials. Samples were heated for 30 min in a boiling water bath then cooled for 5 min in a room temperature water bath. Samples were transferred to centrifuge tubes and centrifuged at 1500 g for 10 min. The supernatant was removed and the optical density at 532 nm measured using a spectrophotometer with the 0 ţM MDA standard used as a blank.

Figure 5.3 shows the standard curve for aqueous samples, with the measured TBARS values for the test and control samples highlighted. Figure 5.4 shows a similar chart for the organic phase samples (i.e. the standard curve corresponding to the lipid fraction).
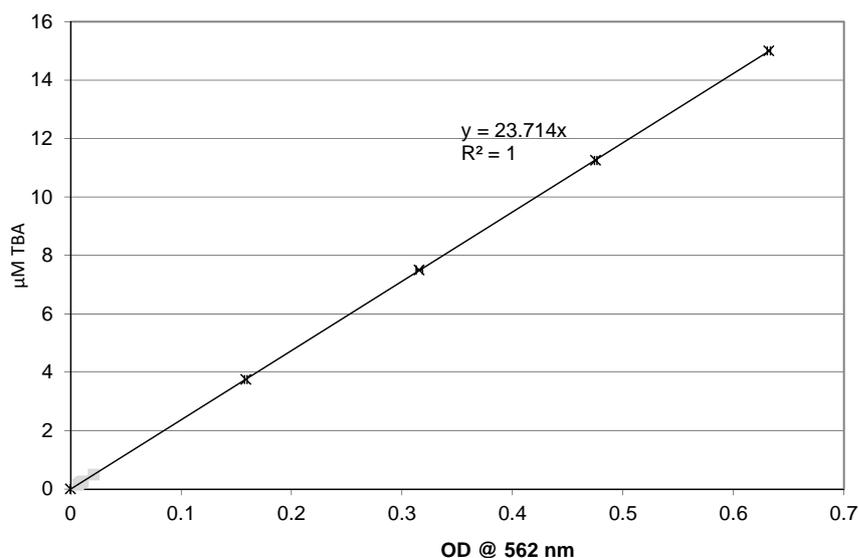
Figure 5.3: Standard curve for the aqueous phase of the two-phase TBARS assay. Test and control sample values are shown in grey.
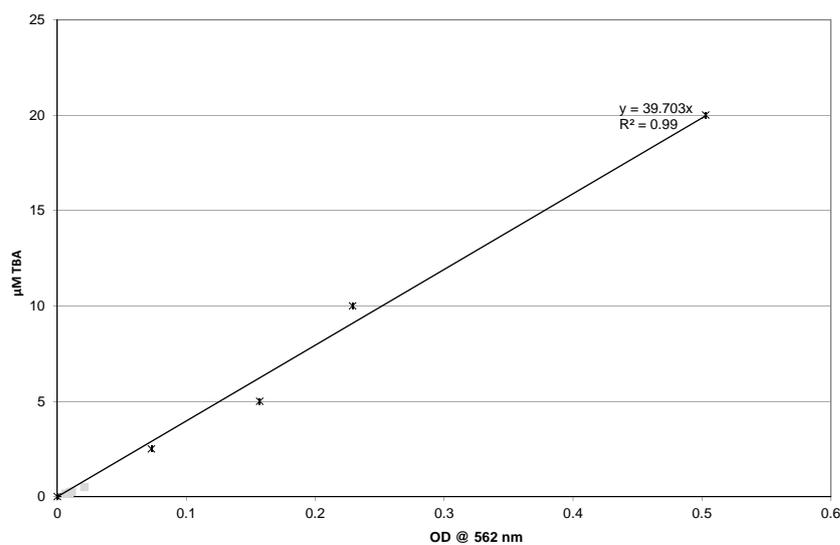
Figure 5.4: Standard curve for the organic phase of the two-phase TBARS assay. Test and control sample values are shown in pink.

As with the single-phase TBARS assay, no measurable amount of MDA was produced in either phase. It should be noted that the two-phase assay was investigated before further investigation of the single-phase assay suggested that an increase in cell concentration was required to produce measurable amounts of MDA on lipid peroxidation.

5.2.2.3   Lipid hydroperoxide assay - ferric thiocyanate method.

The second assay investigated to measure lipid peroxide content was ferric thiocyanate method as described by Mihaljević *et al.*, (1996).

A total of 100 mL of *E. coli* cultured overnight in TSB was pelleted, washed and resuspended in 10 mL deionised water. The resuspended culture was divided into two 5 mL aliquots, and 5 mL of deionised water added to one aliquot to give a negative control sample whilst 5 mL of 1.7 % H_2O_2 solution was added to the second aliquot. Samples were incubated for 45 s before the reaction was halted by addition of 40 mL neutraliser. Cells were recovered by centrifugation at 4000 x g for 15 min, the supernatant discarded and the pellet washed twice with deionised water. Washed pellets were resuspended in 5 mL deionised water and crude cell extracts prepared as before.

Lipids were extracted using the Blythe Dyer method as described for the two-phase TBARS assay. The chloroform layer was removed and assayed.

A reagent solution was prepared by mixing equal quantities of 4.5 mM ferrous sulphate in 0.2 M hydrochloric acid with 3 % potassium thiocyanate in methanol, 80 ţL of this reaction mixture was added to 2 mL of chloroform extract in triplicate (or chloroform alone for a blank) and the reaction mixture was incubated in the dark for 10 min at room temperature. Optical density of the samples at 500 nm was measured using a spectrophotometer.

The control samples gave a mean absorbance of 0.746. The test samples gave a mean absorbance of 0.699. This difference was not statistically significant, a t-test performed using Minitab software gave a p-value of >0.05.

It was observed that absorbance of samples and blank had increased substantially approximately 5 min after measurement in spectrophotometer, suggesting that oxidation of ferrous sulphate was proceeding rapidly in cuvettes. The results from this assay were inconclusive, as it was not possible to de-gas the reagent and sample solutions before assaying. Due to these difficulties, the decision was taken to use the two-phase TBARS assay to estimate peroxide number, as this assay was relatively facile.

5.2.2.4   Sulpho-phospho-vanillin total lipid assay.

The performance of the assay was assessed using the method as described by Izard & Limberger (2003).

A crude cell extract was prepared from 50 mL of overnight *E. coli* culture as previously described. A standard lipid stock was prepared by dissolving

50 mg of triolein in 10 mL chloroform. Standards of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL were prepared by diluting the stock with chloroform. The solvent was evaporated from the samples by incubating at 37řC for 1 hour under nitrogen, and the standards resuspended in 100 ţL deionised water.

A reagent solution was prepared by dissolving 120 mg vanillin in 20 mL deionised water, the volume was then made up to 100 mL with 85 % phosphoric acid.

Standards and samples were prepared in duplicate by adding 2 mL of sulphuric acid to 100 ţL standard or sample in glass vials and incubating for 10 min in a boiling water bath. Vials were cooled in a room temperature water bath for 5 min and 5 mL vanillin reagent was added. Vials were incubated at 37řC for 15 min then cooled for 10 min in a room temperature water bath. The optical density at 530 nm of the samples was read using a spectrophotometer with the 0 mg/mL triolein standard as a blank.

Figure 5.5 shows the standard curve for the sulpho-phospho-vanillin assay.
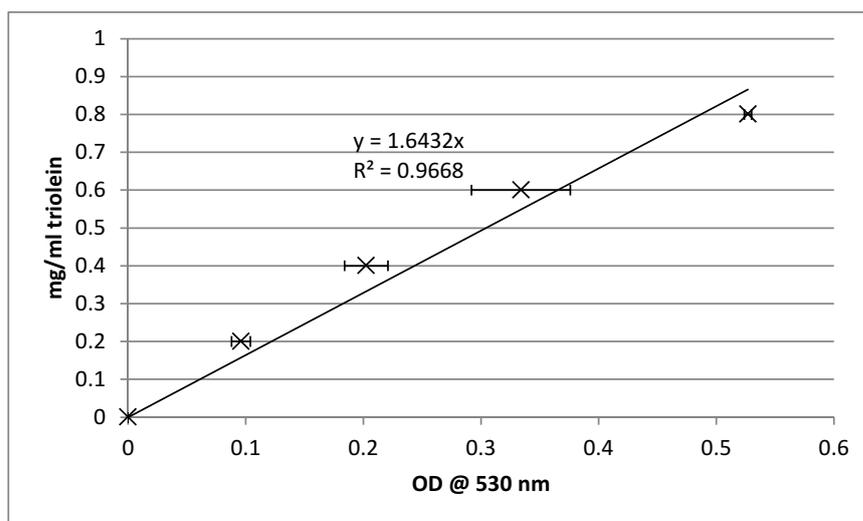
Figure 5.5: Standard curve for triolein standards assayed using the sulpho-phospho-vanillin assay. Error bars show the standard error of the mean of three replicate measurements.

The crude cell extract sample gave a mean absorbance of 0.112, corresponding to an equivalent of 0.218 mg/mL triolein. When the assay was repeated with cells being resuspended in half the previous volume of deionised water, the lipid concentration was approximately doubled. This suggests that 50 mL of overnight culture produces approximately 1 mg of lipid. The assay can be successfully used to estimate total lipid content of crude cell ex-

tracts, and thus to measure any changes in lipid content following hydrogen peroxide treatment.

5.2.2.5   LIVE/DEAD Baclight stain kit.

A bulk *E. coli* culture was produced by inoculating a 250 mL Erlenmeyer flasks each containing 100 ml TSB with culture from a working slope using a culture loop. This was then incubated overnight at 37°C with shaking (100 rpm) and used immediately.

Cell suspensions were transferred into 50 mL centrifuge tubes, centrifuged at 4000 x g for 15 min, and the supernatant discarded. The pellets were washed three times with deionised water, and resuspended in 2.5 mL deionised water. All resuspended pellets were pooled and vortex mixed.

Membrane permeant positive controls were produced by adding 1 mL of resuspended culture to 20 mL of 70 % isopropyl alcohol solution and incubating at room temperature with shaking for 1 hour.

A live negative control was prepared by 1 mL of resuspended culture to 20 mL of TSC and incubating at room temperature with shaking for 1 hour.

The treated suspensions were centrifuged at 4000 x g for 15 min, and the supernatant discarded. The pellets were washed three times with deionised water, and resuspended in 10 mL deionised water.

The optical density at 670 nm of the two suspensions was measured using a spectrophotometer, and adjusted to 0.12. A set of standards was prepared by mixing the two suspensions according to table 5.1.

Ratio negative:positive	mL negative	mL positive
0:100	0	2.0
10:90	0.2	1.8
50:50	1.0	1.0
90:10	1.8	0.2
100:0	2.0	0

Table 5.1: Volumes of positive and negative control suspensions used to produce standards.

Stock 35 % H_2O_2 solution was diluted in deionised water in 5 mL centrifuge tubes to give 0.5 mL of 1.7, 3, 6 and 20 % H_2O_2 solutions. Cells were exposed to H_2O_2 by adding 0.5 mL of H_2O_2 solution to a 0.5 mL aliquot of cell suspension to give the desired final concentrations of 0.85, 1.5, 3 and 10 %. A control was prepared by adding 0.5 mL of deionised water alone. The treatment mixtures were incubated for 45 s before the H_2O_2 was quenched

by addition of 5 volumes of 5% sodium thiosulphate and 0.2 M sodium citrate solution. The mixture was then centrifuged at 4000 x g for 15 min, the supernatant discarded and the pellet washed three times with deionised water. The washed pellet was resuspended in 0.5 mL of deionised water, the absorbance at 670 nm recorded using a spectrophotometer and adjust to 0.12.

The manufacturer's protocol was followed to carry out the live/dead staining - briefly, a 2x working solution of the stain was prepared by dissolving the contents of one component A pipette and one component B pipette in 5 mL deionised water.

A 96 well microtitre plate was prepared by adding 100 L of each bacterial suspension to separate wells in triplicate. The cells were then stained by adding 100 L of the 2x working solution to each well and mixed by pipetting. The plate was then incubated in the dark for 15 mins.

The plate was then read using a Fluostar microplate fluorometer with excitation 485 nm and emission 530 nm, and again at excitation 485 nm, emission 630 nm.

The data were analyzed by dividing the fluorescence intensity of the stained bacterial suspensions at 530 nm by that at 630 nm. A standard curve was plotted using these values for the standards and used to calculate the percentage of cells with damaged membranes from the treated and control samples. Figure 5.6 shows these calculated values.
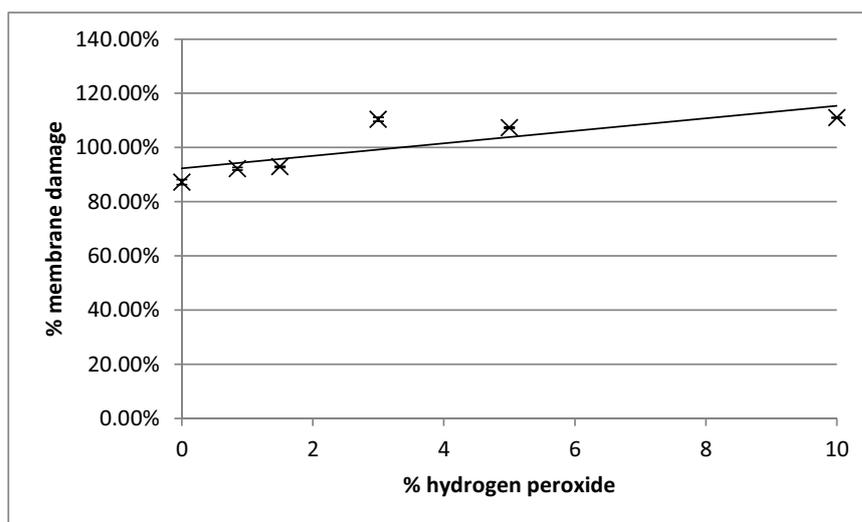
Figure 5.6: Mean percentage of membrane damaged cells from *E. coli* suspensions treated with increasing concentrations of liquid hydrogen peroxide as assayed using the Baclight LIVE/DEAD stain kit.

Initial results showed that all samples, those treated with H_2O_2 and the untreated control, gave a high percentage of cells with damaged membranes. It was hypothesised that this might be due to the presence of sodium citrate in the neutralising solution, and so the procedure was modified for the final study to use sodium thiosulphate alone as the neutraliser. This modification meant that it was impossible to use this method for VHP treated cultures.

5.2.2.6   Liposome model of membrane leakage - asolectin liposomes.

A 10 mg/mL asolectin solution was prepared by dissolving 100 mg asolectin in 10 mL chloroform. This was stored at -20řC until required. A 50 mM carboxyfluorescein solution was prepared by adding carboxyfluorescein to a 10 mM tris-HCl buffer, and adjusting the pH to 7.4 by addition of 10 M NaOH.

A 5 mL round-bottomed flask had 2 mL of asolectin solution added, and the chloroform was evaporated on a rotary evaporator for a total of 1 hour at 37řC. The lipids were resuspended by adding 2 mL of 50 mM carboxyfluorescein solution and mixing on a vortex mixer for 5 min. The resuspended lipid mixture was then divided into two aliquots and small, single-lamellar liposome prepared by sonication with a probe sonicator for 10 cycles of 60 s with a 60 s cooling period between each cycle, or by sonication for 1 h in a bath sonicator at 37řC. Liposome solutions were then allowed to re-anneal by incubating at 37řC for 1 h.

Unencapsulated carboxyfluorescein was removed by passing the liposome solution through a Sephadex G25 column, and washing liposomes out with tris buffer, pH 7.4.

Cuvettes were prepared containing 4 mL of tris buffer, pH 7.4 to which 100 ţL of liposome solution was added. The cuvette was transferred to a fluorometer, excitation wavelength 492 nm, emission wavelength 517 nm. The fluorometer was zeroed and a reading taken every 10 s for a total of 1 h. Liposomes were disrupted by addition of 100 ţL 100 mM Triton X-100 solution, and the fluorescence recorded.

Liposomes were found to be stable for one hour (no increase in fluorescence recorded) and produced fluorescence values of approximately 50 arbitrary units on addition of Triton X100, so this value was chosen as the maximum for the y-axis in the charts.

This method was a facile and fast method for producing simple liposomes, so was used for further testing.

5.2.2.7   Liposome model of membrane leakage - liposomes compatible with membrane proteins.

In order to test whether protein oxidation could cause membrane leakage in a model system, liposomes containing a suitable transmembrane protein were produced. The chloroform evaporation method was not suitable for producing liposomes containing membrane proteins, as the carboxyfluorescein solution used to resuspend the lipids following evaporation of chloroform would require the addition of detergent in order to solubilise the membrane proteins.

An alternative method, whereby both lipid and protein was solubilised by detergent, and liposomes formed by the removal of the detergent was investigated. Two approaches were tested for the removal of detergent, one based on the addition of Biobeads to adsorb the detergent, followed by the removal of the beads, and one using dialysis.

Biobeads method of detergent removal.

A 200 mM carboxyfluorescein solution was prepared by adding carboxyfluorescein to a 10 mM tris-HCl buffer, and adjusting the pH to 7.4 by addition of 10 M NaOH. Following dissolution of the dye, sodium cholate was added to a concentration of 3 %.

A 20 mg/ml asolectin solution was prepared by adding 20 mg of asolectin to 1 ml of the prepared carboxyfluorescein/cholate solution.

Bio-beads were added to the solution in a stepwise manner, and shaken according to the scheme in table 5.2.

mg Biobeads	Temperature (°C)	Time on shaker (mins)
60	4	20
60	4	20
60	4	20
120	4	60
120	RT	60
240	RT	60
240	RT	20
240	RT	60

Table 5.2: Scheme for addition of Biobeads for detergent removal.

The Bio-beads were then removed from the solution by filtration, and excess cholate and carboxyfluorescein removed by passage through a Sephadex

G25 column. A second Bio-bead addition was performed, with 360 mg of Bio-beads added, and the mixture was shaken at room temperature for 40 minutes. Bio-beads were removed by filtration, and buffer exchange of the liposomes performed on a Sephadex G25 column.

This method was found to produce poor results as the Bio-beads bound carboxyfluorescein, leading to formation of liposomes containing a low concentration of carboxyfluorescein. The process was also extremely time-consuming and so was not tested further.

Dialysis method of detergent removal.

A 200 mM carboxyfluorescein solution was prepared by adding carboxyfluorescein to a 10 mM tris-HCl buffer, and adjusting the pH to 7.4 by addition of 10 M NaOH. Following dissolution of the dye, sodium cholate was added to a concentration of 3 %.

A 20 mg/ml asolection solution was prepared by adding 20 mg of asolection to 1 ml of the prepared carboxyfluorescein/cholate solution.

The solution was then transferred to a Float-a-Lyser dialysis device and dialysed against 1 litre of 10 mM Tris-HCl buffer, pH 7.4 containing 200 mM sodium chloride to preserve osmolarity for 4 hours with stirring. The buffer was then changed, and dialysis overnight at 4 řC. This was repeated for three changes of buffer.

A rapid buffer exchange column was prepared by reswelling Sephadex G25 overnight in 0.9 % sodium chloride solution, loading into a 5 ml syringe blocked with a glass fibre disc, and centrifuging at 1000 x g for 5 minutes. More Sephadex was added and centrifugation repeated until the column was fully loaded.

Excess carboxyfluorescein was removed from the liposomes by adding them to the column and washing through with 10 mM Tris-HCl + 200 mM sodium chloride, pH 7.4. Liposomes were washed through the column before the dye.

This method was found to produce stable liposomes containing a high concentration of carboxyfluorescein that fluoresced intensely on addition of Triton X-100, and so was used for further studies.

5.2.3   Methods.

5.2.3.1   Preparation of liquid hydrogen peroxide treated bacterial suspension.

Bulk *E. coli* cultures were produced by inoculating three 250 mL Erlenmeyer flasks each containing 100 mL TSB with culture from working slopes using a culture loop. These were then incubated overnight at 37°C with shaking (100 rpm) and used immediately. Cell suspensions were transferred into 50 mL centrifuge tubes, centrifuged at 4000 x g for 15 min, and the supernatant discarded. The pellets were washed three times with deionised water, and resuspended in 2.5 mL deionised water. All resuspended pellets were pooled, vortex mixed and five 2.5 mL subaliquots taken in 50 mL centrifuge tubes.

Stock 35 % H_2O_2 solution was diluted in deionised water in 5 mL centrifuge tubes to give 3 mL of 1.7, 3, 6 and 20 % H_2O_2 solutions. Cells were exposed to H_2O_2 by adding 2.5 mL of H_2O_2 solution to an aliquot of cell suspension to give the desired final concentrations of 0.85, 1.5, 3 and 10 %. A control was prepared by adding 2.5 mL of deionised water alone. The treatment mixtures were incubated for 45 s before the H_2O_2 was quenched by addition of 5 volumes of 5% sodium thiosulphate and 0.2 M sodium citrate solution. The mixture was then centrifuged at 4000 x g for 15 min, the supernatant discarded and the pellet washed three times with deionised water. The washed pellet was resuspended in 5 mL of deionised water for further treatment.

5.2.3.2   Preparation of alginate gel embedded bacterial sample and vapourised hydrogen peroxide treatment.

The VHP 1000 instrument runs were started before gels were prepared in order to produce a constant H_2O_2 concentration in the treatment chamber. Treatments were timed such that gels were exposed immediately on production, following 20 min of decontamination cycle.

Bacterial cells suspended in deionised water were prepared as before. The resuspended cells were mixed with an equal volume of 4% sodium alginate solution and 1 mL of sample transferred to the lid of a miniature Petri dish. The gel was then spread thinly by gently lowering the base of the Petri dish into the upturned lid. The alginate was cured by addition of 2 mL of 2% calcium chloride solution with the Petri dish base still in position, the

base was then discarded and excess calcium chloride was removed by rinsing three times with deionised water. A total of five gels was produced for each exposure time tested, along with 5 gels to act as unexposed controls.

Dishes containing gels were exposed to the VHP treatment chamber by means of the sample tube for exposure times of 1, 2, 3 and 5 minutes. Dishes were removed from the treatment chamber, the gels were immediately scraped into centrifuge tubes containing 5% sodium thiosulphate and 0.2M sodium citrate solution and the alginate was completely re-dissolved by vortex mixing. The mixture was then centrifuged at 4000 x g for 15 min, the supernatant discarded and the pellet washed three times with deionised water. The washed pellet was resuspended in 5 mL of deionised water for further treatment.

5.2.3.3   Preparation of crude extracts.

Cells were broken apart by three cycles of 60 s sonication on ice separated by 30 s intervals. Intact cells and debris were removed by centrifugation at 4000 x g for 15 min at 4řC, the supernatant was retained and the pellet discarded.

5.2.3.4   Lipid peroxidation estimation by thiobarbituric acid reactive substances assay.

H_2O_2 treated cell suspensions were prepared as described in sections 5.2.3.1 and 5.2.3.2. In addition to the H_2O_2 treated and negative control sample, a positive control was prepared by addition of 60 mM tBHP solution was added to an aliquot of resuspended overnight culture. This control was incubated for 1 hour at 37 řC before the reaction was halted by addition of 40 mL neutraliser. Crude extracts were prepared from these sample described in section 5.3.3.3.

Protein was removed from the samples by adding an equal volume of 20 % trichloroacetic acid (TCA). The samples were incubated for 15 minutes on ice, centrifuged at 2200 g for 15 minutes at 4 řC. The supernatants were retained and the pelleted protein discarded.

A 500 ţM malonaldehyde stock solution was prepared by dissolving 4.17 ţL 1,1,3,3-tetramethoxypropane (TMP) in 1 mL ethanol before making up to 50 mL with deionised water. This stock was diluted in deionised water to give 0, 0.75, 1.5, 3 and 6 ţM standards.

In triplicate, 1.5 mL of each retained supernatant or standard was mixed with 2 mL of a saturated solution of thiobarbituric acid (TBA) containing butylated hydroxytoluene. The mixtures were then incubated at 100 řC for 60 minutes, followed by immediate chilling on ice. The optical density at 535 nm of a 1.5 mL aliquot of each sample or standard was read using a spectrophotometer.

MDA-equivalent TBARS content of the *E. coli* samples was calculated by reference to the standard curve produced from the TMP samples. The whole experiment was repeated three times, and the mean of the three repeats calculated for each sample.

5.2.3.5 Lipid peroxide number estimation by two-phase TBARS assay.

H_2O_2 treated bacterial suspensions were prepared as described in sections 5.2.3.1 and 5.2.3.2, however only suspension samples treated with 3 % liquid H_2O_2 and alginate embedded samples treated with vapourised H_2O_2 for 5 minutes were tested. Crude extracts were prepared from these sample described in section 5.3.3.3.

Lipids were extracted using the Blythe Dyer method. Methanol and chloroform were added to give the ratio methanol:chloroform:sample::2:1:0.8 and this mixture was incubated on ice for 10 min. One volume of chloroform and one volume of water was added, and the mixture was centrifuged at 3000 g for 15 min. A total of 9 mL of organic layer and 18 mL of aqueous layer were removed and retained from each sample.

An MDA stock was made by dissolving 6.6 ţL TMP in 10 mL of ethanol, then diluting 2.4 mL of the solution with 97.6 mL of deionised water. Aqueous standards of 0, 3.75, 7.5, 11.25 and 15 ţM were made by diluting the stock in deionised water. Organic standards of 0, 5, 10, 15 and 20 ţM were made by diluting the stock in chloroform.

An organic layer reagent was prepared by dissolving 0.875 grams of TBA in 15 mL of deionised water and adding 135 mL glacial acetic acid. This was mixed immediately before use with 0.475 grams of sodium sulphate and 11 mg ferric chloride.

In triplicate, 2.5 mL organic sample or standard was pipetted into glass vials and 4 mL of TBA reagent added. Samples were heated for 30 min in a boiling water bath then cooled for 5 min in a room temperature water bath. Samples were transferred to centrifuge tubes with 3.5 mL 5% TCA

and centrifuged at 1500 g for 10 min. The aqueous layer was removed and the optical density at 532 nm measured using a spectrophotometer with the 0 ţM MDA standard used as a blank.

An aqueous reagent was prepared by dissolving 0.875 g TBA in 15 mL diH2O and adding 135 mL glacial acetic acid, then 2.5 mL of TBA reagent was added in triplicate to 2.5 mL aqueous sample or standard in glass vials. Samples were heated for 30 min in a boiling water bath then cooled for 5 min in a room temperature water bath. Samples were transferred to centrifuge tubes and centrifuged at 1500 g for 10 min. The supernatant was removed and the optical density at 532 nm measured using a spectrophotometer with the 0 ţM MDA standard used as a blank.

MDA-equivalent TBARS content of two phases extracted from the *E. coli* samples was calculated by reference to the standard curve produced from the corresponding phase TMP samples. The whole experiment was repeated three times, and the mean of the three repeats calculated for each sample.

5.2.3.6   Measurement of total lipid content using sulpho-phospho-vanillin assay.

H_2O_2 treated cell suspensions were prepared as described in sections 5.2.3.1 and 5.2.3.2. Crude extracts were prepared from these sample described in section 5.2.3.3.

A standard lipid stock was prepared by dissolving 50 mg of triolein in 10 mL chloroform. Standards of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL were prepared by diluting the stock with chloroform. The solvent was evaporated from the samples by incubating at 37řC for 1 hour under nitrogen, and the standards resuspended in 100 ţL deionised water.

A reagent solution was prepared by dissolving 120 mg vanillin in 20 mL deionised water, the volume was then made up to 100 mL with 85 % phosphoric acid.

Standards and samples were prepared in duplicate by adding 2 mL of sulphuric acid to 100 ţL standard or sample in glass vials and incubating for 10 min in a boiling water bath. Vials were cooled in a room temperature water bath for 5 min and 5 mL vanillin reagent was added. Vials were incubated at 37řC for 15 min then cooled for 10 min in a room temperature water bath. The optical density at 530 nm of the samples was read using a spectrophotometer with the 0 mg/mL triolein standard as a blank.

Lipid content of the *E. coli* samples was calculated by reference to the triolein standard curve. The whole experiment was repeated three times, and the mean of the three repeats calculated for each sample.

5.2.3.7   Spectrophotometric assay of cell leakage.

A 100 mL overnight culture of *E. coli* was pelleted and washed as in section 5.2.3.1. One pellet was resuspended in deionised water and diluted to give an OD of 0.8 at 500 nm when read using a GE 3000 series spectrophotometer. The volume required to dilute the pellet was recorded. Three negative control cuvettes were prepared containing 1 mL of this suspension. Three positive control cuvettes were prepared by adding SDS to 1 ml of suspension to give a final concentration of 2%.

The second pellet was resuspended in a volume of 35 % H_2O_2 solution equal to the volume of deionised water used to prepare the control samples. Three test cuvettes were prepared containing 1 mL of this suspension.

The nine cuvettes were loaded onto the GE 3000 series spectrophotometer and the OD at 500 nm recorded every 12 minutes for 20 hours.

5.2.3.8   LIVE/DEAD Baclight stain kit.

A bulk *E. coli* culture was produced by inoculating a 250 mL Erlenmeyer flasks each containing 100 ml TSB with culture from a working slope using a culture loop. This was then incubated overnight at 37ºC with shaking (100 rpm) and used immediately.

Cell suspensions were transferred into 50 mL centrifuge tubes, centrifuged at 4000 x g for 15 min, and the supernatant discarded. The pellets were washed three times with deionised water, and resuspended in 2.5 mL deionised water. All resuspended pellets were pooled and vortex mixed.

Membrane permeant positive controls were produced by adding 1 mL of resuspended culture to 20 mL of 70 % isopropyl alcohol solution and incubating at room temperature with shaking for 1 hour.

A live negative control was prepared by 1 mL of resuspended culture to 20 mL of TSC and incubating at room temperature with shaking for 1 hour.

The treated suspensions were centrifuged at 4000 x g for 15 min, and the supernatant discarded. The pellets were washed three times with deionised water, and resuspended in 10 mL deionised water.

The optical density at 670 nm of the two suspensions was measured using a spectrophotometer, and adjusted to 0.12. A set of standards was

prepared by mixing the two suspensions according to table 5.1.

Stock 35 % H_2O_2 solution was diluted in deionised water in 5 mL centrifuge tubes to give 0.5 mL of 1.7, 3, 6 and 20 % H_2O_2 solutions. Cells were exposed to H_2O_2 by adding 0.5 mL of H_2O_2 solution to a 0.5 mL aliquot of cell suspension to give the desired final concentrations of 0.85, 1.5, 3 and 10 %. A control was prepared by adding 0.5 mL of deionised water alone. The treatment mixtures were incubated for 45 s before the H_2O_2 was quenched by addition of 5 volumes of 5% sodium thiosulphate solution. The mixture was then centrifuged at 4000 x g for 15 min, the supernatant discarded and the pellet washed three times with deionised water. The washed pellet was resuspended in 0.5 mL of deionised water, the absorbance at 670 nm recorded using a spectrophotometer and adjusted to 0.12.

The manufacturer's protocol was followed to carry out the live/dead staining - briefly, a 2x working solution of the stain was prepared by dissolving the contents of one component A pipette and one component B pipette in 5 mL deionised water.

A 96 well microtitre plate was prepared by adding 100 L of each bacterial suspension to separate wells in triplicate. The cells were then stained by adding 100 L of the 2x working solution to each well and mixed by pipetting. The plate was then incubated in the dark for 15 mins.

The plate was then read using a Fluostar microplate fluorometer with excitation 485 nm and emission 530 nm, and again at excitation 485 nm, emission 630 nm.

The data were analyzed by dividing the fluorescence intensity of the stained bacterial suspensions at 530 nm by that at 630 nm. A standard curve was plotted using these values for the standards and used to calculate the percentage of cells with intact membranes from the treated and control samples.

The whole experiment was repeated three times and mean values for the three repeats calculated.

5.2.3.9   Asolectin liposome leakage assay.

A 10 mg/mL asolectin solution was prepared by dissolving 100 mg asolectin in 10 mL chloroform. This was stored at -20řC until required. A 50 mM carboxyfluorescein solution was prepared by adding carboxyfluorescein to a 10 mM tris-HCl buffer, and adjusting the pH to 7.4 by addition of 10 M NaOH.

A 5 mL round-bottomed flask had 2 mL of asolectin solution added, and the chloroform was evaporated on a rotary evaporator for a total of 1 hour at 37řC. The lipids were resuspended by adding 2 mL of 50 mM carboxyfluorescein solution and mixing on a vortex mixer for 5 min. The resuspended lipid mixture was then divided into two aliquots and small, single-lamellar liposome prepared by sonication with a probe sonicator for 10 cycles of 60 s with a 60 s cooling period between each cycle. Liposome solutions were then allowed to re-anneal by incubating at 37řC for 1 h.

Unencapsulated carboxyfluorescein was removed by passing the liposome solution through a Sephadex G25 column, and washing liposomes out with tris buffer, pH 7.4.

Prepared liposome solution was stored overnight at 2-8řC overnight before assay. Control samples were run in triplicate by adding 100 ţL liposome solution to 3.9 mL tris buffer, pH 7.4 in a cuvette. The fluorometer was zeroed with the cuvette containing the diluted liposomes and measurements were taken every 10 s for 1 h. At the end of 1 h, 100 ţL 100 mM Triton X100 was added to disrupt the liposomes and the fluorescence recorded.

Test samples were run in triplicate by adding 100 ţL liposome solution to 3.9 mL tris buffer, pH 7.4 in a cuvette. The fluorometer was zeroed with the cuvette containing the diluted liposomes and 400 ţL of 35 % H_2O_2 was added (to give a final concentration of 3.5 %) and measurements were taken every 10 s for 1 h. At the end of 1 h, 100 ţL 100 mM Triton X100 was added to disrupt the liposomes and the fluorescence recorded.

As *E. coli* cells contain some amount of ferrous ions that can catalyse the release of hydroxyl radicals from H_2O_2, samples were also run in triplicate as for the test samples with the addition of 1.25 mM ferrous sulphate and 1.25 mM EDTA (in order to prevent precipitation of ferric ions).

5.2.3.10   *E. coli* phosphatidylethanolamine liposome containing cytochrome C leakage assay.

A 200 mM carboxyfluorescein solution was prepared by adding carboxyfluorescein to a 10 mM tris-HCl buffer, and adjusting the pH to 7.4 by addition of 10 M NaOH. Following dissolution of the dye, sodium cholate was added to a concentration of 3 %.

Two aliquots of 20 mg/ml phosphatidylethanolamine solution were prepared by adding 0.5 ml of the prepared carboxyfluorescein/cholate solution to vials containing 10 mg of purified *E. coli* phosphatidylethanolamine.

A 0.5 mg/ml cytochrome C oxidase solution was prepared by adding 450 ţL of the prepared carboxyfluorescein/cholate solution to a vial containing 0.25 mg of purified bovine heart cytochrome C oxidase in 50 ţL buffered aqueous solution and a test liposome solution was prepared by mixing one aliquot of 20 mg/ml phosphatidylethanolamine/carboxyfluorescein/cholate solution with this solution.

Control liposome solution was prepared by mixing the second aliquot of 20 mg/ml phosphatidylethanolamine/carboxyfluorescein/cholate solution with 500 ţL of carboxyfluorescein/cholate solution.

The solutions were then transferred to separate Float-a-Lyser dialysis devices and dialysed against 1 litre of 10 mM Tris-HCl buffer, pH 7.4 containing 200 mM sodium chloride to preserve osmolarity for 4 hours with stirring. The buffer was then changed, and dialysis performed overnight at 4 řC. This was repeated for three changes of buffer.

Rapid buffer exchange columns were prepared by reswelling Sephadex G25 overnight in 0.9 % sodium chloride solution, loading into 5 ml syringe blocked with a glass fibre disc, and centrifuging at 1000 x g for 5 minutes. More Sephadex was added and centrifugation repeated until the columns were fully loaded.

Excess carboxyfluorescein was removed from the liposomes by adding them to separate columns and washing through with 10 mM Tris-HCl + 200 mM sodium chloride, pH 7.4.

A black 96 well plate containing samples for fluorometry was prepared. Samples were run in sextuplate by adding 10 ţL liposome solution to 260 ţL tris buffer, pH 7.4 per well.

Positive control samples had 30 ţL of 100 mM Triton X-100 added to disrupt the liposomes, and these wells were used to set the gain on the fluorometer.

Negative control samples had 30 ţL tris buffer, pH 7.4 added. Test samples had 30 ţL 35 % H_2O_2 solution added to give a final concentration of 3.5 %.

Measurements were taken every 10 s for 1 h. At the end of 1 h, 30 ţL 100 mM Triton X100 was added to disrupt the liposomes and the fluorescence recorded.

5.2.3.11   Scanning Electron Microscopy imaging.

H_2O_2 treated *E. coli* suspensions were prepared as in section 5.3.3.1 and 5.3.3.2.

Samples were fixed by resuspending in 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 and incubating for 1 hour. Samples were then centrifuged at 500 x g for 2 mins, and the pellet resuspended in 2:2:1 2 % osmium tetroxide:0.1 M sodium cacodylate, pH 7.4:25 % glutaraldehyde and incubated for 1 hour at room temperature.

Samples were centrifuged again at 500 x g for 2 mins, resuspended in 50 % ethanol in deionised water, and dehydrated by alternatively centrifuging and resuspending in 70, 80 and 95 % ethanol solutions, followed by three washes with absolute ethanol.

Samples were kept at room temperature before dehydration under vacuum on carbon grids, plasma coating with platinum and imagining on a scanning electron microscope.

5.3   Results.

5.3.1   Lipid peroxidation estimation by thiobarbituric acid reactive substances assay.

Figure 5.7 shows the calculated values for nanomoles of TBARS per milligram of lipid for suspension samples tested using a range of H_2O_2 concentrations.
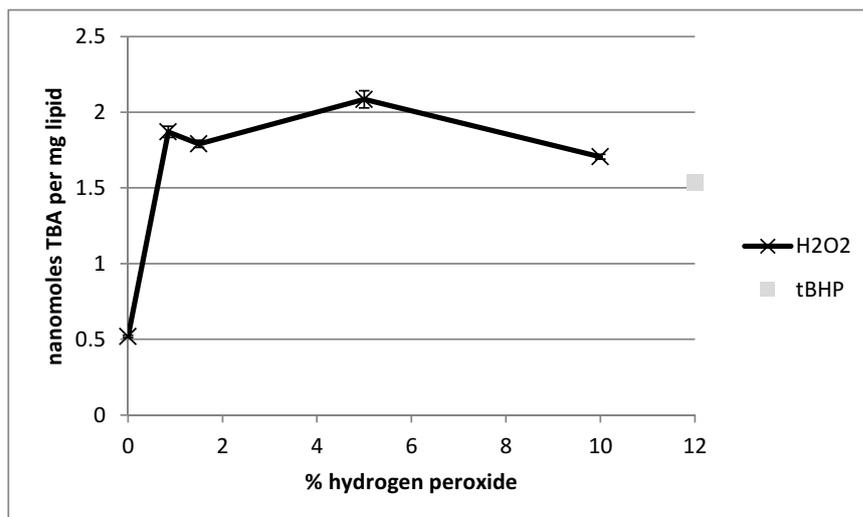
Figure 5.7: Nanomoles of thiobarbituric acid reactive substances per mg of lipid present in suspension samples treated with a range of hydrogen peroxide concentrations or with tert-butyl hydroperoxide. The error bars show the standard error of the mean of measurements taken on three occasions.

Figure 5.   shows the calculated values for nanomoles of TBARS per milligram of lipid for alginate embedded samples tested using a range of exposure times to vapourised H_2O_2.
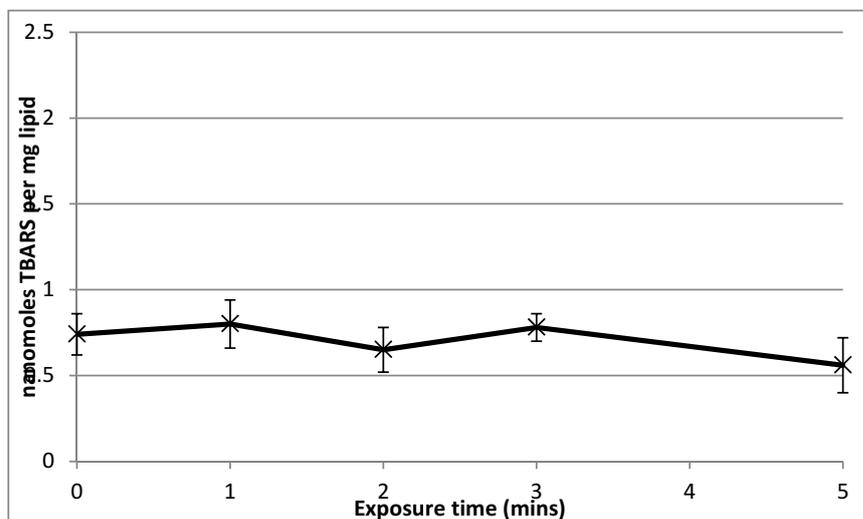
Figure 5.8: Nanomoles of thiobarbituric acid reactive substances per mg of lipid present in suspension samples treated for a range of exposure times with vapourised hydrogen peroxide. The error bars show the standard error of the mean of measurements taken on three occasions.

All the liquid H_2O_2 concentrations tested gave an increase in TBARS compared to the negative control. This difference was statistically significant when analysed using ANOVA ($p < 0.0001$) with Minitab software.

There was no significant difference between the concentrations tested when analysed using ANOVA (p > 0.05), nor was there a difference between the H_2O_2 treated samples and the positive control (tBHP treated).

A smaller increase was seen with the vapour-treated samples compared with the control, and this was not statistically significant when analysed using ANOVA with Minitab software (p>0.05).

These results show that treatment with liquid H_2O_2 does produce an increase in thiobarbituric reactive substances, but the amount of such substances produced does not increase as the concentration used to treat the samples increases.

Treatment with vapourised H_2O_2 does not produce a measurable increase in thiobarbituric acid reactive substances.

All calculated figures are shown as an amount of TBARS per mg of lipid, as the lipid content was measured for each sample. No significant change in lipid content was observed under any of the conditions tested, showing that lipid was not destroyed in large quantities.

5.3.2   Lipid peroxide number estimation by two-phase TBARS assay.

Figure 5.9 shows the measured TBARS content for the aqueous phase extracts from the two samples types compared with their corresponding negative controls. Figure 5.10 shows similar values for the organic phase extracts.
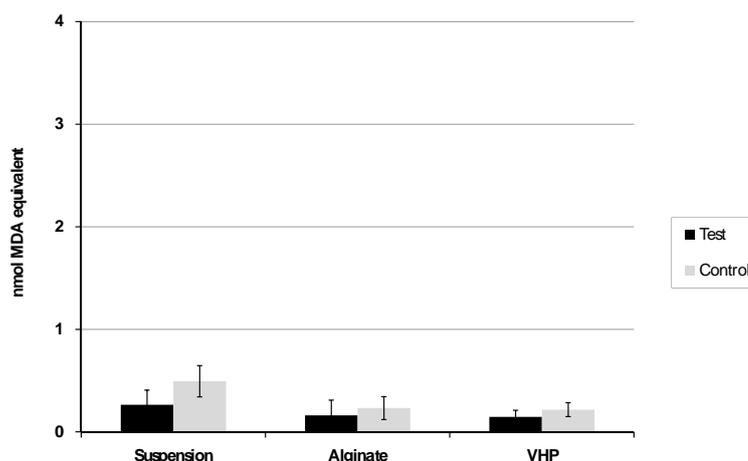
Figure 5.9: Nanomoles MDA equivalent present in the aqueous phases of cell extracts from liquid hydrogen peroxide treated *E. coli* suspensions and vapourised hydrogen peroxide treated samples embedded in alginate gels.
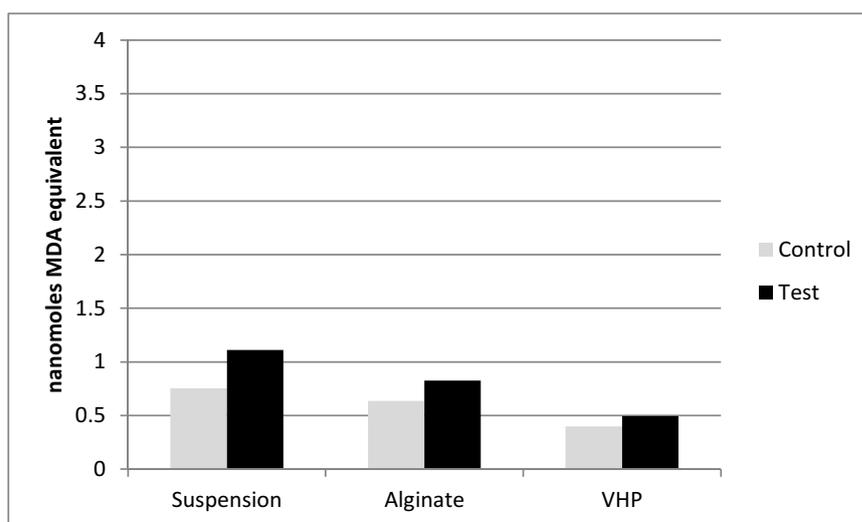
Figure 5.10: Nanomoles MDA equivalent present in the organic phases of cell extracts from liquid hydrogen peroxide treated *E. coli* suspensions and vapourised hydrogen peroxide treated samples embedded in alginate gels.

Neither treatment produced a statistically significant increase in TBARS content in either phase when the results were analysed using ANOVA ($p > 0.05$). This shows that treatment with 0.85 % liquid H_2O_2 or exposure to vapourised H_2O_2 for 1 minute did not result in an increase in lipid peroxide content, though both treatments were sufficient to effect an approximately 1 $\log_{10}$ reduction in CFU/ml count.

5.3.3   Spectrophotometric assay of cell leakage.

Figure 5.11 shows the mean change in absorbance at 500 nm over 10 hours for the control and 35% H_2O_2 treated *E. coli* suspensions.
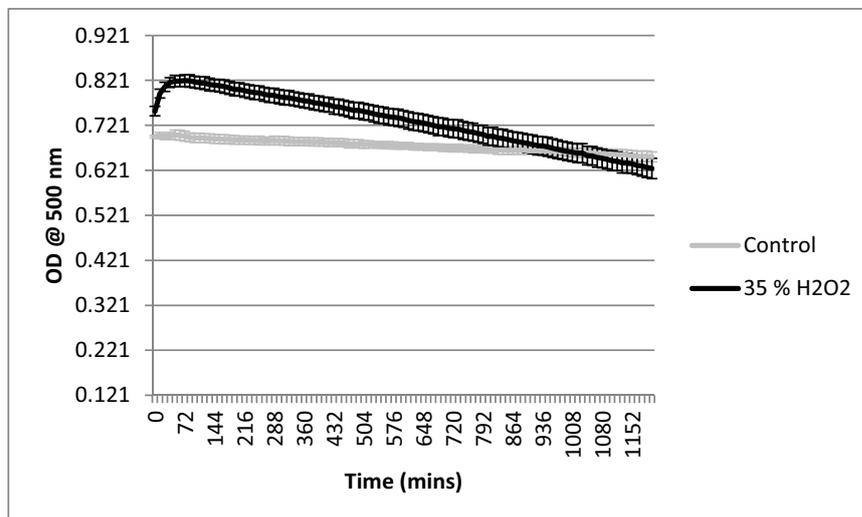
Figure 5.11: Mean absorbance of control (deionised water) and 35% hydrogen peroxide suspended *E. coli* cultures. Error bars show the standard error of the mean of three replicate experiments.

There was a decrease in absorbance observed in the treated samples, however the original absorbance was restored by gently agitating the suspension after incubation, suggesting that cell lysis did not occur.

The positive control reduced to OD to 0.121 (represented by the minimum on the y axis on the chart in figure 5.13) after 36 minutes. The original OD could not be restored by agitation, showing that the reduction in OD due complete lysis is not reversed by resuspending settled cells.

5.3.4   LIVE/DEAD Baclight stain.

Figure 5.12 shows the calculated percentage of cells with membrane damage as assayed using the Baclight LIVE/DEAD stain kit.
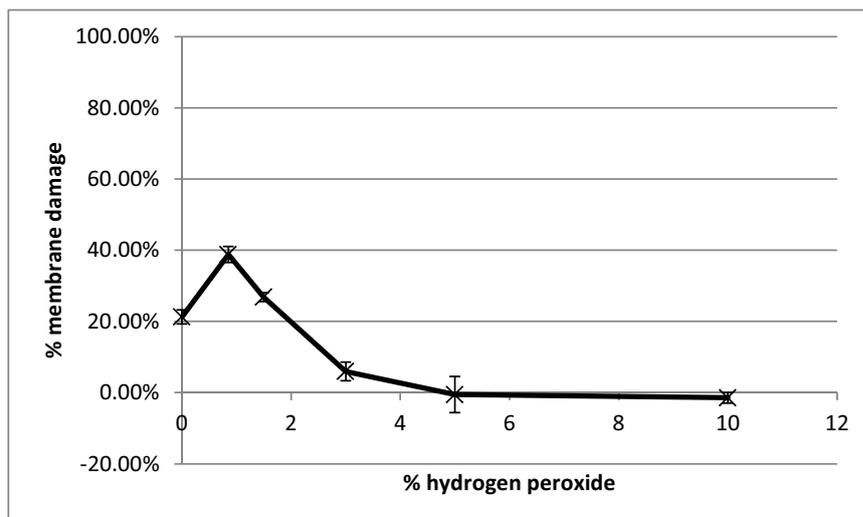
Figure 5.12: Mean percentage of cells with damaged membrane as measured using the Baclight LIVE/DEAD stain kit. Error bars show the standard error of the mean of three replicate experiments.

5.3.5   Asolectin liposome leakage assay.

Figure 5.13 shows the change in fluorescence with time for suspensions of liposomes in control buffer (tris only), H_2O_2 (tris buffer + 3.5% H_2O_2) and Fenton reagent (tris buffer + 3.5 % H_2O_2 + 1.25 mM ferrous sulphate + 1.25 mM EDTA). The maximum value on the y axis shows the average fluorescence produced when liposomes were completely disrupted by the addition of 100 L 100 mM Triton X-100.
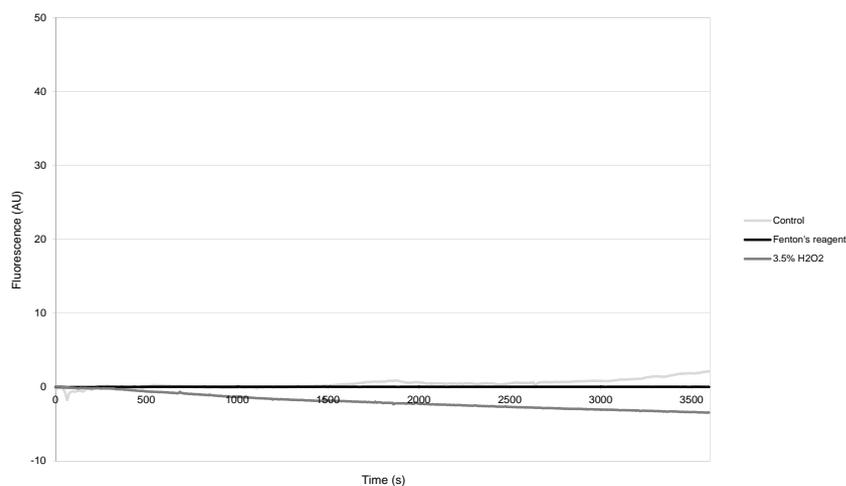
Figure 5.13: Change in fluorescence with time for control (tris buffer only), hydrogen peroxide (tris buffer + 3.5% hydrogen peroxide) and Fenton reagent (tris buffer + 3.5 % hydrogen peroxide + 1.25 mM ferrous sulphate + 1.25 mM EDTA) suspended asolectin liposomes.

Neither treatment produced an increase in fluorescence, showing that these conditions were not able to cause sufficient oxidation of lipids to disrupt the liposomes. The small drop in fluorescence seen with H_2O_2 was consistently produced by all three replicate experiments, and suggests that H_2O_2 might be able to oxidise residual carboxyfluorescein and thus reduce the background fluorescence of the liposome suspensions.

5.3.6 *E. coli* phosphatidylethanolamine liposomes containing cytochrome C leakage assay.

Figure 5.14 shows the change in fluorescence over time for *E. coli* phosphatidylethanolamine liposomes in tris buffer only (negative control) and tris buffer containing 3.5% H_2O_2. Figure 5.15 shows a similar chart for liposomes containing cytochrome c oxidase embedded in the membrane. The maximum value on the y axis represents the fluorescence produced when Triton X-100 was added to disrupt the liposomes.
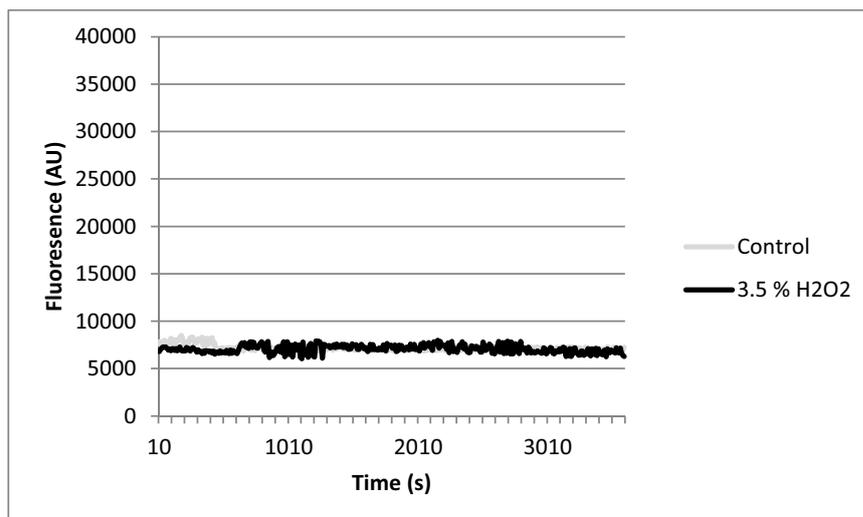
Figure 5.14:   Change in fluorescence over time for *E. coli* phosphatidylethanolamine liposome suspensions in tris buffer only (negative control) and tris buffer containing 3.5% H_2O_2.
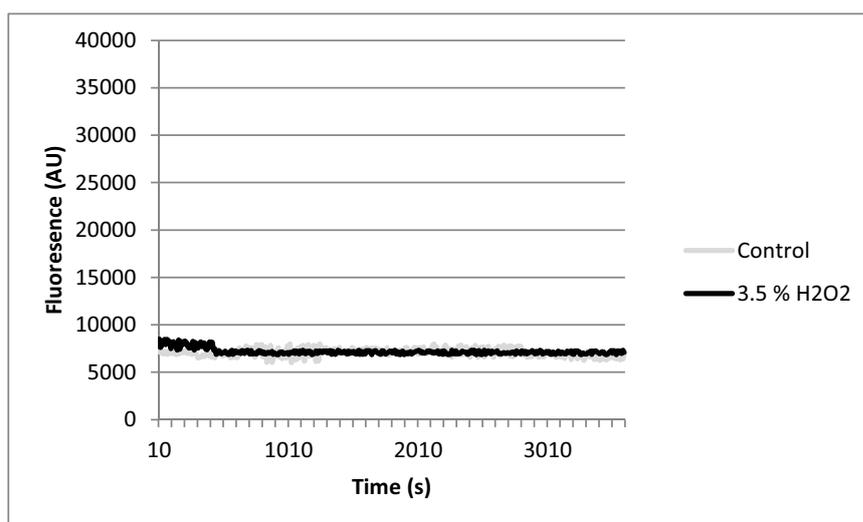
Figure 5.15:   Change in fluorescence over time for *E. coli* phosphatidylethanolamine liposomes containing cytochrome c oxidase embedded within the membrane suspended in tris buffer only (negative control) and tris buffer containing 3.5% H_2O_2.

Exposure to H_2O_2 did not produce an increase in fluorescence with either type of liposome, showing that 3.5 % $H_2O_2$is not able to cause sufficient oxidation of lipids to disrupt the liposomes, nor could this treatment cause sufficient damage to the protein embedded in the membrane to disrupt the liposome.

5.3.7   Scanning Electron Microscopy imaging.

Figure 5.16 shows the SEM images for liquid H_2O_2treated *E. coli* samples, and figure 5.17 shows the images for VHP treated samples.

Some membrane damage could be seen in the liquid treated samples, however this damage was also present in the untreated control suggesting that it was an artifact of the fixing process. The appearance of damage did not increase as the concentration of H_2O_2 increased showing that it was not contributing to the bactericidal action.

No such damage was observed with the VHP treated samples, showing that membrane damage is not part of the bactericidal action of VHP treatment.
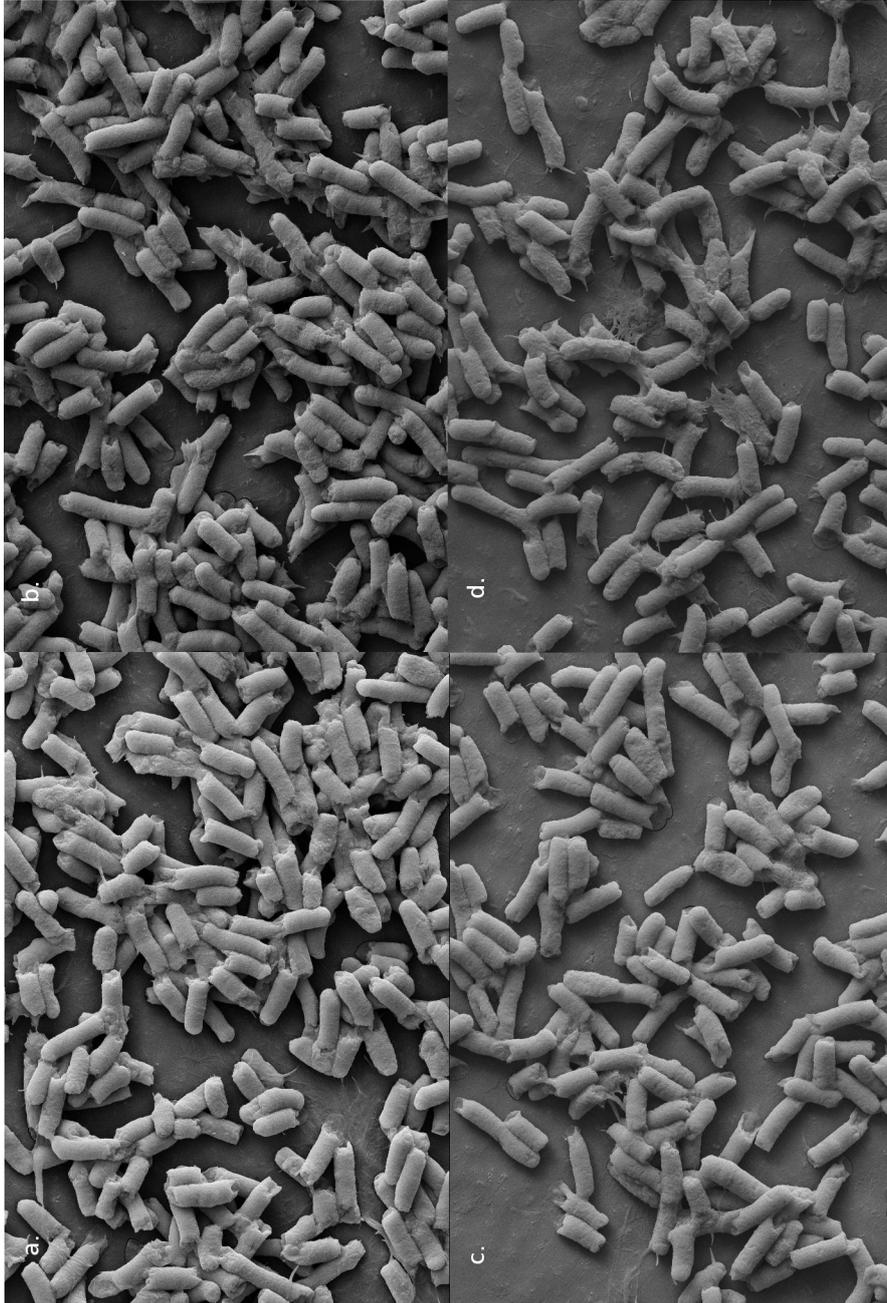
Figure 5.16: SEM images for *E. coli* suspensions treated with a. 0% hydrogen peroxide (control), b. 0.85% hydrogen peroxide, c. 3% hydrogen peroxide and d. 5% hydrogen peroxide.
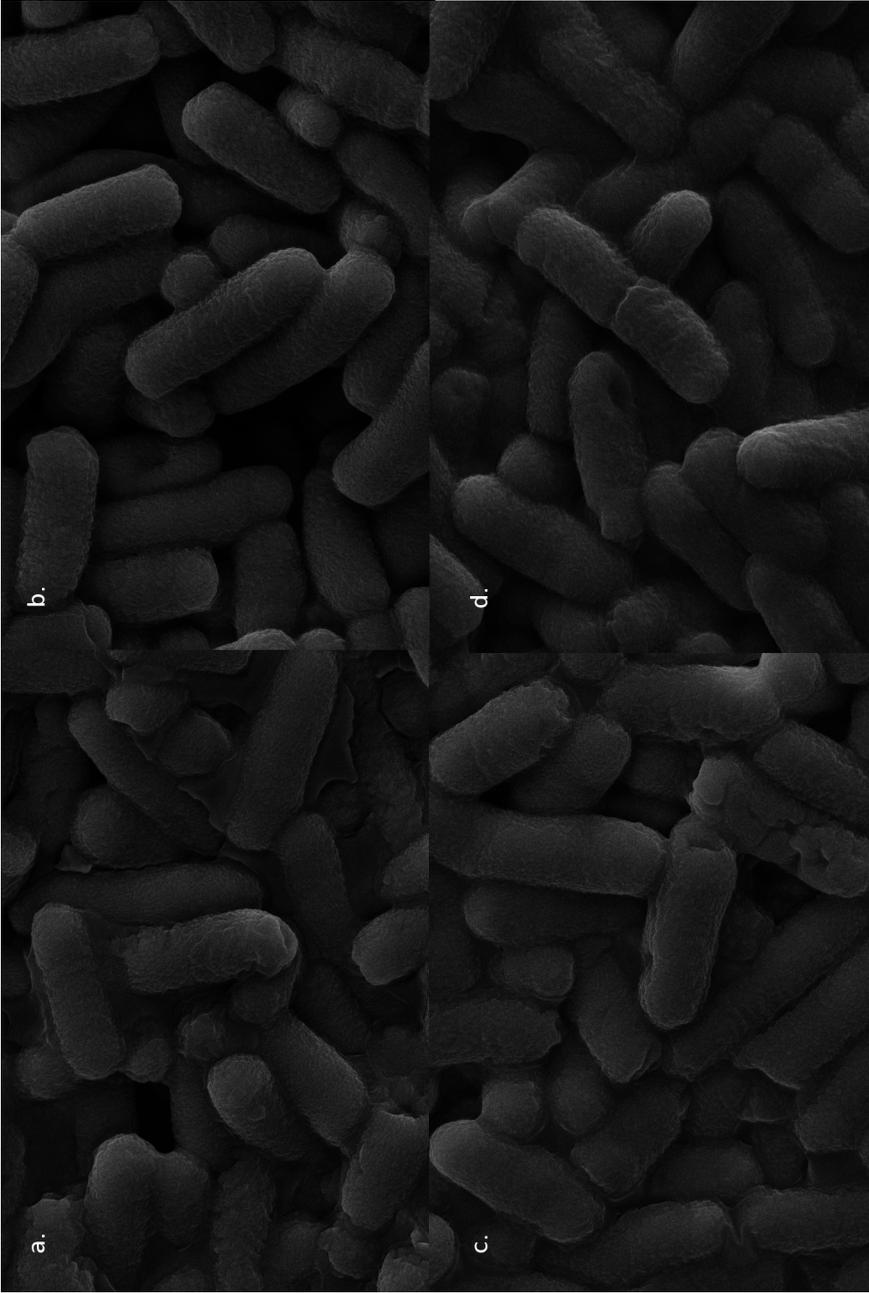
Figure 5.17: SEM images for *E. coli* suspensions treated with vapourised hydrogen peroxide for a. 0 minutes (control), b. 1 minute, c. 3 minutes and d. 5 minutes.

5.4 Discussion.

5.4.1 Lipid peroxidation in hydrogen peroxide treated *E. coli.*

Treatment of *E. coli* cultures with vapourised H_2O_2 did not produce a measurable increase in thiobarbituric-reactive substances. This provides evidence that lipid peroxidation does not occur during VHP treatment, and that neither membrane damage to destruction of lipids, nor the toxic effects of lipid peroxidation end-products, contribute to the bactericidal mechanism of vapourised H_2O_2.

A small amount of TBARS was produced at every concentration of liquid H_2O_2 tested. This amount did not increase as the concentration of H_2O_2 increased, and thus an increased bactericidal effect was not related to an increase in TBARS. The ability of this assay system to measure lipid peroxidation was tested by oxidising asolectin with tert-butyl hydroperoxide, and dose responses to both asolectin starting concentration and reaction time were observed (data not shown).

One interpretation of the fact that a measurable amount of TBARS was produced by treatment with liquid H_2O_2, and that this did not increase as the concentration of H_2O_2 increased, or even if the sample was treated for 1 h at elevated temperature with a strong oxidising agent suggests that there exists within the *E. coli* some small fraction of total lipid which is possible to oxidise, and that this fraction is readily oxidised by addition of H_2O_2. That the amount of MDA-equivalent produced does not correlate with the decrease in colony-forming units observed when higher concentrations of H_2O_2 are used to treat cultures suggests that this lipid peroxidation is not important to the bactericidal mechanism of H_2O_2 at higher concentrations, though it may contribute to the overall bactericidal effect.

There is however, the possibility that some interfering substance (an aldehyde product created by lipid peroxidation other than malonaldehye, for instance) is produced in large quantities, and that this substance reacts with thiobarbituric acid giving a differently coloured product that does not affect the absorbance at 532 nm. Such a substance might mask the true concentration of MDA, leading to an underestimate of the amount of lipid peroxidation that has occurred. However, as has been discussed, this assay has previously been used successfully to measure lipid peroxidation in many studies, and whilst a number of interfering substances have been identified,

the products of the TBA reaction with these substances exhibit some absorbance at 532 nm (Janero, 1990). Indeed, as discussed by Janero (1990), the oxidation of many other cells components, including DNA, proteins and carbohydrates can lead directly to the production of MDA or other TBARS, and so the assay is prone more to false-positives than to false-negatives.

The final interpretation of these results is that the small amount of TBARS measured represents and artefactual result, and is due to production of an interfering substance or MDA itself by oxidation of another cellular component. As discussed in Chapter 4, treatment of _E. coli_ suspensions with liquid H_2O_2 causes extensive double-strand break formation in DNA, almost certainly due to the direct oxidation of the DNA backbone. Such reactions have been shown to produce base propenals which can further breakdown by hydrolysis to yield MDA (Cheeseman _et al._ 1988). The possibility must therefore be considered that the positive result of the TBARS assay with liquid H_2O_2 treated samples is due to DNA oxidation products, especially as the sulpho-phospho-vanillin total lipid assay did not detect a decrease in lipid content in H_2O_2 treated cells, suggesting that lipids are not being destroyed in large quantities.

A previous study by Semchysyn _et al._ (2005) showed an increase in TBARS when _E. coli_ was cultured in the presence of up to 120 ţM (0.00041 %) H_2O_2, with a peak of TBARS at 30 ţM (0.0001%) H_2O_2. They also found that incubating _E. coli_ with 20 ţM (0.00007 %) H_2O_2 caused a time-dependent increase in TBARS, with a peak at 30 minutes, followed by a reduction at 60 minutes to slightly above those at time zero. They did not offer an explanation for this behaviour, though several plausible hypotheses can be advanced. However, their study is flawed in that TBARS production was the only measure they used of lipid damage, no measure was made of membrane integrity, nor was DNA oxidation or growth inhibition measured. As discussed, TBARS alone is a poor measure of lipid peroxidation, and it cannot be concluded from their study that lipid damage does occur, especially when one considers that prokaryotic organisms do not produce PUFAs and so are less susceptible to membrane oxidation.

5.4.2 Membrane damage by hydrogen peroxide treatment.

Though levels of lipid peroxidation may be low, it is possible that such small amounts of lipid damage could cause disproportionate amounts of cell damage by effecting membrane integrity, causing leakage of cell contents.

It is also possible that oxidation of membrane proteins can cause some damage to membrane integrity, though as discussed in Chapter 3, there was no evidence that protein oxidation occurs during H_2O_2 treatment of *E. coli* under the conditions tested.

The direct measurement of cell leakage by spectrophotometric analysis of loss of turbidity showed no loss of turbidity, even with 35 % H_2O_2 over 10 hours. Suspension cultures treated with up to 3.5 % liquid H_2O_2 also showed no increase in the uptake of propidium iodide compared with untreated controls, showing that membrane integrity was not affected by such treatment, even when a large reduction in cell viability occurred. Interestingly, addition of 0.2 M sodium citrate to cells produced a very large increase in propidium iodide uptake, even though it was shown in Chapter 2 that this did not cause any loss in cell viability. This suggests that such an assay cannot be considered as evidence of bactericidal membrane damage in isolation, and also shows that this method is not suitable as measure of cell viability, as culturable cells will undersome circumstances stain as "dead".

Electron microscopy imaging showed some damage to the outer surface of the treated cells, however this damage was also seen in the untreated controls, suggesting that it is an artifact of the fixing process and unrelated to treatment with H_2O_2.

5.4.3 Liposome model of membrane damage.

5.4.3.1 Asolectin liposomes.

The liposome model system using asolectin showed no leakage of carboxyfluorescein on treatment with 3.5 % H_2O_2 for up to one hour, this is interesting as asolectin contains PUFAs and so should be liable to peroxidation. In fact, leakage was not seen even in the presence of ferrous ions and EDTA which should cause Fenton-like production of hydroxyl radicals.

Three possible conclusions can be drawn from the failure to produce leakage of the liposomes by oxidation. The first is that the liposomes used could not be made to release carboxyfluorescein, or that such release would not cause an increase in fluorescence. It can be easily shown that a release of carboxyfluorescein was possible, and that this release was associated with an increase in fluorescence, as the addition of detergent caused an immediate, large increase in measured fluorescence. It was also noted that prolonged storage of the liposome resulted in a gradual increase in background fluo-

rescence, suggesting that destruction of liposomes was measurable in this system.

The second possibility is that the crude asolectin used, despite storage according to recommended conditions, had already been subject to oxidation, and that all PUFAs therein had been oxidised before testing began, leaving only those lipids unsusceptible to such reactions. No attempt was made to assess the lipid content of the asolectin mixture, however, as mentioned in section 6.1, it had been previously shown to be possible to cause lipid peroxidation in the asolectin by incubating with tert-butyl hydroperoxide as measured by TBARS production. Unfortunately, time constraints prevented the testing of tert-butyl hydroperoxide with the liposomes themselves.

The final possibility is that *in vitro*, H_2O_2 alone cannot oxidise lipids in a membrane-like arrangement, and that Fenton-like chemistry cannot be made to occur with high concentrations of H_2O_2, as the catalytic cycle with iron will not take place. Addition of small amounts of ferrous iron caused immediate oxidation to ferric iron, as evinced by the precipitation of insoluble iron. A concentration of ferrous chloride equal to that of the H_2O_2 could not be used as this interfered with the fluorescence of carboxyfluorescein due to the opacity of the solution, however if the iron is acting as a catalyst this should not be important. Addition of EDTA to chelate the iron, and thus prevent precipitation, had no effect on liposome stability. It is beyond the scope of this project to investigate *in vitro* Fenton systems using high H_2O_2 concentrations, but further work with the liposome model might allow such a system to be established and tested.

5.4.3.2   *E. coli* phosphotidylethanolamine liposomes with and without inserted protein.

The *E. coli* lipid liposomes also did not leak on addition of H_2O_2, either with or without addition of cytochrome oxidase c. Unfortunately, temporal and financial constraints prevented exhaustive testing to confirm that the protein was inserted into the membrane and that it was possible to oxidise it. This model, with further development, has the potential to allow the investigation of indirect membrane damage by denaturation of membrane proteins and could provide very valuable information about the action of various biocidal agents.

5.4.4   Conclusion.

The results of this study provide evidence that oxidation of membrane lipids does not occur during killing of *E. coli* cells by H_2O_2. If any small amount of oxidation does occur during treatment with liquid H_2O_2, then is not important to the bactericidal action at the tested concentrations. This result should be expected, as it has been shown that *E. coli* (and the majority of prokaryotic organisms) do not contain polyunsaturated fatty acids, and so oxidation of lipids is unlikely to occur. In fact, it has been shown that PUFAs exhibit bactericidal activity against a range of bacteria, possibly related to the release of their autoxidation products (Sun *et al.*, 2003, Shin *et al.*, 2007). The difficulty in oxidising bacterial membrane lipids might suggest a mechanism for this activity – PUFAs might insert into the bacterial membrane, providing an initiation point for a peroxidation chain reaction for which the bacteria lack chain-terminating antioxidant defences.

Membrane damage, and loss of membrane integrity, has also been shown in this study not to be a component of bactericidal action of liquid or vapour phase H_2O_2. This might be considered more surprising as vapour phase H_2O_2 has been shown to be a potent oxidiser of proteins even in the absence of metal ions (Finnegan *et al.*, 2011). However, it appears that the susceptibility of other cellular components, especially DNA, means that such a mechanism, whilst remaining theoretically possible, is not necessary for the bactericidal action in conditions tested.

Chapter 6

Discussion.

6.1   General comments.

As outlined in Chapter 1, the bactericidal mechanism of H_2O_2 is poorly understood, despite extensive work studying the damage caused to various bacterial macromolecules under conditions of H_2O_2 stress. This is because such studies are not designed to investigate bactericidal action, but rather to understand responses to physiological levels of H_2O_2, elevated oxidative stress, and the killing mechanism of certain types of white blood cell. Inappropriate applications of the findings of these studies to discussion of bactericidal action during sterilisation, which relies on the acute toxic effects of high concentrations of H_2O_2, has led to potentially misleading conclusions as to the mechanism of action of H_2O_2 sterilisation solutions. The mechanism of action of vapourised H_2O_2 remains completely uninvestigated, despite evidence that its mechanism of action might vary significantly from that of the liquid phase.

This study for the first time has investigated the damage caused to a model bacterial organism by typical in-use conditions for liquid and vapour-phase H_2O_2 sterilisation. In most cases it provides the first measurements of damage to macromolecular cell components under such conditions, and is certainly the first such study to simultaneously measure damage to all components and relate them to measures of bactericidal efficacy in order that a greater understanding of the bactericidal mechanism of action of H_2O_2 in both phases can be gained.

It is hoped that the results of this study can be used to help in the rational design of formulated H_2O_2 sterilisation solutions, as well as providing an experimental framework for the study of the mechanisms of oxidising

agents against a range of organisms.

6.2 Summary of results.

6.2.1 Liquid hydrogen peroxide treatment.

Treatment of *E. coli* suspensions with between 0.85 % and 10 % H_2O_2 for 45 seconds caused a decrease in the number of viable colony forming units of between 0.8 and 4 $\log_{10}$ CFU/ml.

Treatment of *E. coli* suspensions with between 0.85 % and 10 % H_2O_2 for 45 seconds caused extensive DNA fragmentation, the amount of which increased as the H_2O_2 concentration used to treat the suspensions increased. Higher H_2O_2 concentrations caused a greater reduction in colony forming units, and thus DNA fragmentation can be said to be correlated with bactericidal action.

Pretreatment of suspensions with an agent that blocked *de novo* protein synthesis did not affect DNA fragmentation following treatment with H_2O_2, suggesting that the DNA fragmentation was not caused by the action of repair enzymes, but rather the direct scission of the DNA backbone by oxidation. Addition of a radical scavenger also did not affect the formation of DNA strand breaks, suggesting that the production of free hydroxyl radicals was not involved in the oxidation of DNA.

Treatment of *E. coli* suspensions with between 0.85 % and 10 % H_2O_2 for 45 seconds caused the formation of a small amount of thiobarbituric-reactive substances, an indicator of lipid oxidation. However, assessment of lipid peroxide formation by an alternative method did not show any increase, nor was this increase correlated in any way with a reduction in colony forming units. It is likely that this increase in TBARS represents an artefactual product, probably from damage to DNA.

Measurement of membrane damage by a reduction in turbidity of cell suspensions did not show any damage, even with 35 % H_2O_2 treatment overnight. An alternative measure of membrane damage, ingress of propidium iodide also showed no damage occurring after treatment with between 0.85 and 10 % H_2O_2. Direct visualisation of the treated cells using scanning electron microscopy showed no changes to the cell surface of treated cells compared to untreated cells.

Testing of a model of membrane damage based on *E. coli*-derived liposomes showed that 3.5 % liquid H_2O_2 could not damage the integrity of these

liposomes sufficiently to cause leakage of the fluorescent dye carboxyfluorescein. Embedding transmembrane proteins in these liposomes did not render them more susceptible to damage by H_2O_2.

Treatment of *E. coli* suspensions with between 0.85 % and 10 % H_2O_2 for 45 seconds caused a decrease in the amount of protein thiols. The amount of protein thiols remaining was lower at higher H_2O_2 concentrations, and this might contribute to the bactericidal effect by either inhibiting the action of repair and respiratory enzymes which contain thiol groups within the active site, or by causing the formation of disulphide bridges between proteins leading to aggregation. Examination of the results suggest that the oxidation of protein thiols is not an important component of bactericidal activity; as discussed in section 3.4.4 the relationship between H_2O_2 concentration, CFU/ml and protein thiol content is such that we can conclude that the decrease in thiol content is merely correlated with, not causative of, bactericidal action.

Measurement of protein carbonyl concentration showed no increase in carbonyl content in either total protein extracts or outer-membrane protein enriched samples from treated suspensions. Measurement of total protein content of these extracts also showed no change following treatment. This suggests that oxidation of amino acids other than cysteine and methionine does not occur, and hence oxidative breakdown of proteins also does not occur.

The damage occurring to the various macromolecular cell targets following treatment of *E. coli* cell suspensions with liquid H_2O_2 is summarised in table 6.1.

Target	Damage
DNA	Extensive fragmentation
Proteins	Thiol oxidation
Lipids/membrane	No lipid oxidation, no change in membrane integrity

Table 6.1: Summary of damage to cell components by liquid hydrogen peroxide.

6.2.2 Vapourised hydrogen peroxide treatment.

Treatment of *E. coli* cultures embedded in alginate gels with ˜0.003 % vapourised H_2O_2 for between 60 seconds and 5 minutes caused a decrease in the number of viable colony forming units of between 0.8 and 6 $\log_{10}$ CFU/ml.

Treatment of *E. coli* gels with ˜0.003 % vapourised H_2O_2 for between 60 seconds and 5 minutes caused extensive DNA fragmentation, the amount of which increased as the exposure time increased. Longer exposure times caused a greater reduction in colony forming units, and thus DNA fragmentation can be said to be correlated with bactericidal action of vapourised H_2O_2.

Treatment of *E. coli* gels with ˜0.003 % vapourised H_2O_2 for between 60 seconds and 5 minutes did not cause the formation of measurable amounts of thiobarbituric-reactive substances, an indicator of lipid oxidation, nor did treatment for 60 seconds cause any measurable increase in lipid peroxides. These results suggest that such conditions do not cause any oxidation of membrane lipids. Direct visualisation of the treated cells using scanning electron microscopy showed no changes to the cell surface of treated cells.

Treatment of *E. coli* gels with ˜0.003 % vapourised H_2O_2 for between 60 seconds and 5 minutes did not cause a measurable decrease in the amount of protein thiols, suggesting that such conditions do not cause oxidation of cysteine or methionine residues in cytoplasmic proteins. The absence of a decrease in thiol content following treatment with vapourised H_2O_2 did not reduce the bactericidal efficacy of the vapour in comparison with the liquid, again showing that this effect is not important to the bactericidal mechanism.

Measurement of protein carbonyl concentration showed no increase in carbonyl content in either total protein extracts or outer-membrane protein enriched samples. This suggests that oxidation of amino acids other than cysteine and methionine does not occur, and hence oxidative breakdown of proteins also does not occur during treatment with these conditions.

The damage occurring to the various macromolecular cell targets following treatment of *E. coli* with vapourised H_2O_2 is summarised in table 6.2.

Target	Damage
DNA	Extensive fragmentation
Proteins	No damage
Lipids/membrane	No lipid oxidation, no change in membrane integrity

Table 6.2: Summary of damage to cell components by vapourised hydrogen peroxide.

6.3   A model of bactericidal action of hydrogen peroxide.

Of the macromolecules measured, only DNA showed extensive damage that was correlated with a reduction in viable colony forming units. This damage was not prevented or decreased by pretreatment with protein synthesis inhibitors. Repair to abasic sites leading to the production of single strand breaks is known to be due to the *de novo* synthesis of DNA repair enzymes (Greenberg and Demple, 1989; Demple and Halbrook, 1983) and so inhibition of protein synthesis will prevent these repair enzymes being manufactured by the cell. This therefore shows that the fragmentation observed is caused by the direct chemical action of the H_2O_2 treatment, not the generation of single-strand breaks by the action of oxidised base excision enzymes. Were free hydroxyl radical production within the cytoplasm to be the major cause of the observed DNA damage, it would be expected that damage would mainly occur to the more readily-accessed base residues, and that a major proportion of strand-breaks would be produced by the action of the repair enzymes.

Support for the hypothesis that DNA damage can be caused by other radicals than hydroxyl is provided by the work of Imlay *et al.* (1988) and Luo *et al.* (1994) as discussed in detail in section 4.4.3.2. Lloyd and Phillips (1999) have also shown *in vitro* using EDTA chelation of iron that damage to DNA, in particular the generation of double strand breaks, is dependent on the close association of iron ions with the DNA backbone.

If free hydroxyl radicals were produced within the cytosol, one would expect extensive damage to both cytoplasmic and outer-membrane proteins, as the hydroxyl radical is one of the most powerful oxidising agents known, and production of these radicals by Fenton chemistry can cause complete breakdown of proteins (for example, see Chapter 3, in which a BSA band can be eliminated from an SDS-PAGE gel by treatment with Fenton's reagent). No such damage was detected however; no increase in protein carbonyls was detected and no decrease in total protein content (which would result from total proteolysis) was detected. Some oxidation of cysteine and methionine thiols was detected following treatment with liquid H_2O_2, however this reaction does not involve a radical-mediated mechanism.

Addition of Tris, which has been shown to scavenge free radicals (Hicks and Gebicki, 1986), had no effect on bactericidal efficacy at H_2O_2 concentra-

tions of 0.85 % and greater, though it did increase the MIC of liquid H_2O_2. Addition of Tris also had no effect on the generation of DNA strand breaks by liquid H_2O_2 at concentrations of 0.85 % and greater. This provides further evidence that the production of free hydroxyl radicals within the cell is not important for the bactericidal action of H_2O_2 at typical sterilant solution concentrations, though such a mechanism might be important at lower concentrations. This effect is easily understood when one considers the ability of H_2O_2 itself to act as a radical scavenger. Such a possibility has been explored by Luo *et al.* (1994), as discussed in section 4.5.3.2, and in fact use of H_2O_2 as a hydroxyl radical scavenger in various systems has been suggested (e.g. Henry and Donahue, 2011) and its effect as such is commonly reported (e.g. Rojas *et al.* 2010; Molina *et al.* 2006). This illustrates the caution with which one must apply findings from studies performed at one range of concentrations to conditions where the concentrations are different by orders of magnitude; one cannot simply assume that the chemistry will remain the same across such a wide range, particularly when one is contemplating an environment as complex as the living cell.

The results obtained provide very strong evidence that the mechanism of bactericidal action of liquid H_2O_2 at concentrations commonly used in biocidal solutions, such as contact lens cleaning solutions, is not due the production of free hydroxyl radicals leading to damage to all macromolecular components of the bacterial cell as commonly reported. Rather, the bactericidal action is due to the specific oxidation of the DNA sugar backbone, a reaction that is more likely to be driven by the production of the ferryl radical.

6.4   Relevance of the study.

This study provides a much improved understanding of the mechanism of bactericidal action of H_2O_2 against vegetative bacteria at typical sterilant in-use conditions. This information is important to the understanding of the differences in susceptibility of bacterial species to H_2O_2 treatment, and to the rational design of sterilisation procedures and solutions.

It has been demonstrated in this study that the main, and perhaps only, target for H_2O_2 in *E. coli* K12 is DNA. Large reductions in colony forming unit numbers can be obtained in short times with DNA fragmentation being the only measurable damage; this shows that the susceptibility of *E. coli*

DNA to oxidation by H_2O_2 is the cause of this strain's sensitivity to H_2O_2 treatment. When investigating the reasons for greater resistance in other species, it would therefore be sensible to initially suspect that such strains possess some mechanism to protect their DNA from damage. This could be accomplished in two ways; either by preventing H_2O_2 from making contact with the DNA molecule, or, as damage appears to be caused by the reaction of H_2O_2 with DNA-associated iron, by regulating the amount of iron bound to the DNA backbone. In fact, it has been shown that *B. subtilis* spores are more resistant to H_2O_2 than the vegetative cells and that this resistance is due to the presence of small, acid-soluble spore proteins (SASPs) which appear to fulfill both of these functions, preventing the reaction of H_2O_2 and the binding of iron to the DNA molecule (Setlow and Setlow, 1993). It is likely that such mechanisms will contribute more to resistance to high concentrations of DNA than the production of enzymes such as catalase which actively destroy H_2O_2 as these higher concentrations will rapidly overwhelm the ability of even an abundantly expressed enzyme to detoxify them.

6.5 Future work.

Whilst this study provides a more thorough understanding of the bactericidal mechanism of liquid H_2O_2 on the level of macromolecular damage, it can only provide indirect evidence of the exact molecular mechanism of action. The investigations performed suggest that free hydroxyl radical production is not important to the bactericidal action, however it was beyond the scope of this project to perform a detailed study of the exact chemistry involved and so further work should be performed to confirm these results. Past studies have made use of spin trapping to reveal the nature of the radicals(s) involved in the damage to DNA by mixtures of H_2O_2 and various metal ions (e.g. Kawanishi *et al.* 1986; Yamamoto *et al.* 1989) and such a study could be performed using physiological concentrations of iron and the higher, typical in-use concentrations of H_2O_2 as used in the this study to determine the exact nature of the radical reaction.

Previous studies have also explored the products of DNA oxidation in great detail (see section 4.5.3.3 for a detailed discussion), but again these have concentrated on physiological and slightly elevated levels of H_2O_2. Such studies performed using typical in-use concentrations of H_2O_2 would again allow a thorough understanding of the exact nature of the reactions

occurring under these conditions.

This study only provides information about the macromolecular damage occurring in *E. coli* K12 following H_2O_2 treatment. This organism was chosen as a model of the action of H_2O_2 on vegetative bacterial cells, and it is likely that the findings of this study can be extrapolated to other bacterial species. However, one of the aims of the this study was to identify a method to analyse macromolecular damage by any number of oxidising agents on a range of organisms, and follow-up studies should now be performed using the protocol described in section 6.4.3 with a number of different organisms. In particular, it would be interesting to compare the results obtained with *E. coli* K12, a Gram negative organism, with those for a model Gram positive organism. It would also be interesting to compare the results with those for eukaryotic organisms, in particular to discover whether the presence of more easily oxidised PUFAs in the eukaryotic membrane means that lipid oxidation and membrane damage contributes to the biocidal action of H_2O_2 against such organisms.

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