Cardiff University
School of Earth, Ocean and Planetary Sciences

THE EMERGENCE OF NUCLEIC ACIDS IN AN IRON-SULPHUR WORLD

By
Bryan Hatton

Thesis submitted for the Degree of Philosophiae Doctor
September 2007

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

One hypothesis for the origin of life suggests that life emerged in a hydrothermal mineral assemblage containing sulphides and hydroxides. The research reported in this thesis shows that these minerals interact with nucleic acid polymers. This has two consequences. Firstly, metal sulphides, particularly iron sulphides, cause the scission of DNA and thus the survival of DNA in this environment is improbable. On the other hand, the reactions at low concentrations could lead to rapid mutations of DNA-like molecules and thus would have affected its evolution. These reactions also have biochemical consequences. For DNA to retain its function as the hereditary molecule it needs to be separated from concentrations of FeS. This also suggests that the results of the molecular ecology of sulfide-rich environments might be affected by reaction with iron sulphides.

The effect that these nanoparticulate sulphides have had on the emergence and survival of nucleic acid polymers has been investigated through plasmid electrophoresis and UV-Vis spectrometry. Metal sulphides interact with DNA in two ways: scission of the DNA backbone and binding with sulphide particles. Treatment of pDNA with FeS, CuS, Fe(II), and Cu(II) breaks the ribo-phosphate backbone of the DNA molecule. This occurs through sulphur-based free radicals produced through redox reactions between S_2^{2-} and Fe(II) and Cu(I) ions. Migration of oligomeric DNA in electrophoresis is retarded by FeS, CuS and ZnS which is attributed to the binding of nanoparticulate sulphides to the DNA.

DNA, RNA, adenine, deoxyadenosine and deoxyadenosine monophosphate bind to FeS, CuS, ZnS and Fe(II)/Fe(III) hydroxides. This occurs through the nucleobase rather than the phosphate. Longer DNA molecules adsorb more readily

than oligomers, RNA or monomers. Thus the hypothesis that iron-sulphide minerals could adsorb and concentrate nucleic acid constituents promoting nucleic acid formation and then polymerisation is supported, although the hypothesis that the phosphate group emerged to allow this binding is not necessary

In the absence of sulphide, Fe(II) causes similar effects. Scission of the DNA occurs through hydroxyl radicals produced through a reaction with trace amounts of O_2 also DNA adsorbed onto precipitated green rusts (Fe(II)/(III) hydroxides). Sulphide, in the absence of metal ions, induced no effect on DNA. In the absence of redox, transition metals oxyhydroxides adsorb to DNA but do not act to break up the molecule. Therefore, non-redox active metal sulphides, such as zinc, could have provided some of the functionality of iron sulphide especially with regards to nucleic acid formation, but without producing free radicals and therefore, simultaneous nucleic acid destruction.

Acknowledgements

I would like to thank my supervisors, Professor David Rickard and Professor Andrew Weightman and my pastoral supervisor, Robert Riding for their support, encouragement and advice. I would also like to thank Professor David Knight for useful discussions and support. I would like to thank my colleagues in the Cardiff Sulphide Research Group, Dr Ian Butler, Chloe Heywood, Shanshang Huang, Anthony Oldroyd and Andrew Griffiths for practical assistance and constructive comments. Thanks to Dr Gordon Webster for his assistance and instruction in the molecular biology lab.

Many thanks to my fellow PhD students at Cardiff Earth Sciences, both those who welcomed me to the department and those who have joined us more recently, for their friendship, support and so much more, especially, Alan, Cathal, Cat, Dave, Helena, Julia, Lizzie, Martin, Rich, Rhodri, Sarah and Simon. Special thanks go to Päivi, you have been a great source of comfort and joy and I'm not sure if I would have gotten through this without you, although, I am sure it wouldn't have been as much fun.

I am grateful to Asta and Sarah for making me feel at home in Cardiff very quickly, for giving me someone to talk to who wasn't a geologist and for giving me a relaxing and enjoyable environment to come home to after work.

Finally, I would like to thank my family, especially my parents David and Pauline, my brother, Lee and my grandparents for their constant support throughout my education.

Contents

Abstract i
Acknowledgements iii
Contents iv
Preface ix

Chapter 1 **Introduction** 1
1. Background 2
2. Project aims 6
3. Explanation of experimental approach 8
4. Thesis Summary 10

Chapter 2 **The FeS-world origin of life hypothesis: A critical review** 12
1. Introduction 13
2. Hydrothermal sulphide minerals 14
3. Carbon fixation and metabolism in an FeS-world 16
3.1. Pyrite-pulled carbon fixation 17
3.2. Fischer-Tropsch synthesis 19
3.3. Formation of the first carbon-carbon bonds 20
3.4. Origins of metabolism 21
3.5. Pyrite-pulled metabolism 21
3.6. Autocatalytic carbon cycles: fidelity of replication 25
3.7. Carbon fixation and metabolism in FeS compartments 26
3.8. Conclusion of carbon-fixation and proto-metabolsim 28
4. Evolution from an Autocatalytic Cycle 29
5. Origin of cellularisation 33
5.1. Wächtershäuser's semi-cellular structures 35
5.2. Pre-formed inorganic cells 40
5.2.1. Self cotained redox 42
5.2.2. Evidence for FeS compartments 42
5.3. Conclusion of cellularisation section 43
6. Origin of nucleic acids 44
6.1. Origin of nucleic acid-amino acid interaction in FeS Cells 44
6.2. Genome evolution in an inorganic cell 46
6.3. Conclusion of origin of nucleic acid 50
7. Conditions and Resources in an FeS world 51
7.1. Iron and Sulphur Resources 52
8. Conclusions 54

Chapter 3 **An electrophoretic study on the effects of Fe (II) and Fe (III) on plasmid DNA** 57
Abstract 58
1. Introduction 58
2. Materials and methods 61
2.1. Plasmid DNA preparation 61
2.2. Sample preparation 62
2.3. Electrophoresis 63
3. Results 64

4. Discussion....70
4.1. Iron(II)-induced scission of DNA....70
4.2. Adsorption of DNA onto iron hydroxides....71
4.3. Implications for the origin of life....71
5. Conclusions....73

Chapter 4 **An electrophoretic study on the effect of aqueous sulphide on plasmid DNA**....75
Abstract....76
1. Introduction....76
2. Materials and methods....78
2.1. Plasmid DNA preparation....78
2.2. Sample preparation....79
2.3. Electrophoresis....80
3. Results....80
3.1. Effect of alkalinity....85
4. Discussion and conclusions....88
4.1. Implications for the origin of life....90

Chapter 5 **FeS-Induced Radical Formation and its Effect on Plasmid DNA** 92
Abstract....93
1. Introduction....93
2. Materials and methods....98
2.1. Plasmid DNA preparation....98
2.2. Sample preparation....89
2.3. Electrophoresis....102
2.4. FeS_{aq} clusters....102
2.5. Molecular O_2....103
2.6. Free radicals....104
3. Results....105
4. Discussion....117
4.1. Radicals....118
4.2. Surface interactions....122
5. Conclusions....122

Chapter 6 **The effect of transition metal redox on DNA in sulphidic systems**
....124
Abstract....125
1. Introduction....125
2. Materials and methods....128
2.1. Plasmid DNA preparation....128
2.2. Sample Preparation and reactant characterization....128
2.3. Electrophoresis....130
3. Results....130
4. Discussion....136
4.1. Copper-induced pDNA scission....136
4.2. Metal-DNA binding....138
5. Conclusions....139

Chapter 7 **Adsorption of nucleic acid constituents on nanoparticulate transition metal sulphides in aqueous solutions**.....141
Abstract.....142
1. Introduction.....142
2. Materials and methods.....144
2.1. Sample preparation.....145
2.2. Analysis.....146
3. Results.....148
3.1. Chromosomal DNA.....148
3.2. Oligomeric DNA.....150
3.3. RNA.....150
3.4. Adenine and derivatives.....150
4. Discussion.....156
5. Conclusions.....160

Chapter 8 **Discussion and conclusions**.....162
1. DNA scission by metal-promoted hydroxyl and sulphur radical production.....163
1.1. Implications for the origin of life.....164
1.2. Other effects.....170
2. Adsorption of nucleic acids onto sulphides and hydroxides.....172
3. Further work.....173
4. List of conclusions.....175

References.....177

Figures

Chapter 2
Figure 1. Extant reductive citrate cycle. 23
Figure 2. Wächtershäuser's pyrite-pulled thio-analogue of the RCC 24
Figure 3. Formation of semi-cells 37
Figure 4. Proposed origin of life scenario in a mineral assemblage 41

Chapter 3
Figure 1. The reaction between iron ammonium sulphate and pDNA 65
Figure 2. The reaction between iron (III) chloride and pDNA 66
Figure 3. Effect of titanium citrate on Fe(II)-pDNA reaction 68
Figure 4. Effect of DMSO on Fe(II)-pDNA reaction 69

Chapter 4
Figure 1. The reaction between Na_2S and pDNA 81
Figure 2. Reduction in pDNA concentration by Na_2S 82
Figure 3. Total amount of pDNA and abundance of each form 84
Figure 4. The effect of $(NH_4)_2S$ on pDNA 86
Figure 5. The Effect of NaOH on plasmid DNA 87

Chapter 5
Figure 1. Confirmation of FeS structure by XRD 101
Figure 2. The reaction between FeS and pDNA 106
Figure 3. FeS-induced relaxation of pDNA 107
Figure 4. FeS-induced reduction in pDNA concentration 108
Figure 5. FeS-induced relaxation of pDNA against pDNA concentration 110
Figure 6. Percentage of pDNA relaxed against pDNA concentration 111
Figure 7. Reduction in DNA concentration against initial DNA concentration 112
Figure 8. The reaction between FeS and pDNA in an anoxic environment 114
Figure 9. The reaction between FeS and pDNA with titanium (III) citrate 115
Figure 10. Effect of DMSO on FeS-pDNA reaction 116

Chapter 6
Figure 1. Effect of copper sulphide on pDNA 132
Figure 2. Effect of zinc sulphide on pDNA 133
Figure 3. Effect of zinc sulphate on pDNA 134
Figure 4. Effect of copper sulphate on pDNA 135

Chapter 7
Figure 1. Adsorption of cDNA onto FeS, CuS and ZnS 149
Figure 2. Adsorption of oDNA onto FeS 152
Figure 3. Adsorption of RNA onto FeS 153
Figure 4. Adsorption of RNA onto FeS 154
Figure 5. Adsorption of adenine and derivatives onto FeS 155

Tables

Chapter 3
Table 1. pH of $FeSO_4$ samples 62

Chapter 5
Table 1. pH of FeS samples 100
Table 2. Effect of catalase on the FeS-pDNA reaction 113
Table 3. Effects of DMSO and CH_2O on FeS-DNA reaction 115
Table 4. Efect of ultraviolet light on FeS-pDNA reaction 116

Preface

This thesis is arranged formally as a series of chapters. However, the chapters are written in the form of research journal papers. These research papers are bookended by an introductory chapter (Chapter 1) which provides the background to the thesis and a summary discussion and conclusions chapter (Chapter 8) which pulls all the threads together. References are collected at the end of the thesis. The chapters are all formatted consistently and do not have the variability required by diverse journals.

Chapter 1

Introduction

1. Background

Within 1 Gyr of its formation, living organisms, capable of metabolism, growth, reproduction and evolution, developed on the Earth from inorganic chemicals. The study of how this occurred is not only a fascinating challenge but can provide insights into the nature of living systems with the potential to inform a variety of fields of study from biomedicine to astrobiology.

After hypothesising that all living things stemmed from a common ancestor Charles Darwin went on to speculate how that ancestor may have arisen from "some warm little pond, with all sorts of ammonia and phosphoric salts, light, heat, electricity, etc. present" (Darwin, 1888). This theory was further expanded by Haldane (1929) and Oparin (1938) who hypothesised a pathway in which microorganisms could arise from an increasingly concentrated solution of organic chemicals. For this to happen an environment is required which is conducive to the production of simple organic chemicals. Miller (1953), using the best estimates of atmospheric composition at the time and an electric spark as an energy source, found that a suite of biologic chemicals could be produced on the early earth.

This "prebiotic broth" theory dominated thinking on the origin of life for several decades and, although it still has proponents (De Duve and Miller, 1991; Miyakawa *et al.*, 2002; Lazcano and Miller, 1996), it has been criticized in recent years on a number of grounds. The atmosphere is now thought to have been more oxidising and thus less conducive to organic synthesis and survival and the ocean is thought to be too dilute for a significant concentration of organics to have emerged. The condensation and polymerisation of organic chemicals in the prebiotic broth

hypothesis also poses a problem as, in an aqueous environment, hydrolysis is favoured over condensation.

Alternative theories on life's emergence on earth have been proposed, one of these, commonly called the FeS-world hypothesis, was developed by Gunter Wachtershauser (1988; 1990; 1992; 1994; 1997; 1998; 2003; 2006) who proposed that life did not emerge heterotrophically in an aqueous environment, as proposed in the *prebiotic soup* hypothesis, but autotrophically as a 2-dimensional organic monolayer on the surface of a mineral at a hydrothermal submarine location in which the oxidation of mackinawite (FeS_m) to pyrite (FeS_2) is coupled with and provides energy for the fixation of gaseous carbon and primitive metabolic reactions which occur on the pyrite surface. Other researchers have noted the potential for hydrothermal systems to promote organic synthesis (Corliss *et al.*, 1981; Shock, 1996; Shock *et al.*, 1998; Shock and Schulte, 1995).

Russell and co-workers (Russell and Arndt, 2005; Russell and Hall, 1997; 2002; Russell *et al.*, 2005; Russell *et al.*, 1988; Russell *et al.*, 1990; Russell and Martin, 2004; Russell and Turner, 1989) produced an alternative FeS-world hypothesis in which life emerges within a hydrothermally precipitated mound of FeS and other similar minerals formed at the interface of sulphide-bearing submarine seepage and iron-bearing oceanic water. Instead of a 2-dimensional surface reaction they propose that the substructure of the mound contained microcavities which acted as primitive cellular membranes in which organic synthesis and development occurred. The energy source for these reactions is not oxidation of the mineral but the presence of an electrochemical gradient across it.

The precipitate initially formed from the meeting of sulphidic vent solution with iron-rich oceanic water at the low temperatures where nucleic acid polymers are

more stable, is not stable pyrite (FeS_2), or pyrrhotite ($Fe_{1-x}S$). Rather it is an iron(II) monosulphide (FeS_m). FeS_m is a nanoparticulate material with a tetragonal structure similar to mackinawite (Evans *et al.*, 1964; Ohfuji and Rickard, 2006; Rickard, 2006). I use the convention that FeS refers to any undefined iron(II) monosulphide and subscripts such as FeS_m refers specifically to mackinawite

In both FeS-world theories the proto-organism is thought to emerge from the mineral only when developed into fully free-living cellular organisms with DNA replication and DNA-protein translation/transcription. The minerals are thought to enhance DNA evolution by adsorbing nucleotides. However, there is good reason to believe that the chemical environment at these hydrothermal sites would have been highly detrimental to the evolution of nucleic acids and other biopolymers.

DNA and RNA are polymers of nucleotides. A single nucleotide consists of: a nucleobase, a ribose sugar and a phosphate. Polymerisation is achieved through a phosphodiester bond between the phosphate group of one monomer and the ribose of another. Mechanisms of DNA damage have been extensively studied in biomedical research as it leads to carcinogenesis and mutagenesis (Kawanishi *et al.*, 2001; Kawanishi *et al.*, 2002; Rodriguez *et al.*, 1997; Balasubramian *et al.*, 1998). Redox active metal ions, particularly copper and iron, are capable of reacting with oxygen (Cu(I) and Fe(II)), reducing agents (Cu(II) and Fe(III)) and hydrogen peroxide (both oxidation states) to produce oxidative free radicals, notably hydroxyl radicals ($OH\cdot$) which react with DNA and RNA at a number of locations which can cause breaking of the phosphodiester bond (Oikawa and Kawanishi, 1998; Rodriguez *et al.*, 1997; Rozenberg-Arska *et al.*, 1985; Toyokuni and Sagripanti, 1992; Toyokuni and Sagripanti, 1993). Pyrite (FeS_2) can also produce $OH\cdot$ radicals at surface defect sites (Cohn *et al.*, 2003; Cohn *et al.*, 2006).

Metal ions also bind to DNA molecules, especially iron (Oikawa and Kawanishi, 1998; Yaffee *et al.*, 1996), copper (Frelon *et al.*, 2003) and zinc (Berg, 1990; Johnston, 1987). The binding of metals to DNA can affect the molecule's shape, biology, chemistry and physical properties (Duguid *et al.*, 1993).

Precipitation of iron and sulphide produces nanoparticulate FeS_m which could potentially react with nucleic acids because of its highly reactive surface. The reaction also produces aqueous FeS clusters with the formula Fe_xS_x ($x = 2 - 4$) (Luther and Rickard, 2005). These clusters are thought to be highly reactive and could potentially react with nucleic acids.

Hydrothermal vents are submarine fissures in the Earth's surface where geothermally heated water is vented into the ocean. They were first discovered in 1977 (Corliss *et al.*, 1979) along with a vast community of animal and bacterial species. They are an environment rich in reducing chemicals where extremely hot water containing hydrogen sulphide, iron sulphides, hydrogen, methane and trace metals are emitted from a crevice in the sea floor. The ecosystems at modern vents are rooted in chemosynthetic bacteria in contrast to photosynthetic organisms in most other ecosystems. This geochemical energy also has potential to fuel the reactions leading to the origin of life.

It has been suggested that the original truly replicating cells probably contained approximately twenty or so elements all of which are available at submarine hot springs (Da Silva and Williams, 1991). Organic compounds would be produced at hydrothermal vents through the reduction of carbon dioxide (Corliss *et al.*, 1981; Ferris, 1992; Hall, 1986; Shock, 1992; 1996; Shock and Schulte, 1995).

Due to changes in the Earth's geochemistry the dramatic black smokers that exist today would probably have been absent. Russell's hypothesis originally placed

the emergence of life at an interface between hydrothermal (150°C), extremely reduced, alkaline, bi-sulphide bearing submarine seepage water and acidic, relatively cooler (90°) iron bearing Hadean ocean (Russell and Hall, 1997). However, in Russell and Martin (2002) these temperatures are re-evaluated with 60°C stated for the ocean and 70-100°C for the exhalate. They claim that the actual temperatures are of minor importance provided the exhalate is hotter than the oceanic water.

2. Project Aims

The FeS-world hypotheses postulate the emergence of an autotrophic metaboliser and its subsequent evolution into a well developed cellular organism whilst still restrained to an iron sulphide mineral. Whilst reviews of these hypotheses have been written (Cody, 2004) and specific issues have been raised against them (Bada and Lazcano, 2002; De Duve and Miller, 1991; Orgel, 2000), these have mostly been concerned with the earliest stages of the hypothesised sequences such as carbon fixation and emergence of primitive metabolic pathways and they are yet to receive a comprehensive critical review. As they are metabolism-first hypotheses the emergence of the nucleic acid-protein system and lipid membranes have received less attention.

There remain many claims and aspects of the FeS-world hypotheses which have not been fully considered or tested. Both hypotheses suggest particular interactions between organic chemicals and FeS-minerals which have not been demonstrated, such as the adsorption of different organic molecules onto the mineral surface. More experimental work has been conducted with pyrite (FeS_2) than with the iron(II) monosulphide which is proposed to be the principal component of the

inorganic substructure in the theories of Russell and co workers. The emergence and survival of biopolymers in this potentially harsh chemical environment has not been tested.

This project aims to examine the validity of the FeS-world hypotheses. In Chapter 2 the proposed sequence of events from carbon fixation on the mineral up to the development of cellular life capable of escaping from the mineral substrate is critically reviewed. This project is mostly concerned with FeS (Chapter 5 & 8). However, it also examines the interaction of DNA with: Fe(II) and Fe(III) in non-sulphidic solutions (Chapter 3), aqueous sulphide (Chapter 4), Cu(II) and Zn(II) in sulphidic and non-sulphidic solutions (Chapter 6). These were conducted, in the case of Fe(II) and aqueous sulphide, as controls for the FeS reaction and, in all cases, because they can provide additional information on the FeS reaction and because they are all chemicals that DNA would have been exposed to during its development in a hydrothermal environment.

Biomedical research has previously examined the DNA damage by iron and to a much lesser extent sulphide and iron sulphide. Their findings are of some, but limited, relevance to prebiotic scenarios as they have focussed on physiological, rather than natural environmental, conditions. Inversely, I am aware that the results of this research may have implications for biomedical research (e.g. Chapter 4) but this is not the focus of this thesis. Likewise, the environmental and biochemical implications of the research are touched upon (e.g. Chapter 7) but the results are not discussed in detail in this context.

3. Explanation of experimental approach

Electrophoresis is used to separate DNA molecules. Negatively charged phosphate groups cause the DNA to migrate through an aqueous gel towards a positive electrode. For linear DNA molecules the migration rate is inversely proportional to the molecular weight of the DNA. A fluorescent dye in the gel allows the DNA to be visualised under UV light, standards can be used to quantify the amount and molecular weight of the separated DNA.

Plasmid DNA (pDNA) electrophoresis is a commonly used technique for determining if a particular reactant causes scission of DNA molecules (Oikawa and Kawanishi, 1998; Sagripanti, 1999; Toyokuni and Sagripanti, 1992; Toyokuni and Sagripanti, 1993; Yaffee *et al.*, 1996). Plasmid DNA is circular and in its natural state is super-coiled. Scission of the pDNA backbone causes it to unwind into a topology which, although it has the same mass and charge, migrates through the electrophoretic gel more slowly than the super-coiled molecule. This results in a band of relaxed pDNA appearing above the super-coiled pDNA band. The amount of relaxed pDNA compared to super-coiled indicates the extent of the reaction. Extraction of plasmid DNA results in a small fraction of it being converted to the relaxed form, positive controls are used to establish the proportion of relaxed pDNA in the sample. With the exception that plasmid DNA is circular and thus has no terminal positions, the chemistry of pDNA is assumed to be the same as linear DNA. As well as its analytical usefulness pDNA has also been proposed to be an early development in the FeS-world (Wächtershäuser, 2006).

A technique involving centrifugation followed by UV-Vis spectroscopy was used for assaying the extent of adsorption of nucleic acids onto minerals. Centrifugation removes the solid mineral from the solution along with any adsorbed nucleic acids. The supernatant was then removed and analysed with UV-Vis absorption spectroscopy (260 nm) to quantify the nucleic acids remaining in solution.

The physical and chemical conditions of the Hadean world are not well constrained (Chapter 2). This project aims to examine the interactions between metal ions and their sulphides and DNA at room temperature and pressure and at the optimal pH for DNA stability (~8). The effects of temperature, pressure and pH can be considered if the mechanisms are determined. A range of prebiotically feasible sulphide concentrations are used.

One possible reason why FeS has received less attention than pyrite in studies into the FeS-world origin of life hypothesis is the difficulty of working with it. Iron(II) monosulphide is much more soluble than pyrite resulting in a high concentration of aqueous Fe^{2+} and HS^- which can interfere with many analytical techniques. Many spectroscopic techniques which are used to examine interactions have proven to be difficult with FeS. Due to the adsorption of nucleic acids onto FeS (Chapter 7) the products of the reaction between FeS and DNA are difficult to remove and analyse. Fe(II) is sensitive to oxidation as well, in sulphidic systems the sulphide is more prone to oxidation and protects Fe(II) from oxidation to some extent (Wolthers *et al.*, 2005). However, it is still necessary to store the solutions and conduct experiments in an anoxic chamber and to use deoxygenated water.

4. Thesis Summary

Chapter 1: Introduction.

Background information and project aims.

Chapter 2: Critical Review of the FeS-World hypothesis.

A review and critique of the proposed sequence of development from inorganic chemicals to free-living cells in both of the FeS-world hypotheses.

Chapter 3: An Electrophoretic Study on the Effects of Iron (II) and Iron (III) on DNA.

An investigation into whether iron ions in prebiotic environments would have affected the emergence of nucleic acid polymers.

Chapter 4: An Electrophoretic Study on the Effects of Sulphide on DNA.

An investigation into whether sulphide ions in prebiotic environments would have affected the emergence of nucleic acid polymers.

Chapter 5: FeS-Induced Radical Formation and its Effect on DNA.

An investigation into iron sulphide promoted free radical production and consideration of the effect this would have on the emergence of nucleic acid polymers in a prebiotic environment.

Chapter 6: The Effect of Transition Metal Redox on DNA in Sulphidic Systems.

Copper and zinc ions in sulphidic and non-sulphidic solutions are investigated to determine the effects of oxidation and reduction in metal-sulphide induced DNA damage.

Chapter 7: Nucleic acids bind to nanoparticulate transition metal sulphides in aqueous solutions.

An investigation into the binding of a variety of nucleic acids with precipitated iron sulphide nanoparticles.

Chapter 2

The FeS-world origin of life hypothesis: A critical review

1. Introduction

Gunter Wächtershäuser and his co-workers in a series of papers (Wächtershäuser, 1990; 1988; 1992; 1994; 1998; 2003; 2006; 1997; Huber *et al.*, 2003; Huber and Wächtershäuser, 1997; 1998) envisag ed the emergence of a 2-dimensional, proto-organism in a hot, volcanic, submarine location bound to an FeS/pyrite mineral assemblage which grows autotrophically fuelled by the oxidative conversion of FeS to pyrite. This is referred to, throughout this review, as Wächtershäuser's hypothesis.

The second FeS-world hypothesis which is reviewed is the "Russell hypothesis" and is due to Russell and his co-authors (Martin and Russell, 2003; Russell and Arndt, 2005; Russell and Hall, 1997; 2002; Russell *et al.*, 2005; Russell *et al.*, 1988; Russell *et al.*, 1990; Russell and Martin, 2004; Russell and Turner, 1989) who place the origin of life at the interface between hot, reduced, alkaline, sulphide-bearing submarine seepage sites and the cooler, acidic iron-bearing ocean. These fluids are prevented from equilibrating with each other due to the continuous precipitation of an iron sulphide "membrane" between the two. This produces a disequilibrium in pH and Eh which is compared to the potential across a living cell and, as it is in extant life, can be a source of energy for organic synthesis. Additionally, it is postulated that the iron sulphide membrane would consist of cell-like microcavities in which organic chemicals could be sequestered and concentrated. This hypothesis combines the mineral surface theory and the prebiotic broth theory; detached constituents remain in the cavities and are available for further reaction whilst in Wächtershäuser's hypothesis detached constituents are irretrievably lost.

Both of these hypotheses postulate the emergence of an autotrophic metaboliser and its subsequent evolution into a well developed cellular organism whilst still restrained to an iron sulphide mineral. Whilst reviews of this hypothesis have been written (Cody, 2004) and specific issues have been raised against it (Bada and Lazcano, 2002; De Duve and Miller, 1991; Orgel, 2000), a comprehensive critical review is yet to be written. The purpose of this review is to consider the validity of the hypothetical evolution from inorganic chemicals to a free living organism in this locale.

2. Hydrothermal sulphide minerals

The three sulphide minerals which feature most in the FeS-world hypotheses are mackinawite, greigite and pyrite. The precipitation of iron and sulphide produces stoichiometric (Rickard, 2006), nanaoparticulate (Ohfuji and Rickard, 2006) iron (II) monosulphide with the tetragonal structure of mackinawite (FeS_m) (Berner, 1962) in which Fe(II) is linked to four equidistant sulphur atoms (Rickard and Morse, 2005). Mackinawite is soluble in mineral acids, prone to oxidation, metastable and has semi-conductor properties.

Pyrite is an iron (II) disulphide with a cubic structure. It is produced from mackinawite oxidation with sulphide as the electron acceptor producing bisulphide (S_2^{2-}). This transition is not solid-state but involves dissolved [FeS] intermediates (Rickard and Luther, 1997). The formation of these aqueous FeS species, and therefore that of pyrite as well, is inhibited by the presence of aldehydic carbonyl groups (Rickard *et al.*, 2001). The importance of this reaction is that it demonstrates

that FeS species, in particular FeS_{aq} clusters, react with common organic moieties. Pyrite is not readily soluble at any pH, is stable and is also a semi-conductor.

Greigite is the thiospinel of iron (Vaughan and Craig, 1978) with an inverse spinel structure. It is formed from the oxidation of two-thirds of the Fe(II) in mackinawite and has the formula $Fe^{II}Fe^{III}_2S_4$. Russell *et al.* (2005) claims the formula of greigite is Ni-S_2-[Fe_4S_4]-S_2-Fe and references Vaughan and Craig (1978) in support of this. However, Vaughan and Craig (1978) give the formula of greigite as Fe_3S_4 in agreement with Krupp (1994). The confusion probably stems from Russell's use of the term *greigite* to describe nickeliferous iron thiospinels. Vaughan and Craig (1985) recommend that all nickeliferous iron thiospinels are referred to as *violarite*, with reference to the ideal end-member violarite composition, $Fe^{II}Ni^{III}_2S_4$. Vaughan and Craig (1985) report that there appears to be a complete solid solution between end-member Fe_3S_4 and Ni_3S_4 although this is restricted synthetically. They note that natural nickeliferous iron thiospinels appear to be metastable products of the oxidation of pentlandite, $(Fe,Ni)_9S_8$, the most common terrestrial nickel sulphide mineral. Pentlandite itself is usually formed from immiscible sulphide-silicate melts under normal mantle and crustal conditions. Pentlandites have been identified in serpentinites and gabbros on the Mid- Atlantic Ridge at 30°N associated with the Lost City hydrothermal vent field serpentinites and gabbros (Delacour *et al.*, 2005). Cobaltian pentlandite has been reported in deep ocean vent sulphides (Mozgova *et al.*, 1996). Thus oxidation products, such as violarites, might be expected to form in these systems although, as far as we know, such occurences have not yet been reported.

Huber and Wächtershäuser (1997) sulphidised a solution of Fe^{2+} and Ni^{2+} with Na_2S for use in their experiments. Huber and Wächtershäuser (1997) did not characterise their precipitate. However, current work in the Rickard laboratory shows

that this synthesis produces a mixture of FeS and NiS with no Fe-Ni bonds, and not a mixed (Fe,Ni)S phase. Although the composition and structure of the FeS is well established, as noted above, the structure and composition of the NiS component has not been determined.

It is important to note, therefore, that griegite is a pure iron sulphide and is formed through the solid state oxidation of mackinawite. It is therefore relatively common in modern sulphidic environments (Rickard and Morse, 2005). It is stable relative to mackinawite and could therefore be expected to be found in Hadean systems. By contrast, the nickeliferous varieties, are violarites which are formed by the oxidation of pentlandites. Although probably present in modern systems, their distribution is limited, and their formation in a low-oxygen Hadean system appears to be less probable.

3. Carbon fixation and metabolism in an FeS-world

The initial step in any origin of life scenario must be the fixation of carbon. In the Hadean carbon probably existed mostly as carbon monoxide and carbon dioxide gasses in the atmosphere and ocean. Carbon fixation refers to the reduction in the oxidation state of carbon atoms and the formation of carbon-carbon bonds (Reaction 1). This reaction is not thermodynamically favourable and therefore, it requires a source of energy.

$$nCO_2 + nH_2 \rightarrow -(CHO)n- + OH \quad \text{.....(1)}$$

The prebiotic broth theory asserts and requires that the atmosphere (or a region of it) was conducive to the fixation of carbon; Wächtershäuser and Russell both challenge this assumption and offer alternative theories for the emergence of organic chemistry.

3.1. Pyrite-pulled carbon fixation

Wächtershäuser's initial hypothesis (Wächtershäuser, 1990; 1988) involves the reduction of CO_2 to small organic molecules particularly thiolated methanoic (formic) acid $HSCH_2OOH$. The source of reducing power for this is the oxidation of a submarine hydrothermally produced mackinawite to pyrite (reaction 2);

$$FeS + H_2S \rightarrow FeS_2 + 2e^- + 2H^+ \quad \text{..... (2)}$$

The electrons on the right hand side come from the S^{2-} ions in FeS. Two S^{2-} are oxidised forming the S_2^{2-} ion in pyrite. The reaction would, it is suggested, couple to CO_2 reduction;

$$CO_2 + H_2 \rightarrow HCOOH \quad \text{..... (3)}$$

With the overall reaction system being;

$$FeS + H_2S + CO_2 \rightarrow FeS_2 + HCOOH \quad (4)$$

Wächtershäuser (1990) calculated that reaction 4 would be thermodynamically favourable as reaction 2 is sufficiently exergenic to overcome the unfavourable thermodynamics of reaction 3. However, his calculations have been criticised as being

limited to standard state conditions rather than more feasible prebiotic or hydrothermal conditions and by using the standard free energies of the reactants rather than the change in free energy of the reaction (Shock and Schulte, 1995; Shock, 1990) and for not considering kinetic barriers in the reaction mechanism.

Schoonen *et al.* (1999) experimentally tested Wächtershäuser's claims on the thermodynamics of this reaction and showed that Wächtershäuser was correct in his assertion that reaction 4 would be exergenic at standard state. However, they also found that the reducing power of reaction 2 is dependent on the activity of H_2S/HS^-, the pH and that it decreases with increasing temperature. These findings have implications for Wächtershäuser's hypothesis and constrain the environment in which it could occur. The influence of both hydrothermal temperatures and H_2S on the origin of life is discussed below.

Another issue is that Wächtershäuser's hypothesis involves a direct electron transfer from the valence band of FeS to the lowest unoccupied molecular orbital of CO_2. Schoonen *et al.* (1999) found that this transfer is severely kinetically inhibited and thus cannot occur. This is a considerable blow to the theory, as the reduction of CO_2 to methanoic acid is the first step in Wächtershäuser's mechanism, without primary carbon fixation no further reactions can occur. Although Wächtershäuser's mechanism may be unfeasible it has been shown that CO_2 fixation can be promoted by reaction 2 and by other minerals found in hydrothermal exhalative systems.

Wächtershäuser's hypothesised "fuel" is the oxidation of FeS to pyrite. However, pyrite is not formed through a solid state transition from FeS but rather FeS dissolves supplying iron and sulphur species from which pyrite can form (Rickard, 2006). Pyrite formation is inhibited by trace amounts of aldehydic carbonyl by

Rickard and Butler (2001) who suggested the same would be true for any organic with an aldehyde group.

3.2 Fischer-Tropsch synthesis

Heinen and Lauwers (1996) found that a $FeS+CO_2+H_2S$ system did indeed produce organic molecules and pyrite. The molecules produced were small (1-5 C) alkane thiols not carboxylic acids. They also detected small amounts of CS_2 and COS which were expected to be a reaction intermediate. Cody (2004) discusses these findings.

Cody (2004) argues that the alkane thiols observed in Heinen and Lauwers (1996) are the products of a Fischer-Tropsch type reaction involving pyrite-bound CO and H_2. The Fischer-Tropsch reaction is

$$(2n+1)H_2 + nCO \rightarrow C_nH_{2n+2} + nH_2O \quad \text{.....(5)}$$

and is catalysed by iron and/or cobalt compounds. The reduction of CO_2 is speculated to be the source of CO and it is this reaction which is coupled to the pyrite forming reaction rather than the synthesis of organics. Zinc sulphide which is also found in hydrothermal systems can promote the reduction CO_2 to CO (Kanemoto *et al.*, 1992). The atmosphere may have been a source of CO. However, the concentration and fate of CO in the early ocean is uncertain (Kasting, 1990). Kanemoto *et al.* (1992) demonstrated ZnS-induced the reaction

$$CO_2 + H_2 \rightarrow HCOOH \quad \text{.....(6)}$$

in which CO may have been an intermediate.

An alternative mechanism for the production of the thiols by Heinen (1996) involves the conversion of CO_2 to COS or CS_2 as a first step. This process involves no change to the oxidation state of the carbon but COS and CS_2 more readily accept electrons than CO_2 does (Luther, 2004).

3.3. Formation of the first carbon-carbon bonds: ethanoic acid synthesis

The first organic molecules would contain only single reduced carbon atoms (e.g. methanoic acid, methane thiol etc.). The next step is for additional carbon atoms to be inserted into these molecules, enabling them to extend and form new structures. Huber and Wächtershäuser (1997) did this using methane thiol (the most abundant thiol produced in the Heinen and Lauwers (1996) experiments) and carbon monoxide to synthesise ethanoic acid (acetate) catalysed by sulphide precipitates (Reaction 7).

$$CH_3SH + CO + H_2O \rightarrow CH_3COOH + H_2S \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (7)$$

They found that a co-precipitated iron and nickel sulphide was able to promote reaction 7 with a high yield but only in neutral or mildly acidic pH. The precipitate was formed from iron and nickel sulphates sulphidized with Na_2S. No attempt was made to characterise the precipitate through XRD or any other method and, as discussed above, such a reaction does not produce mixed (Fe,Ni)S phases. Rather the product was probably an unspecified mixture of mackinawite and an unknown nickel sulphide. Huber and Wächtershäuser (1997) reported that the unknown NiS on its own was able to promote ethanoic acid production with a lower yield but over a more acidic pH range. It should be noted that many enzymes contain

nickel as well as iron-sulphur clusters including CoA synthase. Ethanoate attaches strongly to pyrite, enhances the adsorption of ATP onto pyrite (Tessis and Vieyra, 1996) and is an intermediate in extant fatty acid synthesis.

3.4. Origins of metabolism

There are two fundamental processes which all living organisms engage in. One is the replication of information stored as bio-polymers and the other is metabolism: the decomposition and recomposition of organic chemicals with associated release or expenditure of energy. Another division in theories of the processes involved in the origin of life has been over which of the two phenomena arose first. The RNA world hypothesis is a "genes first" hypothesis whilst the FeS-world hypotheses assert that metabolism was the first aspect of life to evolve. The "genes first" hypothesis has emphasised the role of natural selection in improving the replicators efficiency whilst the metaboliser first hypotheses which are inherently more chemically based tend to focus less on selection. This is understandable as natural selection is traditionally thought of as operating on informational biopolymers (De Duve, 2005).

3.5. Pyrite-pulled metabolism

Wächtershäuser's initial 'organism' consists of a pyrite/FeS surface on which a cycle similar to the reductive citric acid cycle (RCC), fuelled by the oxidative production of pyrite, is occurring. The reductive citric acid cycle (Fig. 1) is a means by which carbon dioxide is fixed in some autotrophic prokaryotes. It is a pathway which produces very simple organic molecules which are the starting points for lipid, amino acid and sugar synthesis. Whereas the extant RCC (Fig. 1) has a suite of

enzymes, co-factors and ATP controlling, promoting and fuelling the reduction, Wächtershäuser's thio-analogue of the RCC (Fig. 2) proposes only the conversion of FeS to pyrite facilitating the cycle. Cody (2004) has provided an excellent summary and analysis of the four different types of reaction in Wächtershäuser's proposed RCC-analogue. Some of these reactions such as the formation of thioketals, dithioketals from keto carbonyls are not pyrite pulled (Fig. 2) but do require a high activity of hydrogen sulphide. For some reactions such as reductive carboxylations the thermodynamics are highly unfavourable and must be coupled to pyrite formation to occur (Fig. 2).

Russell and Hall (1997) also invoke the RCC as a primitive carbon-fixation mechanism. However, in later versions of the theory (Russell and Arndt, 2005; Russell and Hall, 2002; Russell *et al.*, 2005; Russell and Martin, 2004) this has been replaced with a linear carbon-fixation pathway.

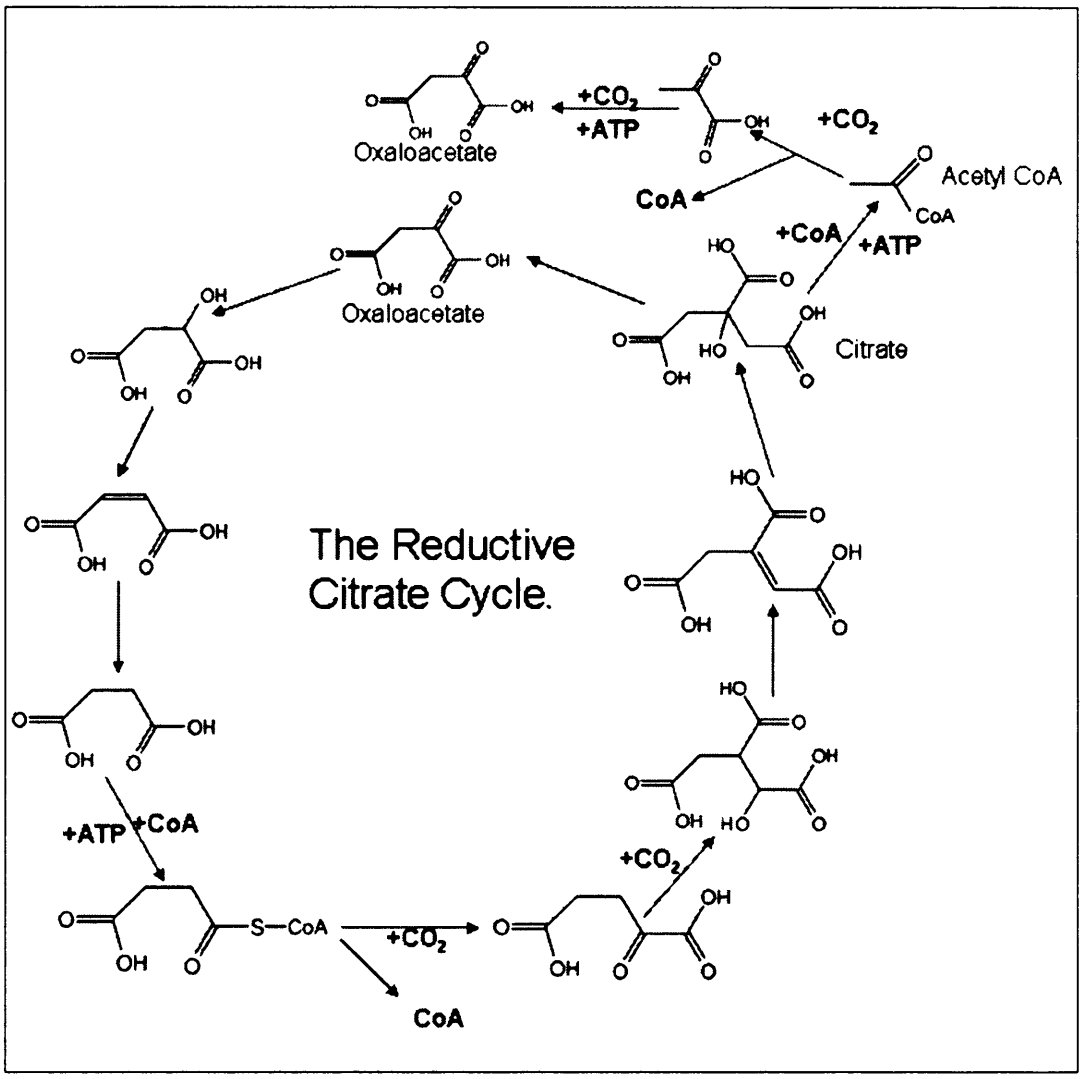


Figure 1: Schematic diagram of the extant reductive (or reverse) citrate cycle (RCC) showing the stages at which CO_2 is fixed and when the reaction is fuelled by adenosine triphosphate hydrolysis (+ATP). CoA refers to the coenzyme acetyl CoA.

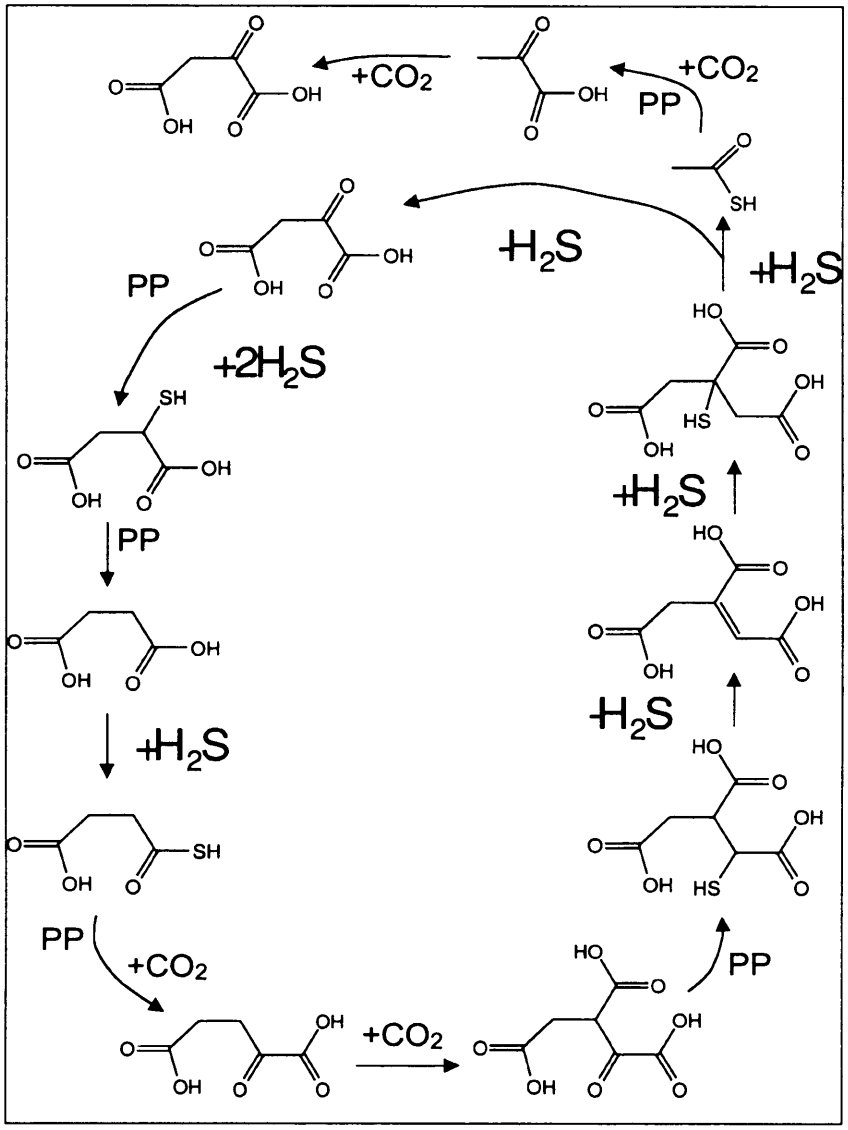


Figure 2: Schematic diagram of Wachtershauser (1988; 1990; 1992) pyrite-pulled thio-analogue of the reductive citric acid cycle, showing the stages at which CO_2 is fixed, where H_2S is involved and where the reaction is pulled by oxidative pyrite formation (PP). Modified from Cody (2004).

3.6. Autocatalytic carbon cycles: fidelity of replication

The RCC is an autocatalytic cycle in which each turn of the cycle produces two molecules of oxaloacetate which can initiate another cycle (Fig. 2). This fact has been used to argue that the RCC or something similar, once initiated, would "dominate the chemical landscape of the prebiotic environment" (Cody, 2004). However, this is only true if the cycle is robust. That is, if every stage in the reaction occurs with (or very near) to 100% conversion from reactant to product. If the conversion is on average 95% (generous estimate, higher than experimental studies would suggest) for each of the 11 steps then each turn of the cycle will produce 1.14 oxaloacetate molecules not 2. At 90% conversion this figure drops to 0.63, at a less generous conversion e.g. 40%, the turnover is 8.4×10^{-5}. In extant biological systems the efficacy of the RCC or any other metabolic cycle is ensured by specific protein catalysts for each step, sometimes organised into multi-enzyme complexes and by having precisely controlled physiological conditions and membranes to prevent diffusion of the substrates.

For each stage in the reaction there is a probability that the molecule will react in the proscribed way and continue the cycle. This will not be the only possible fate for the molecule since it may detach from the surface, it may react in a dead-end manner or it may react in a beneficial novel manner. The RCC or any other autocatalytic carbon cycle will not come to dominate the chemical landscape of the prebiotic world unless the system is highly robust. It will, instead, rapidly collapse.

Orgel (2000) questions the plausibility of the self-organization of the RCC on a mineral surface. He highlights the difficulties faced by synthetic organic chemists in developing very simple autocatalytic reaction cycles. This field is years away from producing a system comparable to the RCC in a "single-pot" reaction system. Orgel

(2000) criticises the concept of non-enzymatic metabolism, particularly in regards to the Wächtershäuser hypothesis. There is no reason to believe that any of the intermediates of the RCC would be catalytic for any of the steps in the RCC or that any single mineral would catalyse these steps producing intermediates in the correct position and orientation to become the substrate of the next reaction.

3.7. Carbon fixation and metabolism in FeS compartments

In Russell's (Russell and Arndt, 2005; Russell and Hall, 1997; 2002; Russell *et al.*, 2005; Russell *et al.*, 1988; Russell *et al.*, 1990; Russell and Martin, 2004; Russell and Turner, 1989) hypothesis, gaseous carbon oxides are also reduced on the surface of an FeS mineral - the internal surfaces of a hydrothermally precipitated 3D FeS membrane. Contrary to Wächtershäuser's theory it is not the oxidation of FeS which provides an energy source but rather pH and Eh gradients across the membrane. The emergence of life, in this theory, is the result of the tendency for a disordered state to migrate towards equilibrium in a scenario in which equilibrium is not possible. The semi-conductive nature of the FeS membrane allows it to act as an electron transfer chain from the exterior of the membrane to the interior driven by a pH gradient between a proposed acidic ocean and an alkaline hydrothermal exhalation. Photolytic ferric iron flocculants aggregated at the mound's exterior acted as a positive electrode Fe(III) accepted electrons ultimately produced by hydrothermal H_2. Filtness *et al.* (2003) demonstrated that FeS precipitated between iron and sulphide containing gels with contrasting pH could maintain the pH gradient. However, the FeS did not act as a membrane but as an active barrier, although a potential of the right order could be produced.

Cody (2004), Russell and Martin (2004) and Martin and Russell (2003) consider the acetyl Co-A pathway to be more appropriate to an FeS driven prebiotic carbon fixation mechanism. The acetyl Co-A pathway is used by acetogenic and methanogenic archaea for carbon fixation. Its inputs are H_2 and CO_2 and it produces a C2 thioester (Reaction 8).

$$4H_2 + 2CO_2 + HSCoA \rightarrow CH_3COSCoA + 3H_2O \quad (8)$$

This is a linear pathway as opposed to the RCC. The acetyl CoA pathway involves simpler intermediates than the RCC which are surface (enzyme) bound in extant pathways. Carbon monoxide dehydrogenase (CODH) is the central enzyme in this system which has (Ni, Fe)S clusters to which CO and methyl bind to and subsequently react.

Cody (2004) bases this preference on the experimental data recorded on metal sulphide facilitated carbon fixation. Russell and Martin (2004) support their claim with comparisons between Fe, Ni sulphide clusters in the enzymes of the extant acetyl Co-A pathway with structures similar to that of the iron thiospinel, greigite. However, as discussed above, their nickeliferous "greigites" are in fact violarites. Russell and Martin (2004) suggest that the sub-units of (Ni,Fe)S thiospinels such as violarites would be capable of fulfilling the catalytic role of CO dehydrogenase. Additionally, they cite the work of Shock (Shock, 1992; 1996; Shock and Schulte, 1995; Shock *et al.*, 1998), concerning hydrothermal synthesis, who calculated that carbon would be mostly in the form of acetate when hydrothermal fluids are mixed with anoxygenic seawater.

The reported occurrence of pentlandites and thus the possible formation of violarites in the Lost City hydrothermal vent field serpentinites and gabbros (Delacour *et al.*, 2005) mentioned above, is particularly interesting in context of the Russell hypothesis. The Lost City hydrothermal vent field is unusual in that the vents are carbonate-dominated, rather than sulphide, and associated serpentinisation is a process generally associated with the formation of strongly alkaline fluids. The interplay between alkaline fluids and acidic ocean water figures centrally in Russell's mechanism for developing the potential gradient necessary to initiate prebiotic organic syntheses.

In the later versions of the hypothesis (Russell *et al.*, 2005; Russell and Hall, 2006; Russell and Martin, 2004) the hydrothermal mound is described as an acetate generator with most of the acetate lost to the ocean, some of it does remain and is the source for all other organic molecules. They argue that acetate production is a thermodynamically favourable reaction whose excess energy could be used to drive the synthesis of other organic molecules.

3.8. Conclusion of carbon-fixation and proto-metabolism

Wächtershäuser's original mechanism involving direct electron transfer from CO_2 to H_2 is probably unfeasible due to kinetic reasons. However, carbon dioxide reduction to organic chemicals has been demonstrated to occur either catalysed by iron (nickel) sulphides or fuelled by FeS oxidation to pyrite (Heinen and Lauwers, 1996) to determine which of these two mechanisms is responsible the experiment could be repeated in presence of formaldehyde. Either way, high temperatures are probably required.

The acetyl Co-A pathway is a simpler carbon fixing pathway than the RCC which may involve too many intermediates to remain stable over time. Although, the acetyl Co-A pathway is not cyclic and most of the acetate/ethanoate produced would be wasted. It has been demonstrated that from thiolated ethanoate C2 structures can be formed however, experimental evidence that larger biomolecules can be produced from this which would be retained and engage in cyclic reactions that could lead to nucleic acids, peptides and lipids is lacking.

4. Evolution from an autocatalytic cycle

Proponents of the "genes first" hypothesis deny that replication and therefore inheritance, selection and evolution could have occurred prior to the emergence of nucleic acid replication. De Duve (2005) states "I shall assume, in agreement with most researchers in this field that the first replicable molecules consisted of RNA." This is an unfortunate assumption and one with which not all researchers would agree. The chemoautotrophic origin of life scenarios posit biogeochemical feedback cycles such as the RCC which involve the replication of organic chemicals which are much simpler than nucleic acids but involve a more convoluted replication. It is envisaged that novel reactions would occur which produce new cycles and reactants. These cycles compete with each other for inputs, territory and fuel. As with any system in which replicators have an inheritable, variable capacity for replication there is the potential for natural selection.

De Duve (2005) points out that the origin of replicating RNA is still unknown. The existence of strands of RNA capable of replicating each other is a significant and difficult advancement which must have involved multiple steps.

Selection of simpler organic chemicals should not be ruled out as a mechanism in the production of the first nucleic acids.

As compartmentalisation is a necessity for selection, De Duve (2005) agrees with Russell that cellularisation must have occurred early in the development of life, prior to the inception of protein and lipid biosynthesis. Therefore, the compartments are said to have formed from abiotically produced lipids which self-assemble into micelles. However, if selection is to play a part at the earliest stages of prebiotic chemistry then a pre-existing, abiotically produced series of compartments becomes a very attractive feature of Russell's FeS-world hypothesis.

As can be seen above, aspects of the iron-sulphur world hypothesis have been experimentally tested and expanded upon. Mostly this has focussed on the earliest stages: carbon fixation, primitive metabolic cycles and polymerisation reactions. Less consideration has been given to the stages after this: the emergence of biopolymers, reproduction, natural selection, cellularisation and the other characterisitics of extant organisms. If an autocatalytic, metabolic replicative cycle can become established in or on a mineral the next step is for it to evolve. For this to occur it is necessary that novel chemical constituents and reaction pathways emerge which are preserved and preferably selected for in some manner. These novelties will be beneficial to the system if they facilitate a step in the replication cycle.

Wächtershäuser (1992) hypothesises on the origin of selection not within a compartment but bound on a 2-D surface. The first selective pressure is the binding capacity of a constituent to the mineral surface. The units of selection are the chemical constituents, products and waste of the surface metabolist which he groups into four categories.

1. Products of decay. (low capacity for adsorption)

2. Metabolic intermediates. (low capacity for attachment but higher capacity for incorporation into surface-bonded polyanions.
3. Reactive polyanions e.g. peptides, polynucleotides (high capacity for binding some loss due to depolymerisation)
4. Inert constituents e.g. carboxylic acids, lipids. (high capacity for binding)

Due to their high capacity for binding types 3 and 4 will accumulate on the mineral over time producing a combination of hydrophilic reactive molecules and inert hydrophobic compounds competing on the mineral surface.

This is a vital aspect of Wächtershäuser's "explanatory theory of biochemistry". It explains, he argues, the ubiquity of polyanions (peptides, nucleic acid, most coenzymes) in biochemistry and why sugar metabolism operates with phosphorylated (anionic) sugars. However, polyanions could be said to exist in life because they are the only way in which genetic information can be stored and transferred to catalysts (proteins) and sugar phosphorylation may have evolved to prevent diffusion through lipid membranes. Wächtershäuser gives no reason why the products of decay would readily desorb or why the metabolic intermediates would more readily polymerise than detach except for the seemingly teleological argument that it is necessary for his hypothesis. It also needs to be pointed out that the interaction between pyrite (and presumably other minerals) and organic chemicals is determined more by chemical selectivity than electrostatic interactions (Bebie and Schoonen, 2000).

Novelty in this system arises from the emergence of new organic chemicals and reaction pathways and can be beneficial to the system however, "Most of these novelties are transient and subject to quick removal by decomposition or detachment"

(Wächtershäuser, 1988). As most mutations/novelties in both extant systems and Wächtershäuser's hypothetical evolving metabolist are deleterious the rate of mutation in both systems must be low. An overly high mutation rate will result in population extinction. DNA replication occurs with a relatively low error rate (1:10,000) even *in vitro* and even lower in living cells with DNA repair and proof-reading enzymes. Novelty is rare in extant systems, and must be likewise in Wächtershäuser's.

Wächtershäuser (1988) postulates a surface organism which is "composed of a self-sufficient subset of surface-bonded constituents" with the capacity to grow through uptake of inorganic nutrients and their subsequent reorganisation into similar constituents. "The surface-bonded constituents of the self-sufficient subset are autocatalytic, promoting the production of constituents of their own kind by their organization in reaction cycles", (Wächtershäuser, 1988). Complex, multi-step reaction cycles are invoked as a substitute for template directed replication. The efficacy of this system would be exceedingly low compared to DNA replication, in other words it would be expected to have a very high rate of mutation.

Growth in this system (i.e. carbon fixation) requires the consumption of FeS. Therefore it must be asked if it is feasible that FeS would be available to fuel reorganisations and metabolism of molecules bound to pyrite. Perhaps growth would be restricted to pyrite/FeS boundaries. Selection at this stage will still favour strongly bound constituents. Reaction systems might then cease as the most strongly bound constituent is selected for with the resultant expulsion of the other metabolically active molecules. A novelty will only be preserved if it results in increased binding capacity for the novel constituent.

It is vital to explain how newly emerging features became predominant in their environment and not merely swallowed in the sea of potential reaction pathways. A mechanism must be required which can preserve and propagate the beneficial results of a chemical novelty. Natural selection only occurs only when interacting systems have variable abilities to compete for limited, essential resources and have the capacity to propagate these abilities. To understand molecular evolution in an FeS world it is thus vital to understand;

1. What are the limited resources?
2. How can chemical novelties in the system lead to changes in the system's ability to exploit resources and propagate themselves?
3. What is the mechanism of propagation of these novelties?

In Russell's papers evolution occurs in the system because a purported alteration to the system seems to bring the system closer to life as we know it. However, no mechanism for the maintenance of these novelties is hypothesised. Novelties only become widespread when they enable a replicating species to produce more offspring than its contemporaries.

5. Origin of Cellularisation

All biological cells are bound by a roughly spherical membrane composed of lipids and proteins. The advantages of cellularisation to an emerging biological system cannot be overstated. The evolution of a cellular membrane brings with it the capacity for homeostasis, holds the metabolic constituents in close proximity, acts as a

selective barrier to the external environment and is the site of many important biochemical reactions.

The traditional, prebiotic broth, thinking on the origin of the cellular membrane has been that the accumulation of lipids in a solution led to self-assembly of lipid-bilayers into spheres in which droplets of the prebiotic broth were captured. The FeS world hypothesis denies that this could occur and presents alternatives in which a hydrothermally precipitated mineral plays some role in confining the biochemistry at the earliest stages of the origin of life.

The first step in the transition from mineral to cellular-bound biochemistry is the production of lipids: the major constituent of cellular membranes. The emergence of pathways for the biosynthesis of lipids is stated to be a late development in both FeS-world hypotheses, although it occurs while the proto-organism is still bound to the mineral. Prior to this, abiotically generated lipids may have played a role. Methane thiol has been synthesised in hydrothermal experiments utilising an iron-nickel sulphide catalyst. From methane thiol and carbon monoxide ethanoic acid has been synthesized via the condensation of a thioester (Huber and Wächtershäuser, 1997) which is analogous to acetyl Co-A which is the root of extant lipid synthesis. Progressive elongation of carboxylic acids, if possible, would result in highly hydrophobic long chain fatty acids which are a lipid and constituent of modern cellular membranes. However, carboxylic acids longer than three carbons are yet to be produced in a valid hydrothermal prebiotic scenario.

Fatty acids bind strongly to the FeS/pyrite due to their hydrophobicity. They therefore accumulate and mix with the bound hydrophilic constituents and the surface would become increasingly hydrophobic. The increase in hydrophobicity promotes

polymerisation reactions including fatty acid extension possibly making lipophilisation a positive feed-back phenomenon.

5.1. Wächtershäuser's semi-cellular structures

Wächtershäuser states that the process of FeS/FeS_2 surface lipophilisation (Fig. 3 a, b) occurred simultaneously with the metabolism of hydrophilic molecules as in the RCC. As the surface lipophilisation progressed, the suggestion is that hydrophilic regions became isolated in two dimensions within a sheet of hydrophobic compounds, essentially forming a 2-D analogue of a cell. There is a selective pressure for the elongation of fatty acids on the mineral surface as the longer the lipid the less soluble it is and the more it will adhere to the pyrite. The invention of a hydrophobic membrane would also facilitate polymerisation through condensation reactions.

The phase of transition in Wächtershäuser's (1992; 1988) evolutionary tale between 2-dimensional monolayer organisation and cellular organisation requires a region of the lipid membrane partially detaching from the mineral in a restricted locale. The region detaches due to loss/neutralisation of the ionic foot group through protonation or association with cations. Although detached from the mineral, the membrane remains cohesive with neighbouring lipids which remain bound and a space for a cytosol to develop is created (Fig 3, d). In the most recent paper (Wächtershäuser, 2006), the cytosolic region is stated to have been formed in a small cavity in the mineral (Fig 3, c) which the lipid membrane spans across, leaving a space, rather than line the cavity (Wächtershäuser, 2006).

Uncharged nutrients (CO, H_2S, NH_3) are able to diffuse across the membrane into the cytosol but the charged constituents of metabolism are prevented from diffusing out through the membrane. Another consequence of the evolution of the

cytosol is the neutralization of the pH which would greatly alter the charges on metabolites and affect charge based interactions. The onset of the semi-cellular structures allows for the emergence of a proton gradient due to the difference between the internal pH and the external pH. This gradient can be used to store energy from or for redox reactions. It is during the semi-cellular stage that "multi-component genetic machinery" emerges.

In Wächtershäuser (1988), a scenario is described wherein hydrophobic lipids accumulate on the mineral surface whilst hydrophilic constituents engage in metabolism. These two different systems expand, progress and diverge whilst in competition with each other for binding sites and for energy produced by the oxidation of FeS. The result of the interaction/competition between these two systems has not been experimentally verified and seems to be highly unpredictable. The progression that he describes is an interesting story but it involves several assumptions. The accumulation of hydrophobic lipids is assumed to compartmentalise the metabolic constituents in two dimensions first and then form an over-layer above them, rather than repel them from the mineral.

If lipids have a high capacity for binding, and the potential to increase that capacity through elongation, they may out compete other molecules, such as those involved in the RCC or any other metabolic cycle, and force them to detach from the surface. Although usually employed to describe interactions between extant species, the competitive exclusion principle may be relevant here. It states that two species requiring exactly the same resources (FeS as fuel and pyrite binding sites) cannot exist in the same locality. Wächtershäuser assumes that the lipids will protect other constituents from hydrolysis and protonation.

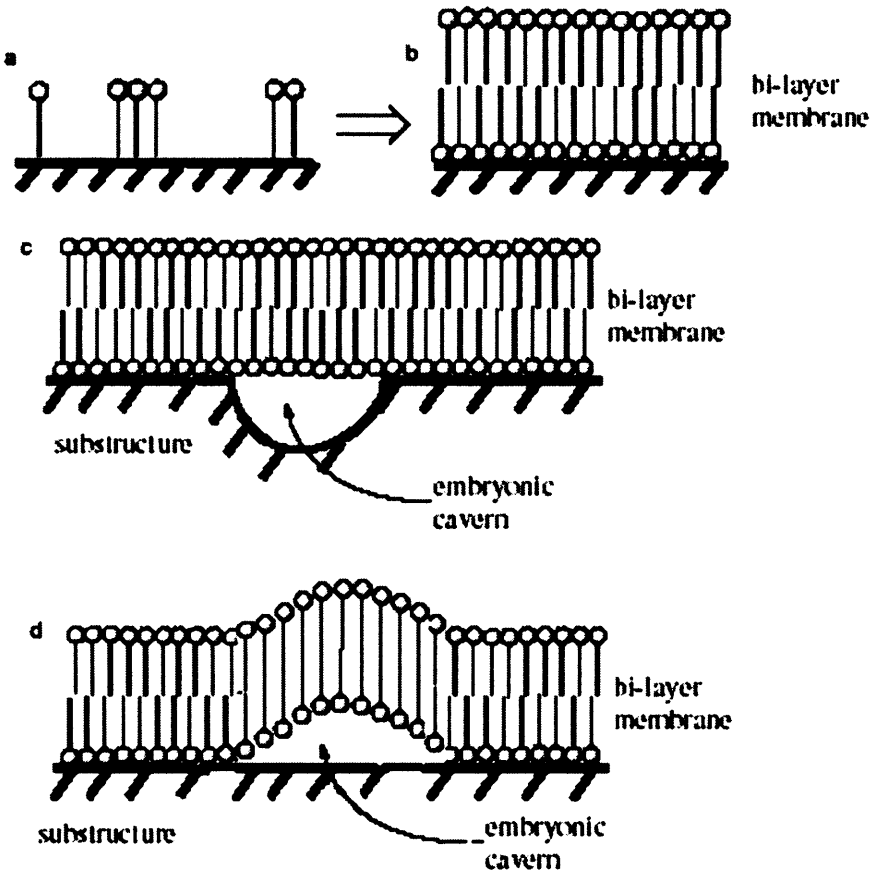


Figure 3: Representation of two hypothetical modes of formation of semi-cells. Lipids accumulate on the surface bound by their aliphatic chains to the mineral substructure (a). After further accumulation a lipid bilayer arises (b). The bilayer either spans a cavity in the mineral (Wächtershäuser, 2006) (c) or partially detaches from the mineral (Wächtershäuser, 1988; 1992)(d). Modified from (Wächtershäuser, 2006).

In order for the semi-cellular state to arise, the lipid membrane must at some point detach, which requires a reduction in its binding capacity. It would be expected that selection for strongly bound molecules would lead to increasing adhesion of the layer of lipids to the mineral. Wächtershäuser does not permit total detachment of the membrane until after the evolution of all the various structures required for cellular life to flourish. The reaction of metal ions and protons with the carboxylic acid head groups of the lipids is stated to be the reason for the loss of attachment strength. If the carboxylic acid is already bound to iron ions in the FeS/FeS_2 mineral they may not be readily displaced by aqueous ions making this transition difficult. The lipids, once their head-groups are neutralized by reaction with aqueous metals may detach individually rather than remain cohesive with attached lipids. Experimental verification of the progression that Wachtershuaser describes needs to be performed to determine its validity, but would be difficult and unreliable due to uncertainties in the conditions.

In Wächtershäuser (1988) a phase is described wherein an "ambience" is proposed to exist which refers to the environment in close proximity to the metabolist which is affected by its detached waste products. This is in contradiction to the claim made by Wächtershäuser (Wächtershäuser, 1992; 1994) and others (Russell and Hall, 1997) that once detached, metabolic constituents become infinitely dilute in the ocean and therefore play no further role in metabolism.

Wächtershäuser (2006) agrees with Kandler's (1995; 1994; 1998) suggestion of a pre-cell stage in the emergence of life and Woese's (1987; 1977) suggestion that the "tree of life" is rooted in a "universal ancestor state" with a high degree of genetic mixing. Wächtershäuser (2006) describes metabolically diverse, free living cells capable of reproduction but unable to limit the exchange of genetic information

between each other as they are frequently undergoing fusion and fission. Wächtershäuser (2003) describes the evolution from this state to the three domains of life in detail, but what is less clear is how the pre-cell state arose from the semi-cellular state. Two differing "idealised" (Wächtershäuser, 1988) transitions have been proposed; in one a detached mineral grain is encased in a "closed cellular membrane envelope" producing a free-living cell which is still dependent on mineral-bound reactions. The second hypothesised transition involves a "semi-cellular" state. In which cytoplasm is encased by the mineral on one side and the lipid membrane on the other. This is said to emerge either through the lipid blistering due to increased cytosolic volume, or by the lipid membrane going over a cavity in the mineral (Wächtershäuser, 2006). The transition from the semi-cellular state to a detached cell is not discussed in detail,

As mentioned above one of the consequences of a lipid membrane is that uncharged nutrients can freely pass into the cytosol but charged components, which make up much of the active metabolism and genetic machinery, cannot diffuse out. However, H_2S in the neutral to acidic conditions proposed in this hypothesis deprotonates to a charged species in alkaline solutions. This is a problem since H_2S is essential for energy production. Fe^{2+} is also unable to pass across the membrane leaving the cell with only the non-replaceable internal mineral grain as a source of Fe^{2+}. Rickard and Luther (1997) demonstrated that the mackinawite to pyrite conversion is an aqueous reaction rather than a solid-state transition, and this has certain implications. For the transition to occur the FeS mineral must dissolve and enter the surrounding solution. It has been stated that detached organics are irretrievably lost to the ocean (Russell and Hall, 1997; Wächtershäuser, 1992; 1994) and dissolved inorganic ions may not be able to promote reactions between organics

attached to the mineral. As this is an aqueous reaction, the effect of a hydrophobic membrane coating the mineral needs to be ascertained as it may prevent dissolution.

5.2. Pre-formed inorganic cells

The hypothesis of Russell *et al.* stands out from Wächtershäuser's and from the prebiotic soup hypothesis in that a abiotically produced cellular envelope exists prior to and throughout the emergence of life. Another significant difference from Wachtershauser's hypothesis is that there is no free-living pre-cell stage. The entire development of living cells occurs within the compartments from carbon fixation to the development of fully-fledged Bacterial and Archaeal cells (Fig. 4).

The first organic components to be added to the inorganic membrane were peptides generated abiotically (Martin and Russell, 2003; Russell and Hall, 2006; Russell *et al.*, 2005) through the Huber and Wächtershäuser (1998) reaction. These peptides were capable of plugging pores in the FeS membranes and encasing catalytic FeS units and phosphate.

The transition from FeS compartments to free living cells with a lipid membrane in Russell's hypothesis begins with the "invention of pathways that catalyse the synthesis of lipids and cell wall constituents" (Martin and Russell, 2003). Lipids bind to the interior surface of the FeS compartment forming a spherical membrane. This is a late development in this hypothesis; requiring the presence of DNA, RNA and proteins. Their evidence for a late development of genetic lipid biosynthesis pathways comes from the observation that Archaea and Bacteria synthesise lipids in completely different ways.

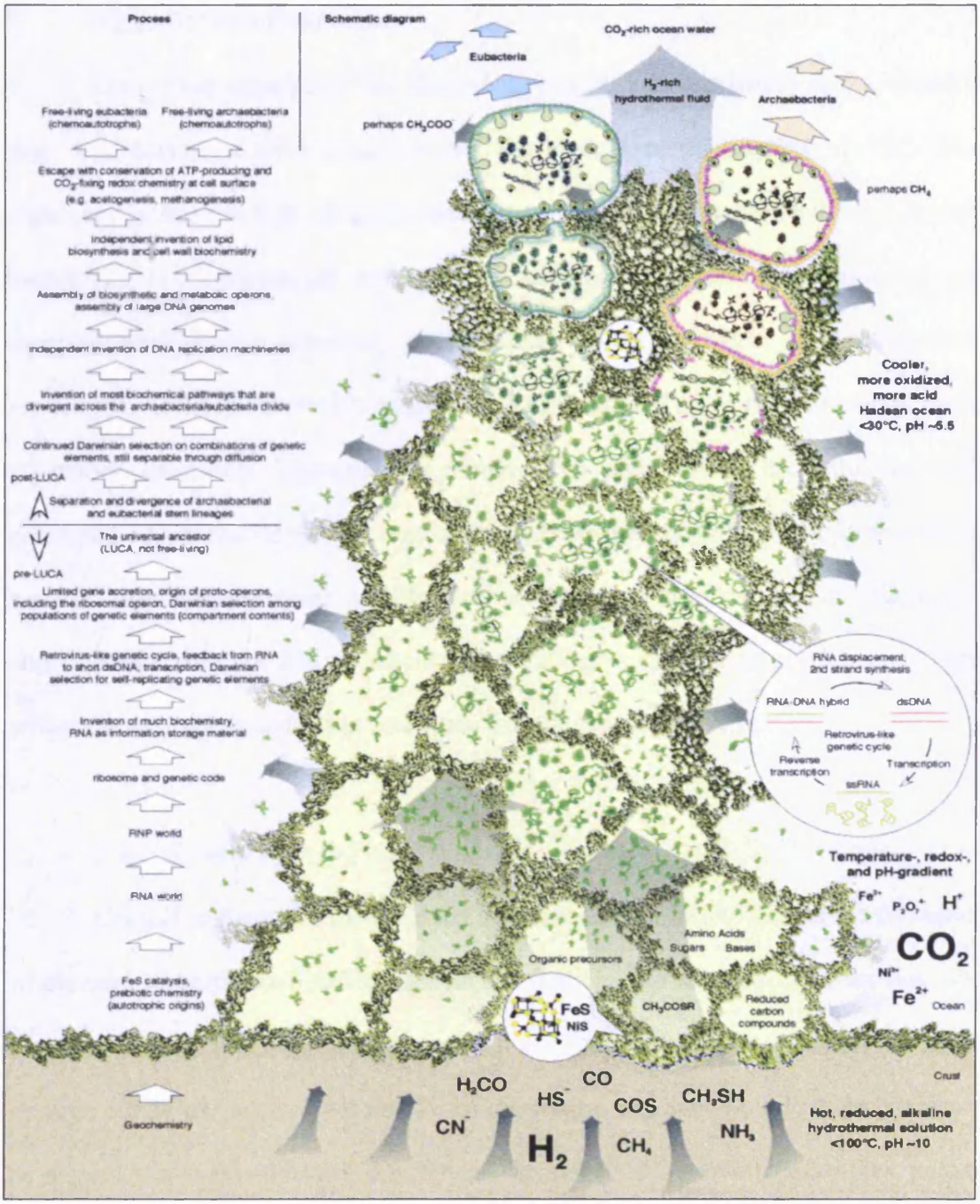


Figure 4: Proposed origin of life scenario from carbon fixation to the independent escape of archaeal and bacterial cells within a naturally forming compartmentalised mineral assemblage consisting primarily of FeS at a Hadean (3.8 Ga old) hydrothermal vent. Gradients of temperature, pH and Eh exist horizontally across the mound. The left hand side of the figure shows the proposed sequence of events (from the bottom to the top) described in the main text; the right portion is a highly schematic drawing illustrating increasingly complex levels of molecular organization within the compartments. The enlarged compartment shows the proposed retrovirus-like genetic cycle of LUCA. Reproduced from Koonin and Martin (2005).

5.2.1. Self contained redox

One of the arguments that Russell uses to support his theory is the assertion that "self contained redox reactions are the most conserved aspect of life". This statement is false and an illogical assumption is drawn from it. Firstly, all known extant life is biochemically very similar with many conserved behavioural and compositional aspects; hereditary nucleic acids, catalytic proteins, lipid membranes, replication, nutrition, excretion, responsive to stimuli and more fundamentally, based on carbon chemistry. Secondly, he assumes the precursor to life exhibited "self contained redox reactions" but was contained inorganically. It is a logical error to assume that the precursor to life had a similar behaviour but an alternative composition to extant life. It would be as fitting to claim that organic chemistry, without containment and redox reactions, was the precursor to life.

5.2.2. Evidence for FeS compartments

Critical to Russell's theory is the location. It must be shown that the formation of the compartmentalised FeS membranes is feasible. No identical structure has been observed in nature. These structures would not form today due to differences in the oceanic conditions such as pH and Fe concentration and they would not be preserved as no rocks from this time are. Martin and Russell (2003) provided examples, natural and synthetic, of analogous structures. The 360 Myr FeS chimney from Silvermines, Ireland (Boyce, 1983) and the 360 Myr old pyrite structure, precipitated as FeS, from the Tynagh ore deposit (Banks, 1985) both formed in conditions now believed to be too hot for life's emergence. Alpine magnesite deposits (Fallick *et al.*, 1991; Zedef *et al.*, 2000) have been favoured instead (Russell and Arndt, 2005).

Russell and Hall (1997) produced an FeS "edifice" by injecting a 0.5 M FeCl solution into a 0.5 M Na_2S solution which when freeze dried and photographed contained a system of micromolar compartments. The concentrations used were higher than hypothesised to exist at the relevant sites and Wächtershäuser (2006) has claimed that the compartments were experimental artefacts produced by the freeze drying process.

5.3. Conclusion of cellularisation section

Russell's FeS chambers are a very attractive feature of his hypothesis, solving several problems in the origin of life geologically before the first prebiotic organic reactions. However, their existence in the natural world has not been definitively proven and the experimental data for their existence has been brought into question. Modern seafloor sulphide edifices are cemented by calcium sulphate. Calcium sulphate has reverse solubility and it dissolves as the vent system wanes and cools. This results in the collapse of the material into a mound of porous sulphides, which are subsequently lithified by continued reaction with the hydrothermal solution. In the Hadean ocean, the sulphate concentration would not have been high enough to produce the calcium sulphate cement and thus the original edifices would not form. The result would be a mound of porous sulphides. Although attractive at first sight to the Russell hypothesis, the bulk material of these mounds is usually pyrite (rather than mackinawite), although sphalerite and chalcopyrite-rich varieties are also widespread.

Wächtershäuser has proposed two slightly different modes of cellularisation both of which require extensive abiotic lipid production which has not yet been experimentally verified. In both hypotheses, escape from the iron sulphide mineral occurs as a late development after the appearance of most of the attributes of a living

cell. The difference is that Wächtershäuser posits a pre-cell stage in which most of life's diversity arises whilst Russell has suggested that the three domains of life emerged within the iron sulphide mound.

6. Origin of Nucleic Acids

The third aspect of living organisms is the genome; the hereditary DNA-code for the production of peptides. Some authors assume an early arrival of the genome (Ertem and Ferris, 1993; Ferris, 1999) often consisting of RNA-catalysed RNA replication, known as the RNA-world (Gilbert, 1986). Nucleic acids are a polymer of phospho-ribose units linked together with phospho-ester bonds with heterocyclic bases bound to the ribose moiety. The FeS-world hypotheses both claim that metabolism emerged first with nucleic acids evolving later due to their catalysis of this metabolism. In both theories nucleic acids coevolved along with cellurisation. In Wächtershäuser's hypothesis it is during the semi-cellular stage and in Russell's it is whilst the proto-organism is bound by the FeS "bubbles".

There has been much literature published on the origin of nucleic acids Wächtershäuser (2006) states that this work is not applicable to his hot, volcanic chemoautotrophic origin hypothesis and that phospho-ribose and nucleotides were both formed by carbon fixation reactions and initially served as ligands for metal catalysts. The first condensed "nucleic acid" polymers are said to have had a peptide back-bone bases derived from pendant hydantoin (imidazolidine-2,4-dione or glycolylurea). Huber and Wächtershäuser (1998) have produced experimental evidence that peptide bonds are produced under the proposed conditions of this hypothesis but not for the phosphodiester bonds which emerged later. Further

condensation of monomers into the long polymers required for the next stage is not described.

Primitive translation (RNA-encoded peptide synthesis) is said to have originated with two surface bonded, side by side, aminoacylated proto-tRNA's. These are hairpin structured RNA polymers which are attached to a amino acid activated in the same manner as in the peptide bond formation (Huber and Wächtershäuser, 1998) removing the need for an aminoacyl tRNA synthetase. Two proto-tRNAs bind to the mineral surface side by side and by base pairing attach to a surface-bound proto-mRNA which promotes a peptide bond between the amino acids they carry. How a tRNA and mRNA orientate to interact whilst both of them are surface bound is not described. In extant translation mRNA is bound to a ribosome and free tRNA molecules form hydrogen bonds with the mRNA. If the mineral is to act as a ribosome then there would be no need for tRNA to interact directly with it. Translation is said to have evolved during the semi-cellular stage of life's emergence. Therefore, the cytosol or the membrane may have been the location in which it occurred although this would require some form of proto-ribosome first. This early emergence of translation is in contrast to the more widely accepted RNA-world view in which replication is first and paramount. An early emergence of peptides, with functional and structural properties, could have been more beneficial to a proto-organism within a restricted locale than one in a vast ocean in which peptides would diffuse away from the nucleic acids that synthesised them.

Wächtershäuser (2006) suggests that there is no reason to assume a late arrival of DNA and that early polymerases did not distinguish between RNA and DNA. The genomics of the pre-cells is reconstructed based on comparisons between Bacteria and Archaea although how this emerged from the primitive description given above is not

given. A mechanism for the elongation of nucleic acids and experimental verification of it is essential. The pre-cells are said to have had circular dsDNA chromosomes similar to extant plasmids because linear chromosomes would have been insufficiently stable (Woese, 1998). Whether these large plasmids emerged during the hot, mineral bound semi-cellular stage or during the free-living stage is not mentioned.

Wächtershäuser states that his theory provides an explanation for the phosphorylation of sugars, that the phosphate groups arose to permit binding of the sugar to a mineral substrate. An alternative explanation could be that sugar phosphorylation arose after cellularisation to prevent the leakage of sugars out of lipid membranes. This is the function of sugar phosphorylation in extant biochemistry. In fact this is the reason why so many metabolic intermediates have charges, it could be argued though that this is the reason charged intermediates have been preserved through cellularisation and not the reason for their origination.

6.1. Origin of nucleic acid-amino acid interaction in FeS cells

In Russell and Hall (1997; 2006) nucleic acid bases are surmised to originate from the condensation of HCN on a sulphide surface. The ribose phosphate element of nucleic acids was produced by the reaction between glyceraldehyde and dihydroxy acetone phosphate, both derived from condensation of formaldehyde and pyrophosphate adsorbed on mackinawite. The hetero-cyclic bases and ribose phosphate combined, on the mackinawite surface, to produce RNA monomers. These monomers initially polymerised in part due to the electrochemical gradient across the membrane and in part due to their binding to the membrane.

The electrochemical gradient across the membrane is compared to the proton motive force (PMF), an energy storage and utilisation process existing in all extant cells. The PMF is produced through an electron transport chain and used to produce ATP, life's universal energy "currency". The suggestion of an inorganically derived electrochemical gradient across a membrane is a very attractive feature of Russell's hypothesis because of its similarities to extant membranes and its pre-existence in the earliest stages of life. The capacity for an FeS membrane to maintain an electrochemical potential has been experimentally verified (Russell and Hall, 2002; Filtness *et al.*, 2003). However, this potential has not been experimentally demonstrated to promote polymerisation of nucleic acids nor is there experimental evidence for the binding of RNA monomers or short chains to mackinawite.

The FeS-bound RNA is then said to have "gripped" a side chain of an amino acid, crudely selecting amino acids based on the hydrophilic nature of the side chain and orientated them in a manner that is conducive to the formation of peptide bonds. Huber *et al.* (2003) demonstrated that peptide bonds can form by activation with carbon monoxide under hot aqueous conditions in the presence of freshly coprecipitated colloidal (Fe,Ni)S without a nucleic acid template. Others have suggested that peptides or peptide nucleic acids may have been the template for nucleic acid polymerisation (Henr y, 2005; Nelson *et al.*, 2000) be cause peptides bonds form more readily under plausible prebiotic conditions and because of the instability of ribose in warm conditions.

6.2. Genome evolution in an inorganic cell

Martin and Russell (2003) state that provided the necessary monomeric building blocks are continuously supplied, something like an RNA-world could arise.

At a later stage but still within the FeS mound the evolution of DNA is said to have occurred. Koonin and Martin (2005) expand upon this and propose a retrovirus-like genetic cycle in which dsDNA is synthesised from an RNA-DNA hybrid within FeS compartments (Fig 4, expanded cell). RNA codes for a small number of peptides whose function is to facilitate the replication cycle, selection is only for molecular self-replication at this stage.

Koonin and Martin (2005) propose a system of evolution within these compartments;

- Discrete genetic elements are mobile between compartments.
- Compartments will have diverse combinations of these genetic elements.
- Some combinations will replicate themselves more efficiently than others.
- Those that replicate the fastest will spread into neighbouring compartments and newly forming compartments.

A key requirement for this is that diffusion of nucleic acids between cells occurs at a rate fast enough to permit colonisation of new cells but is slow enough not to result in homogeneity between the cells or a fatally low concentration of reactants within them. The abiotic formation of new compartments is analogous to cellular division with horizontal gene transfer as the mode of inheritance. Neither Koonin and Martin (2005) nor the other papers on this hypothesis mention the rate of diffusion, the mechanism of formation of cells or the size of the pores between compartments, all of which are essential for considerations of the validity of this hypothesis.

On the origin of DNA Russell states that it would have taken over from RNA due to its enhanced stability, but a mechanism for this is not provided. DNA genomes evolved within the FeS mound. "We find it unlikely that cells with largely or solely RNA-based genetic storage were capable of existing outside a network of inorganic

compartments" (Koonin and Martin, 2005) although RNA repair strategies were probably already in existence. Lipid membranes are a late genetically encoded development here but geochemically synthesised fatty acids are said to have provided a hydrophobic layer at the compartment's surfaces.

Koonin and Martin (2005) discuss selection only at the level of molecular replication. Nucleic acids fall into two groups: purely selfish replicators as well as "altruistic" replicators which produce the enzymes (or ribozymes) required for their own replication and that of other strands. Biosynthesis of molecules not directly related to nucleic acid replication is discussed little and is assumed to be a late development. However, in a compartmentalised system the products of biosynthesis would be retained better than in a prebiotic broth system and would therefore be more strongly selected for. Also, if it can be shown that these replicating FeS compartments could have existed then they become another type of replicator to which natural selection can also apply. This would create a strong selective pressure for the evolution of biosynthesis of chemical constituents which can effect both the internal environment of the compartments and their capacity for survival and reproduction.

Proteins in Koonin and Martin (2005) are only involved in the replication of nucleic acids. However, this would require fairly long and complex proteins and presumably short peptides must have been the predecessors to these enzymatic proteins. The first peptides would have been too small to have an enzymatic role but the suggestion of FeS compartments gives them the possibility of a structural role as well. Precipitated FeS compartments may face collapse or dissolution, short peptides along with abiogenically produced lipids may have bound to the interior of the compartments stabilising them at first and later developing the ability to influence transport between compartments as occurs in modern organic membranes. The genes

coding for these structural peptides would result in the entire compartment being selected for along with all other replicating nucleic acids in the compartment.

A hydrothermally precipitated FeS mound is a chemically harsh environment. Nucleic acids are damaged by iron and other metal ions in certain conditions (Dore *et al.*, 1972; Galaris *et al.*, 2002; Oikawa and Kawanishi, 1998; Rodriguez *et al.*, 1997; Rozenberg-Arska *et al.*, 1985; Sagripanti, 1999) sulphide ions (Attene-Ramos *et al.*, 2006) as well as extremes of pH (Dore *et al.*, 1972). The early production of short peptides would have been selected for due to a potential role in the preservation of nucleic acids by binding to them, sequestering reactive ions or by coating the inner surfaces of the FeS compartments. Another role of short peptides may have been to bind to FeS and phosphate groups preventing them from crystallisation or dissolution and orienting them in a manner that facilitates their catalytic capacities.

6.3. Conclusion of origin of nucleic acids

Nucleic acids evolved after proto- metabolism, in the FeS-world hypotheses, as catalysts for metabolic reactions. Wächtershäuser (2006) states that the sugar and heterocycle components emerged as ligands for metal catalysts prior to their role in peptide synthesis. In Russell's hypothesis nucleic acid monomers are formed by condensation on the FeS membrane fuelled by the electrochemical gradient across it. In both theories nucleic acids anchored to the mineral and attached to amino acids in a manner that facilitated the formation of peptide bonds between them.

7. Conditions and resources in an FeS world

Russell and Wächtershäuser both stress the importance of the environmental geochemistry in which life emerged. The time frame in which life is usually stated to have emerged is 3.9 - 4.5-Ga during the Hadean eon. The Hadean is usually defined as the period between the formation of the Earth and the appearance of the geological record. As there are no rocks from this period it is difficult to pinpoint the exact time of life's emergence and also difficult to determine the atmospheric and oceanic conditions that prevailed. The oldest minerals on earth are zircons dated at 4.4 Gya (Wilde *et al.*, 2001). This is evidence that at this time there were oceans and continental and oceanic crust.

Due to the lack of rocks from the Hadean other techniques have been employed to ascertain its atmospheric and oceanic conditions. The luminosity of the Sun was less than 75% of its current value (Bahcall *et al.*, 2001) resulting in substantially less solar radiation arriving at the Earth's surface, yet the zircons indicate that liquid water existed at this time. The solution to this paradox has been the suggestion that there existed an intense greenhouse atmosphere, warming the Earth. Greenhouse gasses include but are not limited to carbon dioxide, methane, ammonia and water vapour. All of which may have been produced in large quantities by volcanoes in the Precambrian. The relative abundance of these gasses would have a major effect on the oceanic pH, but as they are not yet clarified the pH of the Hadean oceans remains uncertain.

The Russell hypothesis requires an acidic Hadean ocean which, in turn, would suggest a significant dissolved Fe concentration. Others have suggested that the current pH of the ocean which is approximately 8-8.5 has been constant throughout

geological time due to its immense capacity for self-buffering (Zubay, 2003). Russell *et al.* (2005) provides four references to support the an oceanic pH of 5-6 brought about by atmospheric CO_2 (Kasting, 1993; Macleod *et al.*, 1994; Maisonneuve, 1982; Sedwick and Mcmurtry, 1994). Kasting (1993) and Maisonneuve (1982) agree with the claim of a high partial pressure of CO_2 but neither relate this to a low oceanic pH. In fact, Maisonneuve (1982) points to the ocean's ability to self-buffer and that any reduction in pH would be associated with an increase in dissolved cations: "Owing to the cations oceanic waters were alkalinized and the pH would have been about 8. It could fluctuate and rise to 9." MacLeod *et al.* (1994) and Morse and Mackenzie (1998) agree with Russell concerning the mildly acidic ocean hypothesis.

A mildly acidic ocean is vital for this hypothesis for multiple reasons. For example a pH gradient is required to exist between the ocean and the exhalate and phosphate ions in the presence of transition or alkaline earth metals are insoluble in non-acidic conditions.

7.1. Iron and Sulphur Resources

Österberg (1997) brought up a challenge to any theory requiring an influx of H_2S. Prior to the existence of atmospheric oxygen the oceans contained substantial amounts of dissolved Fe(II). Any local source of H_2S or HS- would immediately form an FeS precipitate removing sulphide from the system. Österberg (1997) states that this would make any involvement of H_2S (or HS^-) in the origin of life an impossibility. This has implications for Wächtershäuser's theory, as a high activity of H_2S is required for high reducing power in the pyrite forming reaction. Huber and Wächtershäuser (1997) used 300 μM H_2S in their production of acetic acid. Russell's hypothesis avoids this problem as the precipitation of an iron sulphide membrane

separating two fluids is a vital aspect of it. However, Russell's theory still requires a continuous supply of sulphide to the site. Quantification of the iron and sulphur in these theories is necessary as it is feasible that a flux of H_2S could be sufficiently intense and prolonged to saturate the Fe^{2+} leading to an excess of sulphide. If the rate at which Fe^{2+} diffuses in to the system is lower than the flux of H_2S it may be possible for a sulphide dominated system to be maintained.

A concentration for Fe^{2+} in the primeval ocean has been estimated to be 0.1 to 0.5 mM (Walker and Brimblecombe, 1985). Russell and his co-workers (Macleod *et al.*, 1994) have suggested a dissolved iron concentration approaching 100 ppm (1.79 mM). Wächtershäuser discusses the site for his hypothesis less than Russell does, merely referring to it as volcanic or hydrothermal so it is difficult to determine what quantity of sulphide may be supplied or how long for. Timing is relevant as sulphide may be required to be available for considerable periods in order to fuel the sequence of reactions prior to the evolution of the exploitation of alternative energy sources. Sulphate was not present in the oceans at this time and therefore, the hydrothermal black smokers did not exist. Russell places the origin of life at an off-ridge alkaline seepage site. Russell and Hall (1997) and Macleod *et al* (1994) state that reduced alkaline fluids from ultramafic rocks could have provided up to 750 ppm (22.5 mM) HS- to these sites. However, this is at 250 °C and the hypothesis has been relocated to a lower temperature site since then. The sulphide concentration at these seepage sites has not been determined or hypothesised, but the iron concentration may have reached as high 10 mM due to their proximity to hot acidic springs which contributed ~ 20 mM Fe^{2+} (Russell *et al.*, 2005). It would be expected that Wächtershäuser's hypothesis could not occur in a locality such as this.

In conclusion there are many essential parameters as yet undetermined to establish whether or not these hypotheses are geochemically plausible. Wächtershäuser needs to find or describe plausible locations in which his hypothesis could occur and Russell needs to demonstrate that his sites could have supplied sufficient quantities of sulphide for a sufficient time.

8. Conclusions

Russell's theory begins with a geochemical conundrum: the existence of an electrochemical gradient in an environment in which equilibrium cannot be achieved due to the continuous precipitation of a barrier. This provides a constant energy source and drive for alteration in the system. It also provides a continuum of differing conditions throughout the mound, potentially promoting a variety of reactions such as carbon fixation at high temperatures and polymerisation at lower temperatures. Although Wächtershäuser (2006) criticises Russell for claiming the origin of all three Domains occurred in one location, his own hypothesis involves evolution to the pre-cell stage in an environment with less variety. In this environment the proto-organism is exposed to very high temperatures throughout its development which may be a problem as life is dependent on different weak chemical interactions which are unlikely to form at high temperatures.

Russell provides a unique geochemical setting in which the origin of life is said to be possible but cites the work of others in describing the procession from this. Wächtershäuser provides a much more unique and detailed sequence, rejects most of the research inspired by the prebiotic broth hypothesis and presents a sequence of reactions firmly rooted in the chemoautotrophic hypothesis.

Russell's hypothesis has a strong advantage in that cells are pre-existing prior to prebiotic chemistry; this allows the chemistry discussed by Wächtershäuser to be involved but reduces or eliminates the problem of detachment. This is, however, dependent on the existence of the FeS-compartments being demonstrated to be possible. Wachterhauser (2006) points out that this has not yet been done. These compartments are only one of the many requirements for both hypotheses that are yet to be demonstrated. Only the very earliest stages such as carbon fixation, simple elongation reactions and peptide bond formation have been experimentally verified. Many of the biological monomers such as nucleobases and ribose, lipids, phosphodiester bonds have not been produced. No autocatalytic cycle has been created or simulated in viable hydrothermal conditions nor has the interaction between a competing hydrophilic metabolism and a hydrophobic membrane been simulated.

The chemists who have worked on the origin of life have produced excellent descriptive explanations. They have described sequences of events leading to the emergence of autocatalytic cycles and in some cases have demonstrated that these steps are chemically feasible and bring the emerging system closer to extant life. The complex multi-step pathway to the origin of life must have involved many improbable steps which although possible were only one possibility out of many. Wächtershäuser often uses the term "explanatory theory of biochemistry". The explanatory theory in biology is natural selection which Wächtershäuser invokes more than Russell does even though Russell's hypothesis has a an additional level at which replication occurs: the FeS bubbles. Any system in which replicators have an inheritable, differential capacity to replicate will experience Darwinian evolution. A hypothesis which involves a pre-genetic emergence of autocatalytic, metabolic cycles (which must be

simpler than the reductive citric acid cycle) must focus on how these cycles are selected for to enable them to survive and propagate.

Chapter 3

An electrophoretic study of the effects of iron (II) and iron (III) on plasmid DNA

Abstract

Although there are many reports of iron ions interacting with and damaging DNA in the biomedical literature, the significance of this to theories on the emergence of nucleic acids in origin of life scenarios has not been considered. Plasmid DNA was incubated with solutions of Fe(II), or Fe(III) and analysed with electrophoresis. Two major effects are observed. Firstly, Fe(II) (0.1-25 mM) and Cu(II) (0.1-150 mM) unravelled the super-coiled plasmid probably through the production of oxidative free radicals which caused scission of the DNA back-bone. Secondly, DNA incubated with either Fe(III) (≥5 mM) or Fe(II) (≥50 mM) did not appear on the gels due to extensive fragmentation of the plasmid and/or its binding to iron hydroxide precipitates which prevented it from migrating out of the well. It has been suggested that life may have emerged in an environment with a high concentration of iron ions. The results suggest that high concentration of aqueous metal ions would have been detrimental to an emerging nucleic acid system. By contrast, lower metal concentrations may have facilitated DNA evolution by breaking the DNA molecules and allowing them to recombine in novel ways. Iron hydroxide minerals could have provided a surface for nucleic acid binding and organisation.

1. Introduction

It has been hypothesised that life emerged on or adjacent to metal-bearing minerals precipitated at submarine hydrothermal systems. (Russell and Hall, 1997; 2002; Wächtershäuser, 1988; 1998; Russell and Arndt, 2005; Russell and Martin, 2004; Wächtershäuser, 1990; 1992). These minerals are thought to have adsorbed

organic molecules and promoted the reactions between them that lead to the evolution of a mineral bound proto-organism. Detachment of the proto-organism from the mineral surface occurs late in these processes, after the development of lipid membranes, enzyme catalysis and DNA translation and transcription. Iron sulphide minerals, such as mackinawite and pyrite are theorised to play an especially important role, but other iron minerals were also present. The main alternative theory in origin of life research is the prebiotic soup theory originally proposed independently by Oparin (1938) and Haldane (1929). In the prebiotic soup scenario life is thought to have emerged in the ocean which, prior to the evolution of oxygen, had a considerably higher concentration of Fe(II) than it does today, possibly in the region of 0.1-0.5 mM (Österberg 1997). In both scenarios, there would have been high Fe concentrations in Hadean aquatic environments. The effect of such concentrations on the emergence of primitive nucleic acid polymers is not well understood.

Metals interact with DNA in a variety of ways. Many proteins contain metal ions which allow them to bind to DNA as part of normal cell functioning. Redox active metals, such as iron and copper, can engage in reactions with other constituents of the cell that lead to the production of reactive oxygen species (ROS) such as the hydroxyl free radical $OH\cdot$. ROS have been studied extensively by biomedical researchers, they can oxidise a range of biochemicals and are a known source of DNA damage which have been implicated in carcinogenesis, mutagenesis, teratogenesis and aging (Sagripanti, 1999; Rodriguez *et al.*, 1997; Oikawa and Kawanishi, 1998).

Fenton reactions are a well-researched source of DNA damage *in vivo* and *in vitro*. The Fenton reaction (Fenton 1893) is the ferrous (Fe(II)) salt decomposition of hydrogen peroxide into hydroxyl radicals. Hydroxyl radicals are one of a suite of reactive oxygen species which are known to attack the ribo-phosphate backbone of

DNA. Fe(III) can also decompose hydrogen peroxide into oxidative free radicals (Oikawa and Kawanishi, 1998; Rodriguez *et al.*, 1997; Toyokuni and Sagripanti, 1992).

$$Fe(II) + H_2O_2 \rightarrow OH^- + OH\cdot \quad \text{.....(1)}$$

$$Fe(III) + H_2O_2 \rightarrow Fe(II) + OOH\cdot + H^+ \text{.....(2)}$$

Fe(II) can damage DNA in vitro without the addition of hydrogen peroxide provided oxygen is present (Toyokuni and Sagripanti, 1992) this occurs via the superoxide radical.

$$Fe(II) + O_2 \rightarrow Fe(III) + (O_2\cdot)^- \text{.....(3)}$$

Superoxide radicals can then react with Fe(II) and two protons; producing hydrogen peroxide and initiating Fenton reactions.

$$Fe(II) + (O_2\cdot)^- + 2H^+ \rightarrow Fe(III) + H_2O_2 \text{.....(4)}$$

Most of the research in this area has looked at Fe(III) induced DNA damage in the presence of reducing agents. There is no known mechanism for Fe(III) to initiate radical production in the absence of hydrogen peroxide but, on reduction to Fe(II) and in the presence of oxygen, it can react as in reactions 3 and 4 (Toyokuni and Sagripanti, 1992; 1993; Oikawa and Kawanishi, 1998; Rozenberg-Arska *et al.*, 1985).

Hydroxyl radicals are non-specific in their interactions with DNA and are short lived so do not migrate far in solution before engaging in a reaction. Yet some researchers have observed site-specific DNA damage caused by iron or copper and hydrogen peroxide (Henle *et al.*, 1999; Kawanishi *et al.*, 2001; Lloyd and Phillips, 1999; Oikawa and Kawanishi, 1998; Yamamoto and Kawanishi, 1989). This has been attributed to Fenton reactions involving metal ions bound to specific sites on the DNA.

Oikawa and Kawanishi (1998) reported site-specific DNA damage in the presence of 20 μM copper(II) or iron(III), reducing agents and hydrogen peroxide.

Despite the abundance of studies on damage to DNA involving iron ions, origin of life researchers are yet to consider the validity of bio-polymers such DNA emerging, surviving and functioning in an environment with a high concentration of iron and/or other metal ions. This paper reports an investigation of the reaction between ferrous and ferric iron and DNA in the absence of reducing agents and hydrogen peroxide and in a low oxygen environment, using plasmid electrophoresis.

2. Materials and methods

2.1. Plasmid DNA preparation

pUC18 plasmid DNA (pDNA) was extracted from cultures of *E. coli* grown in LB broth with 100 mg/L ampicillin using Wizard® Plus SV Minipreps DNA Purification System according to the manufacturer's instructions. pDNA was stored in nuclease free water and at -20°C until required. All reagents were of analytical grade. Sterile, O_2-free deionized water (SD1 H_2O) was used throughout. MilliQ ™ 18 M Ω H_2O was sterilized through autoclaving for 2 h at 120°C and 2 Bars in a *Priorclave*™ and deoxygenated by bubbling O_2 free N_2 for at least 1 h. Studies of the resultant dissolved O_2 concentration (Butler *et al.*, 1994) shows that the resultant solution contains measurable O_2 in the 1-10 ppmv range. All containers were sterile on purchase or sterilized through autoclaving as above except plastic bottles for Fe(II) and Fe(III) stock solutions which were washed with 2% Decon 90® and ethanol.

2.2. Sample preparation

Stock solutions of 0.5, 5, 50, 500 mM Fe(II), or Fe(III) were prepared with analytical grade $(NH_4)_2Fe(SO_4)_2.6H_2O$ or $FeCl_3$ (Fisher Chemicals™) dissolved in SD1 H_2O. In a Labmaster *130*™ anoxic glove box, filled with O_2 free N_2, 50 µL of iron salt solution and pDNA were prepared in 0.2 mL PCR tubes along with Tris buffer, a buffer of pH 8 used commonly in DNA experiments. Iron concentrations were varied from 0.1 mM to 150 mM, the pH of these solutions is given below. The source of iron (II) was Mohr's salt ,$(NH_4)_2Fe(SO_4)_2.6H_2O$), as it less prone to oxidation than other Fe(II) salts.

The pH of the iron sulphate solutions was measured on a Jenway 2030 digital pH meter. Suitably small micro-electrodes were not available to measure the 50 µL samples so 50 mL replicates were made with identical concentrations of iron sulphate and Tris and dH_2O was substituted for DNA. Table 1 shows the pH of these solutions.

Table 1. Sample pH

Fe(II) concentration (mM).	pH
0.1	8.2
1	8
10	7.7
25	7.8
50	7.5
100	7.2
150	6.7

The Eh was controlled by adding Ti(III) citrate to the system. The equilibrium potential for Ti(III) citrate is -450mV (Zehnder and Wuhrmann, 1976) and addition of varying quantities to the reaction can be used to poise the Eh to below a particular level (e.g. below -250mV). In this experimentation, Ti (III) citrate was used to provide an

O_2-free solution, poising the Eh below 250 mV and reducing the possibility of Fe(II) oxidation. Ti(III) citrate was prepared according to the method of Rickard (1997) and Butler and Rickard (2000).

Dimethyl sulphoxide ($(CH_3)_2SO$, DMSO) from Fisher Chemicals™ was used as a free radical trap. It is commonly used with oxygen-derived free radicals but also reacts to trap nitrogen and sulphur free radicals. (Kimura and Szent-Gyorgyi, 1969). The use of DMSO as a general radical scavenger to preserve the integrity of DNA is widely employed in biochemistry.

2.3. Electrophoresis

After pDNA was incubated with the iron salt for 1 h, the samples were removed from the glove box for electrophoretic analysis. 5 μL of the samples were mixed with 1 μL of 5*loading buffer and loaded on a 0.7% agarose gel with Ethidium® bromide added to a concentration of 10 μg/ml. A voltage of 80 V was applied across the gel until the dye had moved sufficiently down the gel. The gels were then photographed with ultra violet light in a Gene Genius Bio Imaging System™. In some cases band intensity was measured using GeneTools™ densitometry from SynGene ™ which computes the quantity of DNA in a band from its intensity relative to a standard marker (Bioline HyperLadder 1).

Plasmid DNA in its natural state is a circular, super-coiled molecule. A common method of investigating damage to DNA is to use plasmids because single strand breaks to the ribose-phosphate back-bone cause an unwinding of the molecule which dramatically affects its migration on an electrophoretic gel (Rozenberg-Arska *et al.*, 1985; Jezowska-Bojczuk *et al.*, 2002; Toyokuni and Sagripanti, 1992).

3. Results

In the positive controls (Fig. 1; +ve) pDNA exists almost entirely in the supercoiled form. Across low concentrations of Fe(II) (Fig. 1) there is a progressive increase in the prevalence of the relaxed forms, with no supercoiled DNA present at 25 mM Fe(II). DNA can be seen to remain in the well at concentrations of 1 mM to 25 mM. At and above 50 mM Fe(II) no DNA was visible on the gel either in bands or remaining in the well. Other observations include faint streaks down the gel and a slight increase in mobility of the relaxed DNA at 1 and 10 mM.

Iron (III) chloride caused the disappearance of pDNA signal in electrophoresis (Fig. 2). This effect occurred at a lower iron concentration ($\geq$5 mM) than with Fe(II) (Fig. 1) although there was no increase in the prevalence of the super-coiled DNA. Both the super-coiled and relaxed DNA bands lost intensity to the same degree.

The presence of Ti(III) citrate inhibited the Fe(II)-induced DNA loss (Fig. 3a). The relaxation of supercoiled pDNA by Fe(II) did not occur to the extent that it did in Figure 1. The loss of DNA signal at $\geq$ 50 mM Fe(II) was also inhibited although there is little if any DNA visible at 150 mM. The DNA observed in the wells was caused by the presence of Ti(III) citrate. No Ti (III) citrate was present in the positive control in Figure 3a but controls (Fig. 3b) without Fe(II) and with Ti (III) citrate show this same effect.

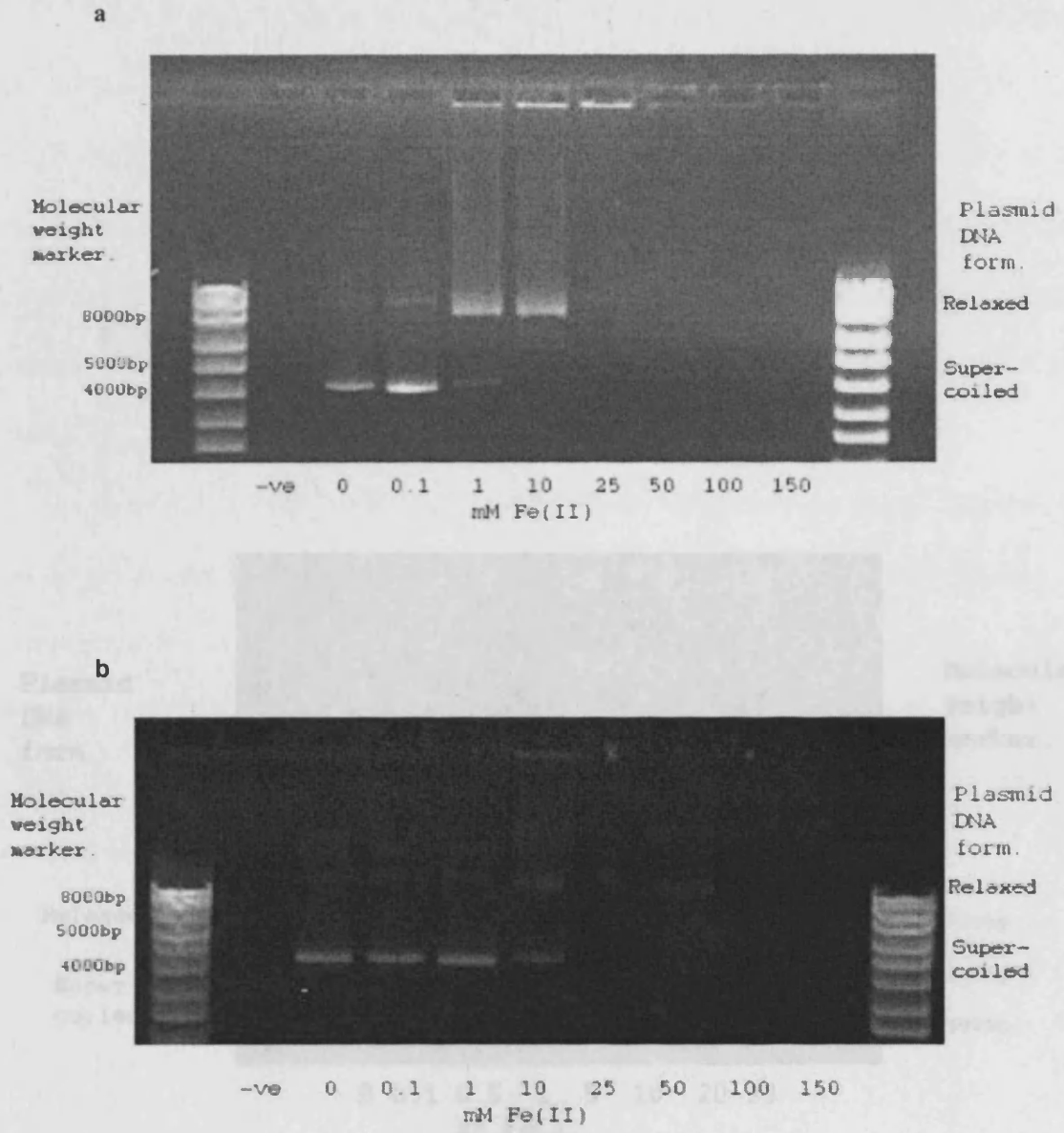


Figure 1. **a** A typical gel electrophoresis result of the reaction between iron ammonium sulphate and pDNA. The quantity of pDNA measured with densitomentry in the positive control was 53.6 ng which corresponds to a concentration of plasmids of 6.1 nM. Iron concentrations are given beneath the gel. The positions of the relaxed and super-coiled plasmid and the size of the molecular weight markers are indicated. **b** Second gel electrophoresis result of the reaction between iron ammonium sulphate and pDNA included to demonstrate repeatability.

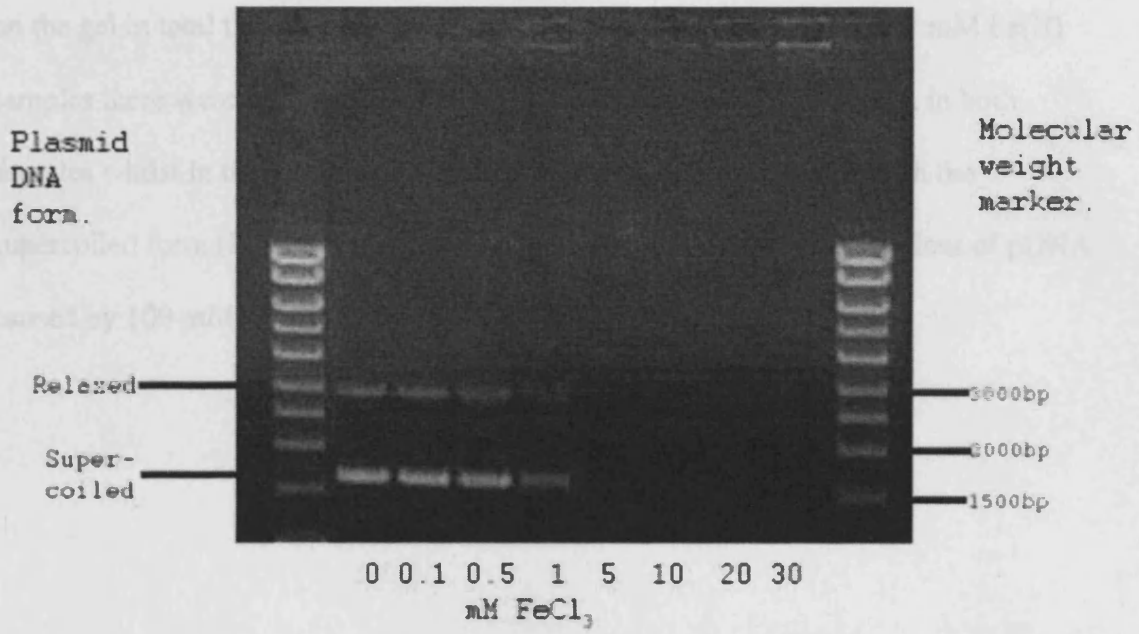
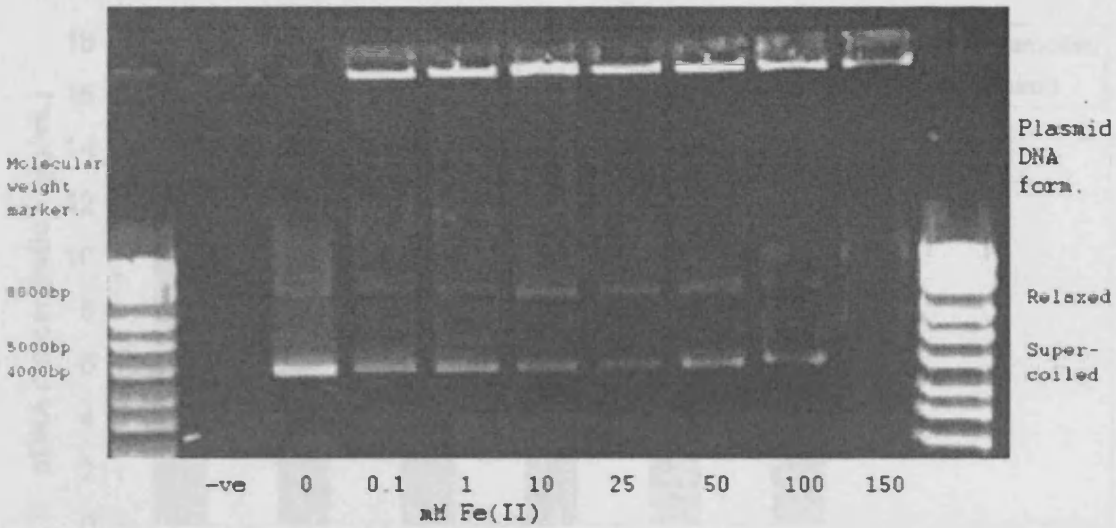


Figure 2. A typical gel showing Fe(III)-induced loss of plasmid DNA. The iron chloride concentrations are given below the image of the gel. The positions of the relaxed and super-coiled plasmid and the size of the molecular weight markers are indicated. The positions of the relaxed and super-coiled plasmid and the size of the molecular weight markers are indicated.

Both the relaxation and loss of pDNA caused by Fe(II) were partially inhibited by dimethyl sulphoxide (DMSO) (Fig. 4). Samples of 0.1, 1, 10 and 100 mM $FeSO_4$, TRIS buffer and 13 μg/mL pDNA were prepared with and without DMSO at a concentration of 2.82 M (20%) and ran on the same gel according to the above electrophoresis method, dH_2O was substituted for DMSO in the controls. Two samples of each condition were prepared and analysed, the results are the mean of these two.

In the 0.1, 1 and 10 mM samples with DMSO there was more pDNA detected on the gel in total than in the samples without DMSO (Fig. 4). In the 0.1 mM Fe(II) samples there were approximately equal amounts of super-coiled pDNA in both samples whilst in the 1 mM and 10 mM samples there was more DNA in the supercoiled form (Fig. 4). 2.82 M DMSO did not prevent the complete loss of pDNA caused by 100 mM Fe(II).



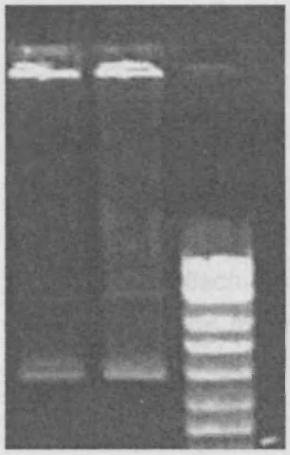


Figure 3; a) Gel showing the inhibition of Fe(II) induced pDNA relaxation by reducing agent titanium citrate. b) Ti(III) citrate and pDNA controls incubated under the same conditions as Fe(II) and pDNA samples. Both lanes contain the same amount of pDNA and titanium citrate and are included to show repeatability.

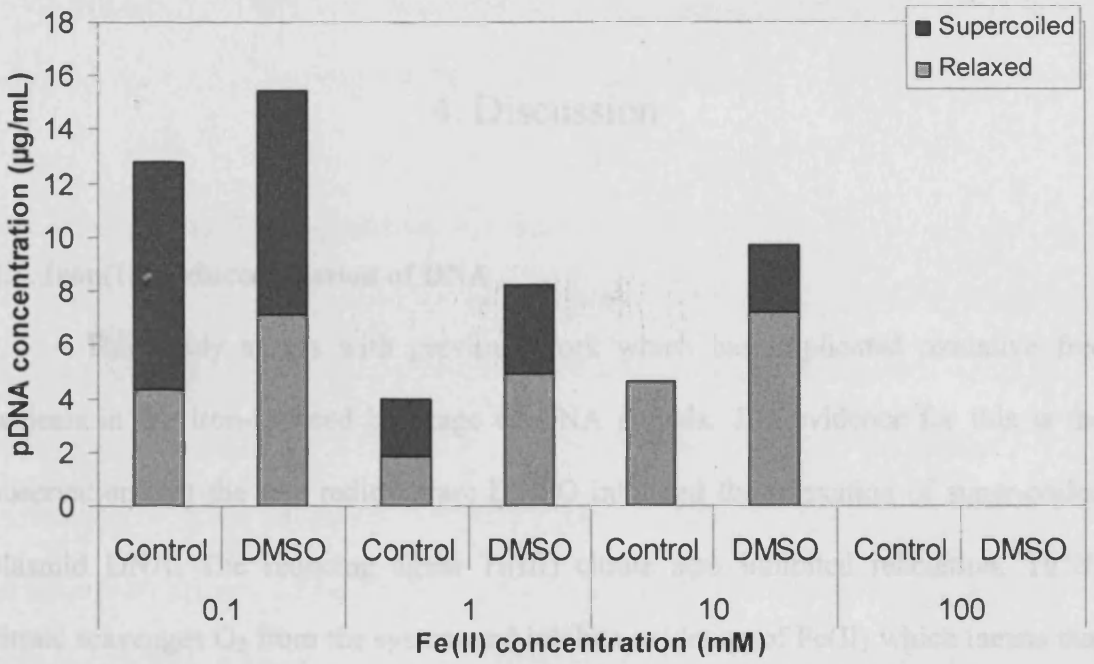


Figure 4. The inhibtion of Fe(II) induced pDNA relaxation by dimethyl sulphoxide. The DMSO concentration was 2.8M (20%). The bars show the total concentration of pDNA (µg/ml) and the abundance of each topology. DNA concentration was quantified from an electrophoretic gel; each value is the mean of two identical preparations. Raw data for this chart is provided in Appendix 1

4. Discussion

4.1. Iron(II)-induced scission of DNA

This study agrees with previous work which has implicated oxidative free radicals in the iron-induced breakage of DNA strands. The evidence for this is the observation that the free radical trap: DMSO inhibited the relaxation of super-coiled plasmid DNA. The reducing agent Ti(III) citrate also inhibited relaxation. Ti(III) citrate scavenges O_2 from the system and inhibits oxidation of Fe(II) which means that the oxidation of Fe(II) must be a vital step in this reaction. However, Fe(III) is not the reacting species as iron(III) chloride did not cause pDNA-relaxation and a reaction between DNA and Fe(III) would not be expected to be inhibited by DMSO. An oxidizing radical must be produced via the oxidation of Fe(II). There are two ways in which iron may be oxidized in this system. Firstly Fe(II) hydroxide is unstable and capable of autoxidation in the absence of oxygen by reacting with water. This reaction produces hydrogen and is not linked to any known mechanism for producing radicals although it has not been studied in regards to radical generation. Secondly the reaction mechanism involves the reduction of molecular oxygen, present in the glove box to a concentration of ≤ 10 ppmv, to the superoxide radical which leads to the generation of hydrogen peroxide which is then decomposed into hydroxyl radicals by Fe^{2+} in the Fenton reaction as described above. 2.82 M DMSO was insufficient to completely inhibit DNA damage induced by millimolar amounts of Fe(II). Although the kinetics of ROS production in this system are not known, this is a surprise since there should be

an abundance of DMSO to neutralise hydroxyl radicals unless, as noted in the introduction, the reaction may involve Fe(II) bound to DNA.

4.2. Adsorption of DNA onto iron hydroxides

The simplest explanation for the disappearance of the plasmid DNA bands ≥ 50 mM is that the same mechanism which caused scission of the backbone at lower Fe(II) concentrations occurred to such an extent that the pDNA was fragmented so extensively that the oligomers that were produced were too small to be retained or visualised on the gel. This is evidenced by Ti(III) citrate inhibiting the reaction even at high Fe(II) concentrations. It is clear, however, that the reaction between Fe(II)/(III) and DNA involves more than the radical-mediated reaction described above. Non-migrated DNA can be seen in the wells with treatment of 1, 10 or 25 mM Fe(II) (Figure 1). The solubility of iron is low, thermodynamic modelling indicates that in these conditions layered Fe(II)/(III) hydroxides, mostly Green Rusts, would precipitate from these solutions. Green rusts can reduce organic and inorganic molecules (O'loughlin and Burris, 2004) and also have positive surface charges which could bind and hold DNA in the well. Fe(III) also prevented the appearance of pDNA on the gels although there is no evidence of the back-bone being severed, indicating Fe(III) can react with DNA in an alternative manner. This could be a reaction between Fe^{3+} and the phosphate of the DNA back-bone but could also be due to the DNA binding to a Fe(III) hydroxide precipitate. Which is compatible with the observation Ti(III) citrate inhibited Fe(II)-induced DNA loss.

4.3. Implications for the origin of life

The results bring into question the development and survival of DNA in a prebiotic environment with a high concentration of dissolved iron. The current concentration of particulate iron in ocean water is 0.1-0.3 nM (Rickard and Luther, 2007). However, prior to the emergence of photosynthetic life and the subsequent development of an oxygenated atmosphere, Fe(II) was considerably more abundant. It has been estimated that the dissolved iron concentration in the Hadean (pre-Archean) ocean was above 0.1 mM (Walker and Brimblecombe, 1985). If life emerged in the Hadean ocean as thought by some proponents of the prebiotic soup theory then a minor reaction with DNA would have occurred. This could be detrimental for the emergence of DNA translation and transcription but alternatively low frequency scission of DNA may have aided early evolution. Repeated breaking of the molecule and subsequent rebuilding of it would allow a more rapid emergence of novel sequences and functionality.

At higher Fe(II) concentrations ($\geq$ 50 mM) the effect on DNA is major. This degree of scission would make it improbable that unprotected nucleic acids could survive or form at all in an environment with this amount of Fe(II) such as the hydrothermal vents cited in the FeS-world theories. Present-day hydrothermal solutions venting from 'black smokers' contain Fe^{2+} in millimolar concentrations and appreciable amounts of Cu^{2+} as well (Bebie and Schoonen, 2000). Russell and Arndt (2005) state that "the highly reduced acidic fluids would have carried about 20 mM of Fe^{2+} to the ocean" quoting data from Von Damm (2000), Douville *et al.* (2002) and Allen and Seyfried (2003). this seems to be around the limit at which major DNA damage occurs. Russell and Hall (2005) found that 100 mM Fe(II) was required to produce the chamber-like FeS structures involved in their theory. These figures approach the concentration at which a major DNA-damaging reaction occurs.

In the absence of hydrogen peroxide, oxidation is essential for Fe(II)-mediated ROS generation and produces Fe(III) which reacts with DNA more readily than Fe(II) does. The oxygen concentration in this study was in the order of 10 ppm (0.3 mM) which although low is sufficient for the production of reactive oxygen species. The strong evidence for the low concentration of molecular oxygen in the pre 2.5 Gyr atmosphere has been summarised by Canfield (2005). Currently, the major evidence is the presence of sedimentary pyrite retaining a mass-independent sulphur isotope fractionation signature in rocks older than 2.45 Gyr (Papineau *et al.*, 2007). This requires a low partial pressure of molecular O_2 , not exceeding 10^{-5} bar (Pavlov and Kasting, 2002) which is equivalent to a concentration of 10 ppmv. Carver (1981) estimated an O_2 concentration of 10^{-3} to 10^{-1} PAL (200 – 20000 ppmv) which is supported by Canfield's (2005) estimation of 10^{-3} PAL. This approaches the upper limit of the oxygen-free atmospheres used in this experimentation and is far higher, by orders of magnitude than the concentrations of molecular oxygen in the presence of Ti(III) citrate. It is thus likely that the oxygen level in the Hadean atmosphere was similar to or higher than that which the samples have been exposed to in this study.

5. Conclusions

Treatment of pDNA with as little as 0.1 mM Fe(II) induces scission of the ribose-phosphate chain in a proportion of plasmids without hydrogen peroxide or reducing agents. This effect is proportional to the concentration of Fe(II) between 0.1 mM and 25 mM. At higher concentrations the intensity of DNA bands on the gels is reduced. Fe(II) at 50 mM is sufficient to cause a complete loss of the pDNA signal. The cause of back-bone scission is likely to be the production of hydroxyl radicals at

pDNA sites where iron ions have bound. The loss of pDNA from the gel may be related to the scission of the back-bone; at higher Fe(II) concentrations the DNA may be broken into monomers or oligomers too small to be retained or visualised on the gel. Alternatively, the loss of DNA from the gel may be due to a direct interaction between pDNA and Fe(II) or Fe(III) or the binding of DNA to iron hydroxide precipitates and its sequestration in the loading-wells. There is evidence that Fe(III) may be the prevalent reacting species here which caused loss of pDNA signal at a lower concentration than the Fe(II) solutions, approximately a third of which would be autoxidised to Fe(III). Fe(III) reacts with DNA in the absence of reducing agents. This is contrary to what has been reported in biomedical studies which have not examined Fe(III) concentrations high enough to observe this reaction.

This study highlights the fact that an environment with even relatively low concentrations of both Fe(II) and O_2 results in an oxidative stress which can decompose DNA and presumably other biomolecules, especially polymers. In a low Fe(II) environment, this may have assisted DNA evolution by allowing strands to be cut up and recycled. At higher concentrations, including those proposed in the FeS-world hypothesis, or when Fe(III) is present, other reactions occur with Fe(II) or Fe(III) which may have been highly detrimental to an evolving proto-organism.

Chapter 4

An electrophoretic study of the effect of aqueous sulphide on plasmid DNA

Abstract

The reaction between sulphide and DNA *in vitro* at 25°C in aqueous solution has been investigated in light of recent observations that sulphide is genotoxic in living cells and the hypotheses that life emerged in an alkaline sulphidic environment. No evidence has been found for a direct interaction between sulphide and plasmid DNA. When incubated with sodium sulphide, plasmid DNA was irreversibly denatured. That is, the hydrogen bonding between base pairs was disrupted. This is attributed to the alkalinity of the solution as no effect on DNA was observed with the less alkaline ammonium sulphide whilst sodium hydroxide did induce DNA denaturation. The genotoxicity of sulphide must therefore involve interactions between sulphide and components of the cell other than DNA. In a prebiotic scenario, high concentrations of sulphide may have hindered the emergence of a proto-organism. Extreme alkalinity ≥12 would have prevented the base pairing of nucleic acids and possibly severed the ribose-phosphate backbone. Thus there is an upper limit to the pH of the environment in which DNA replication, translation and transcription could have evolved.

1. Introduction

Volcanically derived sulphide has been suggested to have been a source of energy for prebiotic synthesis and metabolism (Russell and Arndt, 2005; Russell and Hall, 1997; 2002; Russell *et al.*, 2005; Russell *et al.*, 1988; Russell *et al.*, 1990; Russell and Martin, 2004; Russell and Turner, 1989; Wächtershäuser, 1988; 1990; 1992; 1994; 1998; 2003). A proto-organism with DNA translation and transcription emerged from this system and was capable of surviving and thriving within this high

sulphide environment. Even after the development of DNA translation and transcription, sulphide would have been abundant in these environments.

Attene-Ramos *et al.* (2006) suggested that the production of sulphide in the human colon, by commensal sulphate-reducing bacteria, may be involved in the development of colorectal cancer and have experimentally demonstrated that sulphide is a genotoxic agent in living cells. Whether this is the result of a direct reaction between sulphide and DNA or a reaction which involves other cellular components is not known.

Given the potential of sulphide to react with DNA (Attene-Ramos *et al.*, 2006) it could be suggested that it may have prevented the emergence of life in the scenario above. If this is the case, DNA in sulphidic, prebiotic scenarios without the protection and repair mechanisms found in living cells would have been susceptible to deleterious reactions. Therefore, the hypothesis of a proto-biont in a sulphidic environment may be brought into question.

Little experimental work has been conducted on the effect of sulphide on DNA. What has been done (Attene-Ramos *et al.*, 2006; Hughes *et al.*, 1984) has demonstrated that hydrogen sulphide is a genotoxic agent in living cells at concentrations of as little as 250 μM, comparable to that found in the human colon. The effect of sulphide on DNA in these studies has been dependent on or enhanced by inhibition of DNA repair mechanisms.

Free sulphide (S(-II)) exists in aqueous solutions mostly in the form of H_2S and HS-, with negligible S^{2-}. The pK_1 (H_2S) is close to 7 (Suleimenov and Seward, 1997) so H_2S dominates the system at acidic pH, whilst the proportion of HS^- increases with the pH of the solution. HS^- and H_2S engage in different reactions. HS^- is highly nucleophilic and is a Lewis base whereas H_2S can act as a Lewis base or

acid and is not nucleophilic. In contrast, H_2S is an excellent electron acceptor, whilst HS^- is not (Rickard and Luther, 2007). This pH dependent speciation of the sulphide is relevant as DNA can be altered by nucleophilic attack. DNA has several electrophilic sites such as the N7 and O6 of guanines and the N3 of adenines, and P-O bonds are also susceptible to nucleophilic attack. Methionine, a sulphur-containing amino acid, reacts with adenosine triphosphate to form S-adenosylmethionine. This occurs by the methionine-sulphide attacking the 5' carbon in ribose and replacing the oxygen and attached phosphates with the methionine group, i.e. the phosphodiester (P-O) bond is broken.

Previous experimentation has been conducted on living cells incubated with sulphide with the DNA subsequently extracted or stained and then analysed (Attene-Ramos *et al.*, 2006; Hughes *et al.*, 1984). This does not indicate a direct reaction between sulphide and DNA. The reported DNA damage could result from an interaction between sulphide and other cellular constituents or inhibition of proof-reading or repair enzymes. To determine if this reaction is relevant in a sulphidic, prebiotic setting and to elucidate further information on the mechanism of genotoxicity it is necessary to remove the complicating cellular elements and incubate DNA with sulphide *in vitro*. In this paper, an experimental investigation of the direct reaction between sulphide and pDNA occurring *in vitro* at sulphide concentrations up to hypothetical prebiotic levels is reported.

2. Materials and methods

2.1. Plasmid DNA preparation

pUC18 plasmid DNA (pDNA) was extracted from cultures of *Escherichia coli* grown in LB broth (tryptone, yeast extract and NaCl) with 100 mg/L ampicillin using

Wizard® Plus SV Minipreps DNA Purification System according to the manufacturer's instructions. pDNA was stored in nuclease free water and at -20°C until required. All reagents were of analytical grade. Sterile, O_2-free deionized water (dH_2O) was used throughout. MilliQ ™ 18 MΩ H_2O was sterilized through autoclaving for 2 h at 120°C and 2 Atm in a *Priorclave*™ and deoxygenated by bubbling O_2 free N_2 for at least 1 h. All containers were sterile on purchase or sterilized through autoclaving or washing with Decon 90® and ethanol when autoclaving was not possible.

2.2. Sample preparation

Sulphide stock solutions of 0.5, 5, 50 and 500 mM were prepared with analytical grade sodium sulphide nonahydrate ($Na_2S.9H_2O$) dissolved in dH_2O. 50 µL of sodium sulphide solutions and pDNA were prepared in 0.2 mL microfuge tubes along with 10 mM Tris buffer, a buffer of pH 8 used commonly in DNA experiments. Sodium sulphide concentrations were varied from 0.1 mM to 150 mM.

In order to separate the effect of S(-II) from that of OH^- and Na^+, similar series of experiments were prepared with aqueous solutions of NaOH and $(NH_4)_2S$. The effect of pH on the Na_2S reaction was investigated by adding NaOH to a sulphide series to a concentration of 0.1 M NaOH. The pH of the samples could not be measured directly due to their small volume so samples were replicated in larger vials with the same concentration of reactants and buffer except dH_2O was substituted for DNA (DNA at these concentrations is not expected to affect pH). pH was measured using a *Jenway*™ 2030 digital pH meter.

2.3. Electrophoresis

All samples were incubated for 1 h in a *Labmaster 130*™ anoxic glove box filled with O_2 free N_2. The samples were removed from the glove box for electrophoretic analysis. 5 μL of the samples were mixed with 1 μL of 5xloading buffer (pH = 8) and loaded on a 0.7% agarose gel with ethidium bromide added to a concentration of 10 μg/mL. Electrophoresis was carried out at 80 V. The gels were photographed with ultra-violet light in a Gene Genius Bio Imaging System™ and band intensity was measured using GeneTools™ densitometry from SynGene ™ which computes the quantity of DNA in a band from its intensity and the size of a DNA band from its rate of migration relative to a standard molecular weight marker (Bioline HyperLadder 1). pDNA extraction produces solutions of different pDNA concentrations on each extraction. A positive control was used in each experiment using the same pDNA stock solution as the experimental samples.

3. Results

As shown in Fig. 1, ≥ 50 mM Na_2S caused a reduction in band intensity of pDNA. No relaxation of the super-coiled pDNA was observed. The quantification of the effect (Fig. 2) showed that the decrease in DNA signal intensity was observed at ≥ 50 mM Na_2S for pDNA at 42.1 and 19.6 μg/mL. The reduction in DNA was proportional to the initial ratio of the reactants, which is consistent with a chemical reaction occurring between pDNA and the Na_2S solution.

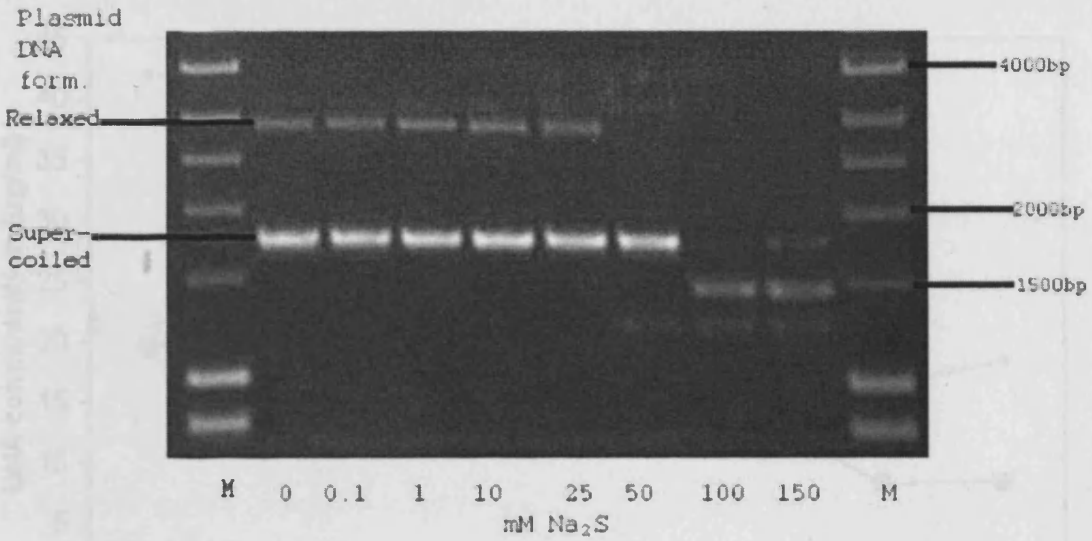


Figure 1. A typical gel electrophoresis result from the reaction of Na_2S with plasmid DNA. Na_2S concentrations are given below the image of the gel, the positions of the relaxed and super-coiled plasmid and the size of the molecular weight markers are indicated.

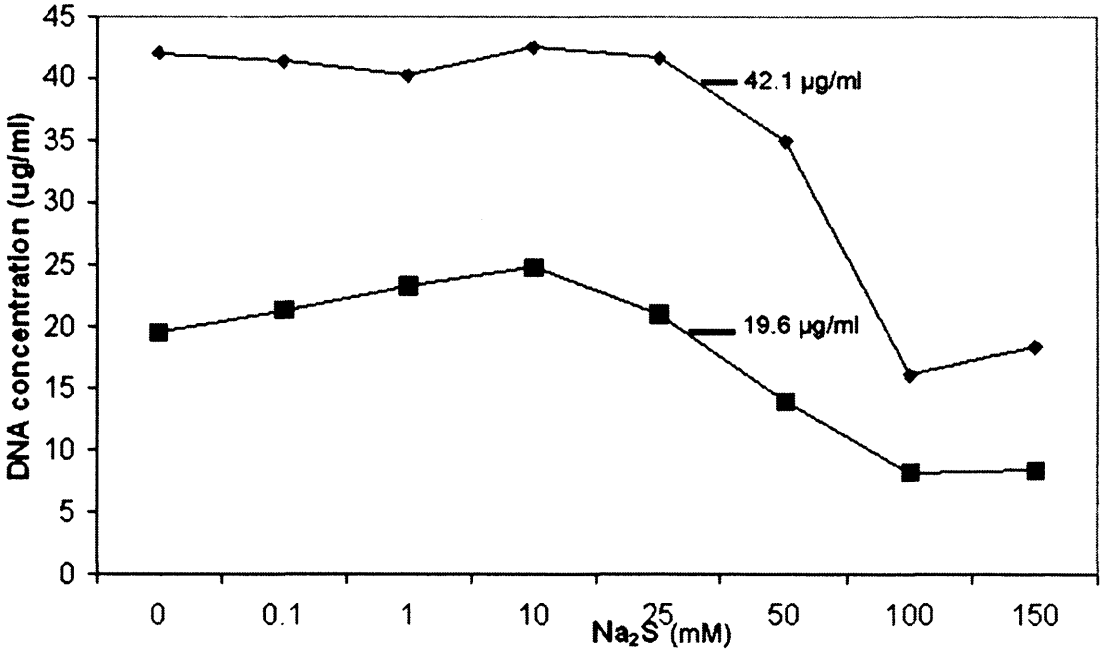


Figure 2. The reduction in total DNA concentration (relaxed + super-coiled + additional bands) with increasing Na_2S concentration. The results for two initial pDNA concentrations and 19.6 and 42.1 µg/mL) are shown. Raw data for this chart is provided in Appendix 2.

For an initial pDNA concentration of 42.1 μg/mL the total amount of DNA at 100 mM Na_2S was reduced to > 50% and exists almost entirely as two different bands (Figure 3). The total intensity of these bands at ≥ 100 mM Na_2S corresponds to < 50% of the total initial concentration of pDNA. The lower of the two bands (Band 2; Figure 3) was found at ≥ 50 mM and coincides with the loss of the relaxed plasmid. The upper band (Band 1; Figure 3) was formed at ≥ 100 mM Na_2S and coincided with the reduction in intensity of the super-coiled band.

The sizes of the bands were estimated from their migration rate using GeneTools™ and are 2550 ± 255 and 2940 ± 294 nucleotides (assuming an accuracy of ± 10%) for bands 1 and 2 respectively. However, these measurements are standardized for linear DNA and the shape of a DNA molecule will affect its electrophoretic migration rate. Birnboim and Doly (1979) showed that the difference in migration rate of equally sized single-stranded linear DNA compared to single stranded circular DNA is small and it is assumed that it is contained within the ± 10% precision.

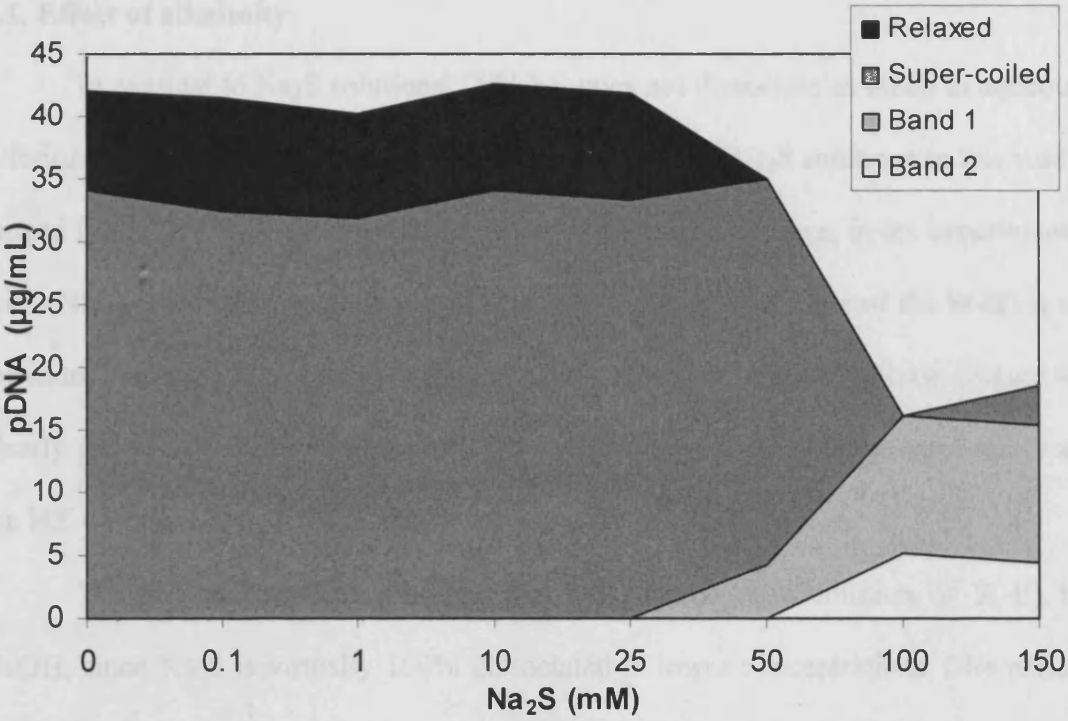


Figure 3. The total amount of pDNA and the abundance of each form or fragment for the experiment 42.1 µg/mL pDNA + Na_2S. Raw data for this chart is provided in Appendix 2.

3.1. Effect of alkalinity

In contrast to Na_2S solutions, $(NH_4)_2S$ does not dissociate as much in aqueous solutions and retains a less alkaline pH. The pH of the $(NH_4)_2S$ solutions in this study ranged from 7.9 to 9.7 for 0.1 mM and 150 mM respectively. Thus, in the experiments with $(NH_4)_2S$ the more nucleophilic HS^- is less dominant and more of the S(-II) is in the more oxidizing H_2S form. The results of the experiments with $(NH_4)_2S$ (Figure 4) clearly showed no effect of aqueous S(-II) in the form of H_2S over the same range as the HS^- dominated Na_2S solutions.

The sodium sulphide solutions can be regarded as a solution of S(-II) in NaOH, since Na_2S is virtually 100% dissociated at lower concentrations. The pH of the solutions varied between pH = 8.2 for 0.1 mM Na_2S to pH = 12.7 for 150 mM Na_2S solution. The degree of dissociation is suggested by comparing the theoretical values for 100% dissociation (pH =10 for 0.1 mM Na2S and pH =13.2 for 50 mM Na_2S) with the measured values. The effect of pH on pDNA was investigated by replacing Na_2S with NaOH. The results (Figure 5) show loss of the relaxed DNA band at 0.1 M and increasing loss of the supercoiled DNA band at higher NaOH concentrations. At 0.3 M NaOH a DNA band below the super-coiled band was observed as seen in the experiments with $\geq$ 50 mM. DNA was not detected on the gel at 0.8 M or 1 M NaOH. The pH of the 1M NaOH solution was found to be 12.2. The results showed that the DNA loss on reaction with Na_2S and the appearance of more-rapidly migrating bands can be most likely explained as a result of the effect of alkaline pH on DNA.

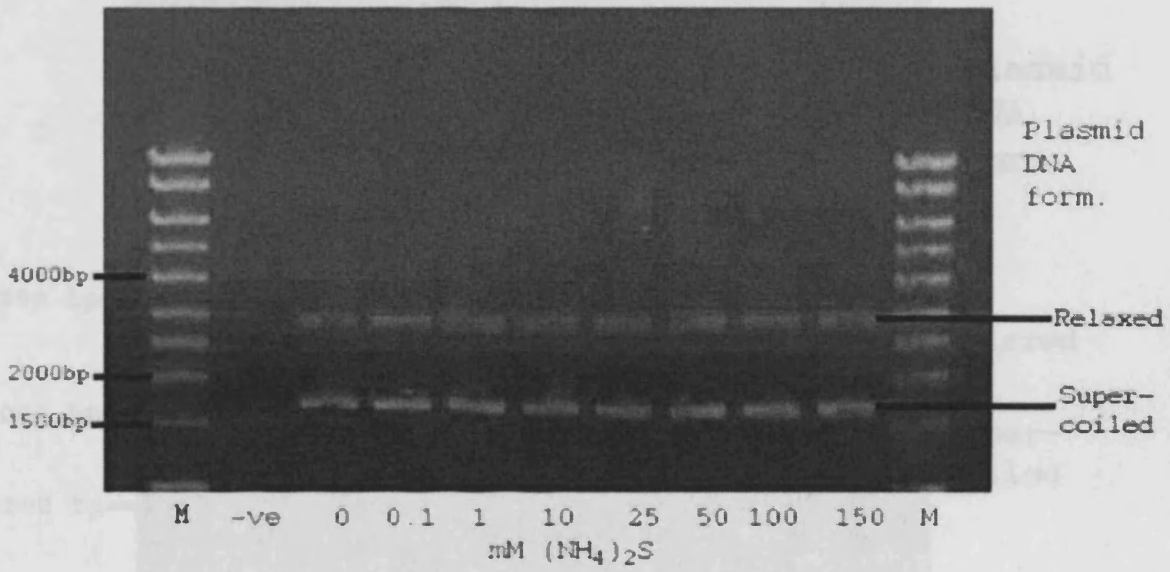


Figure 4. The effect of $(NH_4)_2S$ on pDNA. Ammonium sulphide concentrations are given below the image of the gel, the positions of the relaxed and super-coiled plasmid and the size of the molecular weight markers are indicated.

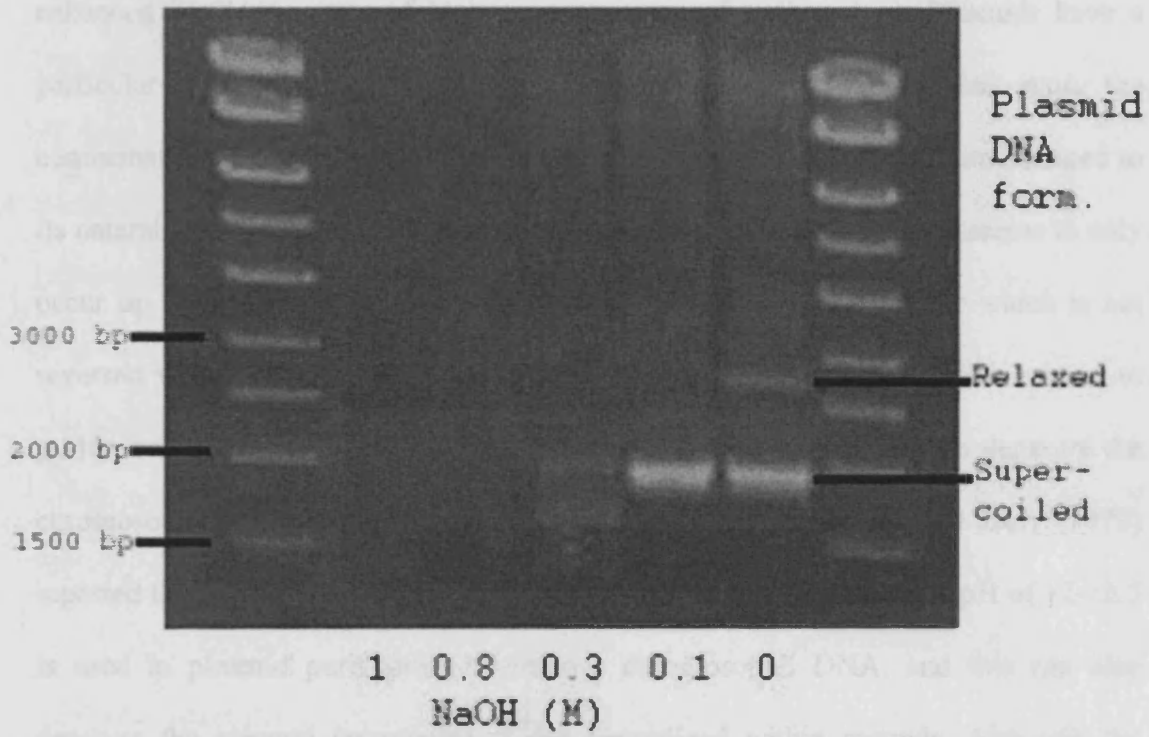


Figure 5. Effect of NaOH on plasmid DNA. Sodium hydroxide concentrations are given below the gel, molecular weight markers and the positions of the two pDNA bands are labelled.

4. Discussion and conclusions

At highly alkaline pH, DNA strands separate from each other, in the process known as melting or denaturation. Dore (1972) demonstrated that this effect is enhanced in the presence of high concentrations of sodium ions. Plasmids have a particular resistance to denaturation, since, due to their super-coiled state, the conformation of the molecule is not altered and the plasmid can return undamaged to its natural form once the solvent is neutralized. However, this resistance seems to only occur up to a point, beyond this the plasmid is denatured in a manner which is not reversed when the solution is neutralized. This feature of plasmid DNA is used to purify it from chromosomal DNA in which a pH of 12-12.5 is used to denature the chromosomal DNA but leave the plasmid DNA intact. Birnboim and Doly (1979) reported that at pH $\geq$ 13 plasmid DNA can be irreversibly denatured. A pH of 12-12.5 is used in plasmid purification to remove chromosomal DNA, and this can also denature the plasmid irreversibly if not neutralized within seconds. Although the irreversible denaturation of plasmid DNA has not been chemically described, it is known that its migration rate in electrophoresis is more rapid than that of natural super-coiled pDNA (Birnboim and Doly, 1979; Thatcher *et al.*, 1997). Band 1 (Figure 5) is most likely irreversibly denatured super-coiled plasmid in which base-pairing between nucleotides on opposing strands has been permanently prevented although the molecule remains in the super-coiled topology. The evidence for this is that it only appears at $\geq$ 100 mM Na_2S (pH = 12.5) which coincides with the loss of the super-coiled plasmid and therefore must be derived from super-coiled plasmids being denatured by the high pH. At 50mM Na_2S only the relaxed pDNA band is reduced in intensity.

Band 2 appeared at ≥ 50 mM Na_2S. Two interesting observations about this are that; (a) the relaxed pDNA band disappeared at this point and that there is no loss of the super-coiled plasmid. This means that this band must be derived from the relaxed pDNA. Given that the relaxed plasmid is not as resistant to denaturation as its super-coiled counter-part, it may be concluded that this band is likely to be single-stranded DNA produced by the separation of the double-helix of the pDNA. The mass of this band was calculated using GeneTools™ as corresponding to 2550 ± 255 nucleotides (assuming an error of ±10%). A complete single strand of the plasmid has 2686 nucleotides which is within this range. Its migration will be affected by whether it is circular or linear, however. Birnboim and Doly (1979) reported that this difference is minimal. Treatment with nuclease enzymes can be used to distinguish between linear and circular DNA but as these bands are produced by alkalinity and not sulphide this has not been pursued in this study.

The total intensity of these two bands corresponds to only approximately 50% of either initial pDNA concentration at ≥ 100 mM Na_2S. This loss of intensity was also seen with NaOH and is therefore also the result of alkalinity rather than sulphide. Birnboim and Doly (1979) observed the production of linear single-stranded DNA from plasmids treated with solutions of high pH. This implies that OH^- can break single stranded DNA which could explain the apparent disappearance of the pDNA. The pDNA may be fragmented by OH^- into oligomers which are too small to be retained and/or too low in concentration to be visualised on the gel. However the results of this study show no evidence of fragmentation by OH^- as it is unknown if either band 1 or 2 are linear. It should also be noted that irreversibly denatured DNA by alkalis has not been chemically described so it cannot be ruled out that this effect may have inhibited the intercalation and/or fluorescence between the DNA-dye

ethidium bromide and the pDNA. This would mean that the pDNA remains intact but not fluorescing on the gel. The possibility that DNA was somehow prevented from migrating through the gel by being neutralised, for example, is unlikely since no fluorescence was seen in the well.

S(-II) does not react with DNA at concentrations from 0.1-150 mM either as HS- or H_2S. These results provide no evidence for a direct reaction between S(-II) and DNA at physiological sulphide concentrations. Therefore the sulphide-induced DNA damage reported by Attene-Ramos *et al.* (2006) must involve reactions between S(-II) and other cellular components or some secondary feature of the reaction chemistry. Cellular DNA damage is often attributed to radical production. Sulphide can enhance radical production in cells via precipitation with Fe(II) to FeS which is thought to decompose hydrogen peroxide more efficiently than Fe(II) alone (Berglin and Carlsson, 1986). Also sulphide could act as an Fe(III) reducing agent in the cell, Fe(III) must be reduced to Fe(II) in order to initiate free radical production (Oikawa and Kawanishi 1998).

4.1. Implications for the origin of life

These results do not appear to present any challenges to the hypotheses that life emerged in a sulphidic environment. Some indication of the intended sulphide concentrations are given in these hypotheses. Wächtershäuser (1992) states 100 mM H_2S was required to convert FeS to pyrite. Russell and Hall (1997) calculated HS^- concentrations of 20 mM and used 100 mM to produce compartmentalised FeS structures. (Russell and Arndt, 2005) claim that up to 10 mM HS^- would have been supplied to the seepage sites in which life emerged. These concentrations are within the range of this experimentation where no effect on DNA due to S(-II) was observed.

These results also show that Na^+ concentrations up to seawater concentrations do not affect DNA. In contrast, the results do provide an upper limit to the pH of the environment in which DNA could be formed. Russell and Hall (1997), for example, proposed that the electromotive driving force for prebiotic reactions involved the potential gradient set up at interfaces between an early acidic ocean and alkaline hydrothermal solutions. The results of this experimentation suggest that extreme alkalinity (e.g. pH > 12) would have prevented the base-pairing of nucleic acid molecules and possibly also any interaction with amino acids. If OH^- concentrations were high enough the hostility of the environment to DNA would make the emergence of translation and transcription very difficult unless the nucleic acids were sequestered in a protective environment. An alternative view point would be that if OH^- does severe single stranded DNA then it could have provided a mechanism for the breakage of DNA strands in a manner that allowed them to recombine in novel ways. This cycling of nucleic acids would have provided a high rate of molecular evolution and thus possibly have been a benefit to an emerging organism. OH^- or S(-II) may act in concert with other ions in prebiotic environments, particularly metals, and this is the subject of further study.

Chapter 5

FeS-Induced radical formation and its effect on plasmid DNA

Abstract

The origin of life has been proposed to have occurred at a hydrothermal vent and was facilitated by iron (II) monosulphide precipitated from the sulphidic vent solution and the iron-bearing oceanic water. The effect that this material would have on nucleic acid polymers has been investigated. Plasmid DNA was incubated with solutions of iron (II) monosulphide, precipitated from iron (II) sulphate and sodium sulphide, and analysed by electrophoresis. At $\geq$ 0.1 mM iron sulphide an increase in the proportion of the relaxed plasmid DNA occurred caused by scission of the DNA backbone. The reactant is proposed to be a sulphur-based radical produced from the iron-catalysed decomposition of bisulphide, in a manner analogous to the Fenton reaction. At 25 – 150 mM iron sulphide, a reduction in DNA concentration was observed which was attributed to adsorption onto the surface of the iron sulphide precipitate. This supports the hypothesis that iron sulphide minerals could have adsorbed and sequestered nucleic acids but also indicates they would have been subject to attack by free radicals.

1. Introduction

It has been hypothesised that life emerged on or adjacent to metal-bearing minerals precipitated at submarine hydrothermal vents. (Russell and Hall, 1997; 2002; Wächtershäuser, 1988; 1998; 1990; 1992; Russell and Arndt, 2005; Russell and Martin, 2004). These minerals are thought to have adsorbed organic molecules and promoted the reactions between them that lead to the evolution of a mineral bound proto-organism.

Wächtershäuser (1990; 1988; 1992; 1994; 1998; 2003) proposed an FeS-world hypothesis in which the oxidation of mackinawite (FeS_m) to pyrite (FeS_2) is coupled with, and provides energy for, fixation of gaseous carbon and primitive metabolic reactions which occur on the pyrite surface.

Russell and co-workers (Russell and Arndt, 2005; Russell and Hall, 1997; 2002; Russell *et al.*, 2005; Russell *et al.*, 1988; Russell *et al.*, 1990; Russell and Martin, 2004; Russell and Turner, 1989) place the origin of life at the interface of sulphide bearing submarine seepage water and iron bearing oceanic water. The precipitate initially formed from this reaction, iron(II) monosulphide, is nanoparticulate material with a tetragonal mackinawite structure (Evans *et al.*, 1964). Rickard *et al.* (2006a) showed that this material is stoichiometric FeS. I refer to this material as FeS_m. I use FeS for generic, unspecified iron (II) monosulphides. FeS nanocrystals have excellent catalytic properties (Cody, 2004; Cody *et al.*, 2004).

Mackinawite oxidises through a solid state reaction to form the iron thiospinel, greigite, Fe_3S_4 (Rickard and Luther, 2007). Griegite is more stable than mackinawite and the oxidation reaction is relatively facile at low temperatures in aqueous solutions (Rickard and Luther, 2007; Rickard and Morse, 2005). Russell and Hall (2006) considered that mackinawite provided the inorganic structure and reaction surfaces of the first membrane with the more oxidised greigite as a minor phase.

Theberge and Luther (1997), Rickard (1997), Luther and Rickard (2005) characterised aqueous FeS clusters (FeS_{aq}) and showed that these species were intimately involved in iron sulphide chemistry. The cluster structure is similar to the basic structural moiety of mackinawite and FeS_{aq} clusters have been shown to be involved in the formation of both mackinawite (Rickard and Luther, 2006) and pyrite

(Rickard *et al.*, 2001). Rickard (2006b) showed that FeS_{aq} clusters determined the solubility of nanoparticulate FeS_m at neutral to alkaline pH values.

In both of the iron-sulphur world hypotheses the emergence of most of the characteristics of modern cellular life occurs at the iron sulphide surface whilst it is exposed to very high concentrations of sulphide and metal ions. Detachment of the proto-biont from the mineral surface does not occur until after the development of fully fledged cells with lipid walls, enzyme catalysis and DNA replication, translation and transcription.

Biological macromolecules are sensitive to hydrolysis and oxidation reactions. Free radicals, generated by the redox reactions of intracellular iron and other metal ions, are a cause of cancer and mutations due to their capacity to react with DNA (Berglin and Carlsson, 1986; Imlay *et al.*, 1988; Kawanishi *et al.*, 2001; Toyokuni and Sagripanti, 1992). Mackinawite has a reactive surface (Wolthers *et al.*, 2005) and is associated with iron-sulphide nanoparticles and aqueous clusters both of which have potential to be highly reactive with regards to organic chemicals (Rickard *et al.*, 2001).

Since the FeS-world hypotheses were published there has been some interest in the reactions between iron sulphide minerals and molecules of prebiotic significance. Most of this has examined pyrite (FeS_2) which is prominent in the theories of Wächtershäuser. Bebie and Schoonen (2000) found that certain organic compounds including components of DNA; purine, adenine and d-ribose interact with the surface of pyrite. Iron (II) monosulphide (mackinawite) has been studied less although it plays a more important role in the theories of both Russell and Wächtershäuser.

Pyrite induces hydrogen peroxide formation regardless of the presence of oxygen whilst iron(II) monosulphide (FeS) does not (Borda *et al.*, 2001). Hydroxyl radicals are produced by the reaction between adsorbed water and Fe(III) in a defect site on the pyrite surface. Hydroxyl radicals readily react with DNA causing strand breaks, they can also react with each other to form hydrogen peroxide. Cohn *et al.* (2003) demonstrated that nucleic acids are destroyed in the presence of pyrite due to this reaction. .

Although not studied as much as pyrite, there is a number of ways in which FeS could interact with DNA. Firstly, the dominant dissolved species in an iron sulphide solution are aqueous clusters of the formula Fe_nS_n where n commonly equals 2 or 4 (Rickard and Luther, 2006; Rickard and Morse, 2005). These FeS_{aq} clusters are of interest in the origin of life because they closely resemble the clusters which constitute the active centres of many proteins including the ferredoxins which are an ancient and ubiquitous class of redox enzymes (Beinert, 2000). However, they are highly reactive species which could cause strand-breaks in biopolymers.

Secondly, both iron and sulphide are capable of generating free radicals in certain conditions. Fe(II) in oxic systems and/or in the presence of hydrogen peroxide generates hydroxyl radicals. Sulphur-based radicals may also be generated in oxic sulphide solutions when iron is present to catalyse the oxidation of hydrogen sulphide (Tapley *et al.*, 1999). There is also evidence for the existence of sulphide radicals able to form in entirely anoxic sulphide solutions. This comes from the observation that H_2S oxidises two thirds of the Fe(II) in mackinawite in a solid state reaction to Fe(III) during greigite formation which occurs in the absence of oxygen (Rickard and Luther, 2007). The electron acceptor in this reaction is unknown and may be a sulphide species. Little is known about these sulphide radicals due to the inherent difficulties

of studying highly unstable molecules. However, all free radicals are, by definition, highly oxidising.

Sulphide has been found to be genotoxic in living cells (Attene-Ramos *et al.*, 2006; Berglin and Carlsson, 1986). A proposed mechanism for this is reaction with cellular iron and the formation of iron sulphide, which has been reported to convert hydrogen peroxide to hydroxyl radicals more efficiently than ferrous iron (Berglin and Carlsson, 1986). The mechanism by which FeS decomposes hydrogen peroxide has not been reported and the reaction is only going to be relevant when hydrogen peroxide is present

Thirdly, one of the reasons FeS is thought to be involved in the origin of life is due to its highly reactive surface which is capable of adsorbing and reducing a suite of simple organic chemicals (Cody *et al.*, 2004; Huber and Wächtershäuser, 1997; 1998). However, during the later stages of the origin of life these surfaces may have been a hindrance by promoting hydrolysis or oxidation of biopolymers if they remain in the same locale. In both FeS-world hypotheses double-stranded DNA evolved within or on an iron sulphide mineral assemblage and detachment from the mineral did not occur until after the development of fully developed cells with DNA translation and transcription. Wächtershäuser (2006) hypothesises the emergence of circular dsDNA chromosomes similar to extant plasmids during his "semi-cellular" stage of evolution in which a quantity of cytosol is bound on one side by an FeS/pyrite mineral and by a lipid membrane on the other. Koonin and Martin (2005) building on the hypothesis of Russell *et al.* (Russell and Arndt, 2005; Russell and Hall, 1997; 2002; Russell *et al.*, 2005; Russell *et al.*, 1988) propose a retrovirus-like genetic cycle in which dsDNA is synthesised from an RNA-DNA hybrid within FeS compartments.

This study aims to consider the validity of the hypothesis that DNA was capable of emerging and remaining intact in proximity to FeS. DNA is a relatively stable molecule and the reaction with FeS should provide a guide to the problems of the development of prebiotic macromolecules in an FeS-environment in general. I report the results of an experimental program on the reaction of DNA with FeS at concentrations typical of those in hypothetical FeS-world scenarios.

2. Materials and methods

2.1 Plasmid DNA preparation

pUC18 plasmid DNA (pDNA) was extracted from cultures of *E.coli* grown in LB broth with 100 mg/L ampicillin using Wizard® Plus SV Minipreps DNA Purification System according to the manufacturer's instructions or purchased from Bayou Biolabs at ~1000 μg/mL and diluted with dH_2O. pDNA was stored in nuclease free water and at -20°C until required. Plasmid DNA electrophoresis was used because it is very effective at demonstrating damage due to its natural super-coiled topology. Strand-breaks in the molecule cause it to unwind or "relax" which retards its migration through an electrophoretic gel. Relaxed pDNA can be easily visualized as a band above the band of undamaged super-coiled pDNA. Also, Wächtershäuser (2006) claimed that the first double-stranded DNA chromosomes were circular and similar to pDNA.

2.2 Sample preparation

Stock solutions of 0.5, 5, 50, 500 mM Fe(II) and S(-II) were prepared with analytical grade $(NH_4)_2Fe(SO_4)_2.6H_2O$ (Mohr's salt) and $Na_2S.9H_2O$ (Fisher

Chemicals™) dissolved in dH_2O. In a Labmaster *130*™ anoxic glove box, filled with O_2 free N_2 sodium sulphide solution was added to Tris buffer, a buffer of pH 8 commonly used in DNA experiments, in a 0.2 mL microfuge tube. To this solution, $(NH_4)_2Fe(SO_4)_2.6H_2O$ was added to form an FeS precipitate and finally pDNA solution was added to give a final volume of 50 μL.

All reagents were of analytical grade. Sterile, O_2-free deionized water (dH_2O) was used throughout. MilliQ ™ 18 MΩ H_2O was sterilized through autoclaving for 2 h at 120°C and 2 Bars in a *Priorclave*™ and deoxygenated by bubbling O_2 free N_2 for at least 1 h. Studies of the resultant dissolved O_2 concentration (Butler *et al.*, 1994) show that the solution contains measurable O_2 in the 1-10 ppmv range. O_2 level in the anoxic cabinet was monitored and did not exceed 10 ppm. All containers were sterile on purchase or sterilized through autoclaving as above except plastic bottles for metal sulphate and sodium sulphide stock solutions which were washed with 2% Decon 90® and then rinsed with ethanol.

The FeS_m reactant was similar to that described in some detail by Ohfuji and Rickard (2006), Rickard *et al.* (2006) and Rickard (2006a). It is stoichiometric nanoparticulate FeS with a mackinawite structure (Fig. 1). The particles are between 2 and 5.7 nm thick and between 3 and 10.8 nm in length with a mean size of 3.6 x 5.8 nm. The specific surface area is 380 ± 10 m^2 g^{-1}. In acid to neutral solutions the solubility is described by

$$FeS_m + 2H^+ \rightarrow Fe^{2+} + H_2S \quad \text{.....(1)}$$

where $\log K_{sp,1}$ = 3:5 ±0.25. In neutral to alkaline solutions, the solubility can be closely described by the intrinsic solubility

$$FeS_m \rightarrow FeS^0 \quad \text{.....(2)}$$

where FeS^0 represents the FeS_{aq} cluster monomer and log $K_{sp,\,0}$ = -5.7. Iron sulphide concentrations were varied from 0.1 mM to 150 mM. The pH of the iron sulphide + DNA solutions was measured using a *Jenway 2030* ™ digital pH meter and are listed below.

Table 1. pH measurements of 50 mL analogues to FeS + pDNA preparations. The concentrations of all reactants were kept the same as in the experiments, 10 mL dH_2O was substituted for pDNA.

FeS concentration (mM)	pH
0.1	8.1
1	8.2
10	8.1
25	8
50	8
100	7.5
150	6.8

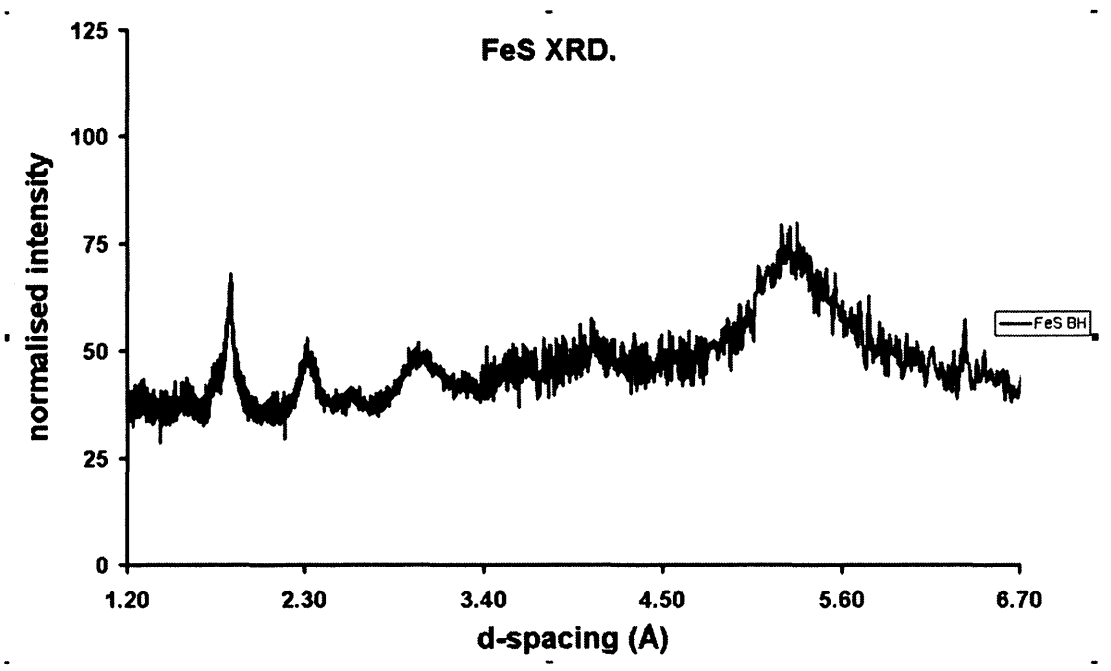


Figure 1. XRD confirmation of FeS_m structure. The d (001) spacing is 0.508 to 0.519 nm which compares with 0.503 nm for anhydrous mackinawite but which is less than the 0.660 to 0.548 nm spacing observed of hydrated mackinawite from LAXRPD.

2.3. Electrophoresis

All samples were prepared and incubated for 1 h in a *Labmaster 130*™ anoxic glove box filled with O_2 free N_2. The samples were removed from the glove box for electrophoretic analysis. A 5 μL aliquot of the samples was mixed with 1 μL of 5*loading buffer and loaded on a 0.7% agarose gel with ethidium® bromide added to a concentration of 10 μg/mL. 80 V was applied across the gel until the dye had moved sufficiently down it. The gels were then photographed with ultra violet light in a Gene Genius Bio Imaging System™. In some experiments the amount of pDNA on the gel was quantified using GeneTools™ densitometry from SynGene ™ which computes the quantity of DNA in a band from its fluorescent intensity relative to a standard marker: Bioline HyperLadder 1 which produces a series of bands with different quantities of DNA in each. The expected quantity of DNA in each band is provided by the suppliers. A single band is selected and assigned the expected DNA concentration which is used by the software to assign DNA concentration to all other bands on the gel. However, to increase accuracy HyperLadder 1 was added to a minimum of two wells on each gel (5 μL) and quantification was performed using at least 4 different bands from both markers and the mean concentration was taken. When initial pDNA concentration is given in the results section this refers to the total amount of pDNA measured on the gel in both the super-coiled band and the relaxed band in the FeS-free positive control for that experiment.

2.4. FeS_{aq} clusters

Formaldehyde is interesting in this context since it has been shown to react with FeS to prevent the formation of FeS_{aq} clusters (Rickard *et al.*, 2001). It is therefore possible to test if these highly reactive clusters are involved in the reaction

with DNA by adding small amounts of formaldehyde to the experimental system. Aqueous formaldehyde solution, purchased from Sigma-Aldrich, was added to four 1 mM FeS + pDNA solutions to a final concentration of 2 mM formaldehyde and run on the same gel along with four 1 mM FeS + pDNA solutions without formaldehyde.

2.5. Molecular O_2

A problem with the standard methodology described above is that although the reagents and reaction were prepared and carried out under anoxic conditions, electrophoresis was routinely carried out, for obvious practical reasons, in a system exposed to the atmosphere. In order to determine if this had any effect on the reaction, the electrophoresis tank was placed in the anoxic cabinet and the samples loaded directly on to it, without being exposed to atmospheric oxygen at any point. All solvents, including electrophoresis buffer, were thoroughly deoxygenated with oxygen-free nitrogen and the anoxic cabinet was monitored at ≤ 10 ppm O_2.

Ti(III) citrate has been used extensively in iron sulphide chemistry as a strong reducing agent (Zehnder and Wuhrmann, 1976). The measured Eh of an aqueous Ti(III) citrate solution at neutral pH approaches - 400 mV. The addition of Ti(III) citrate to the experimental systems removes the possibility of the reaction between Fe(II) and O_2 and the consequent Fenton reactions. Ti (III) citrate was prepared from 50 mL 0.2 M Na citrate and 5 mL 1.8 M $Ti(III)Cl_3$ brought to a pH of 7 with $NaCO_3$. Ti(III) citrate was added after the precipitation of FeS_m and before the addition of pDNA. The final Ti(III) citrate concentration in the reaction vessel was 3.6 mM. Ti(III) citrate was added to a series of FeS + DNA solutions, including the FeS-free control, and run on a gel as described above.

2.6. Free radicals

Dimethyl sulphoxide (DMSO) is a general free radical trap which is widely used for trapping hydroxyl radicals (Jakob and Heber, 1996; Floyd and Lewis, 1983; Imlay *et al.*, 1988; Sagripanti, 1999) but it is a non-specific radical scavenger (Nagel *et al.*, 2006; Nguyenduy *et al.*, 1986) and may also trap sulphide radicals. DMSO (Fisher Chemicals™) w as used to test whether free radicals are involved in the reaction of FeS with DNA, it was added to FeS and pDNA solutions to a final concentration of 2.8 M DMSO. One gel was run with 100 and 150 mM FeS with and without 2.8 M DMSO and one was run with 4 repetitions of each of 1 mM FeS, 2.8 M DMSO and 2.8 M DMSO + 1mM FeS.

Catalase is an enzyme which decomposes hydrogen peroxide (H_2O_2) to H_2O and O_2 preventing OH· formation. Catalase from Sigma-Aldrich was added to four solutions of pDNA + 0.1 mM FeS to a final concentration of 1000 units/mL catalase. These solutions were run simultaneously on a gel as above, quantified with Genetools and the percentage pDNA relaxation was measured and compared against a 0.1 mM FeS + pDNA control without catalase.

Ultraviolet light enhances Fenton reaction-induced DNA damage. To test if the reaction in this study is similarly enhanced 4 solutions of 1 mM FeS + pDNA were incubated under ultraviolet light. The light source was a 4 W mercury UV lamp (Mineralight, UVSL-25, giving 80 /tW/cm^2 at 45 cm height, UV products inc) for 30 min of the 1 h incubation it was set for long wavelength UV light (peak intensity at 366 nm) and for the following 30 min was set to short wave (254 nm). The lamp was placed 10 cm from the samples which were prepared as above except that they were prepared in UV-permeable micro cuvettes. For comparison 4 samples were prepared

with 1 mM FeS + pDNA and wrapped in aluminium foil, 4 pDNA controls were placed under the UV lamp and 4 pDNA controls were wrapped in aluminium foil.

3. Results

An increase in the proportion of relaxed pDNA occurred on incubation with FeS at FeS concentrations as low as 0.1 mM (Figures 2&3). In the untreated control (Figure 2, 0 mM) pDNA existed mostly in the super-coiled form (lower band). At concentrations of FeS as low as 0.1 or 1 mM an increase in the proportion of the relaxed pDNA (upper band) was observed which was proportional to the concentration of FeS. When incubated with 25 mM FeS there was no detectable super-coiled pDNA on the gel. However, super-coiled DNA was present at higher concentrations. At 25, 50, 100 and 150 mM both relaxed and super-coiled pDNA are visible, although the bands are smeared, broken and at a lower intensity than the positive control. Lateral streaks can be seen above the bands at $\geq$ 25 mM.

Over the concentration range 0.1 to 10 mM total DNA concentration remained approximately constant. FeS at concentrations of 25 mM or higher caused a significant drop in the intensity of both the super-coiled and relaxed pDNA (Figs. 2 & 4). When incubated with 100 or 150 mM FeS approximately only 60% of the pDNA could still be detected (Fig. 4).

In other gels, prepared under the same conditions but with a lower pDNA concentration, the pattern was different. Complete relaxation of the pDNA was observed at 25 mM FeS in most cases. However, above this, depending on DNA concentration, a complete loss of both supercoiled and relaxed pDNA often occurred.

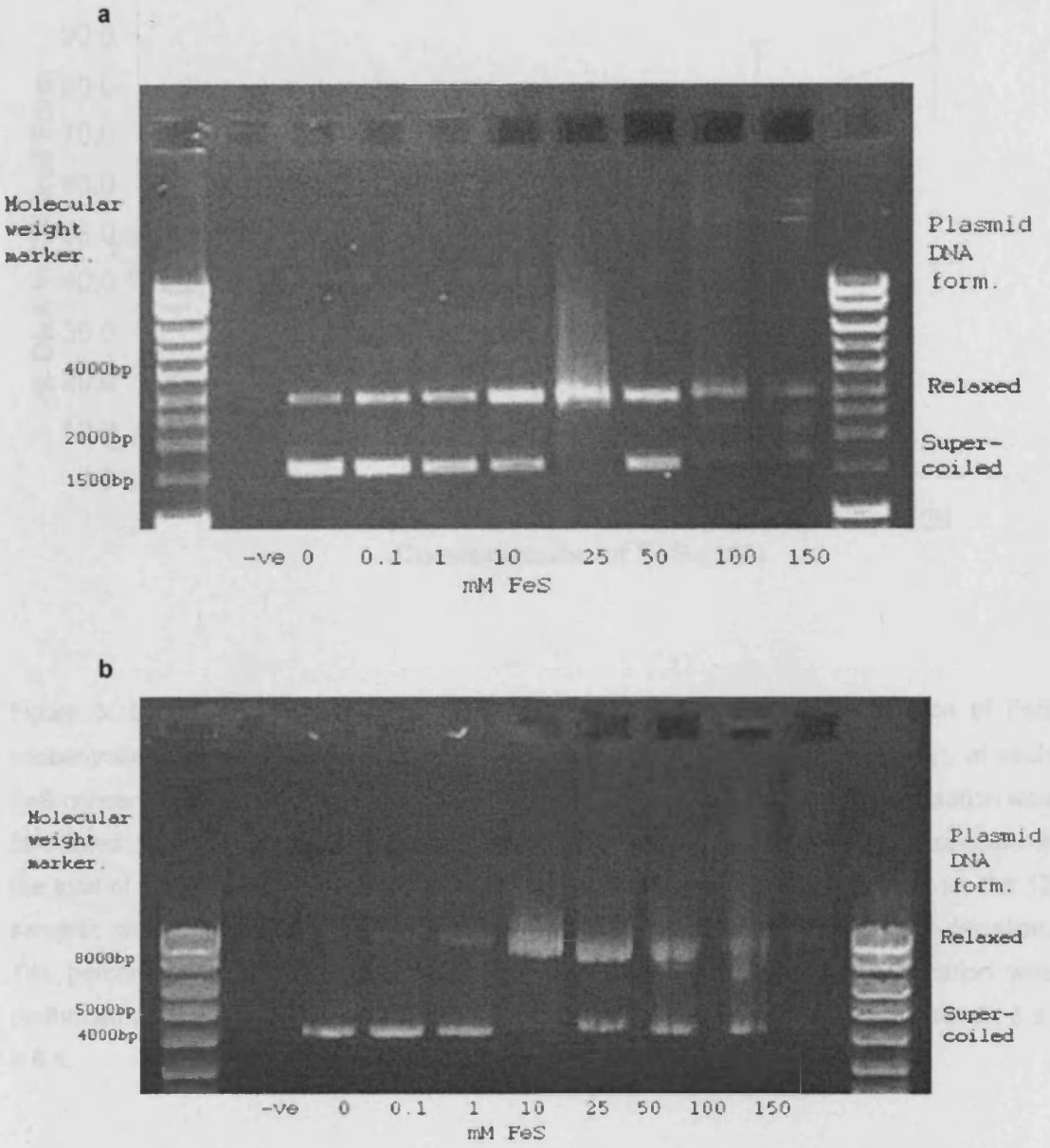


Figure 2: **a**. Image of a typical electrophoretic gel of FeS + pDNA. Iron sulphide concentrations are given below the image of the gel, the positions of the relaxed and super-coiled plasmid and the size of the molecular weight markers are indicated. The concentration of pDNA detected in the positive control (0mM FeS) is 17.2 ug/mL. **b**. Second gel electrophoresis result of the reaction between FeS and pDNA.

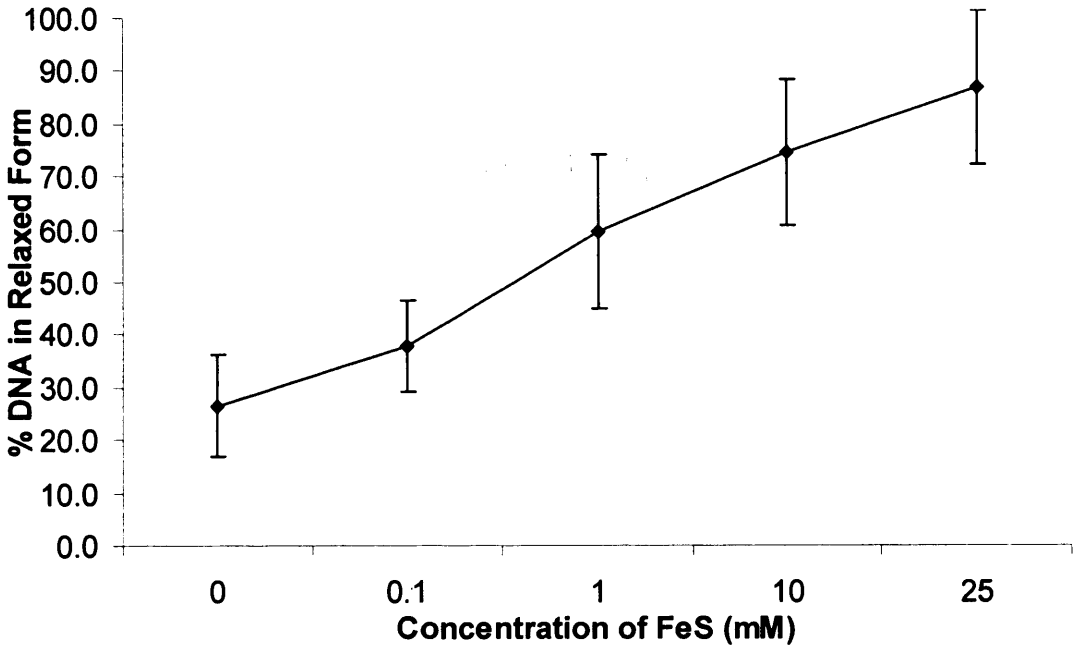


Figure 3: Graph showing FeS-induced relaxation of plasmid DNA as a function of FeS concentration. 12 samples, with differing concentrations of pDNA(1.3-78.5 μg/mL), at each FeS concentration (0.1, 1 10, 25 mM) were run on 6 different gels. Percentage relaxation was calculated, for each sample, from the amount of pDNA in the relaxed state as a percentage of the total of both the relaxed and super-coiled bands. Each point shows the mean for the 12 samples plotted against concentration of iron sulphide, error bars are ± standard deviation. The percentage of pDNA relaxed increased with FeS concentration. Quantification was performed using BioLine hyper ladder 1. Raw data for this chart is provided in Appendix 3 a, b & c.

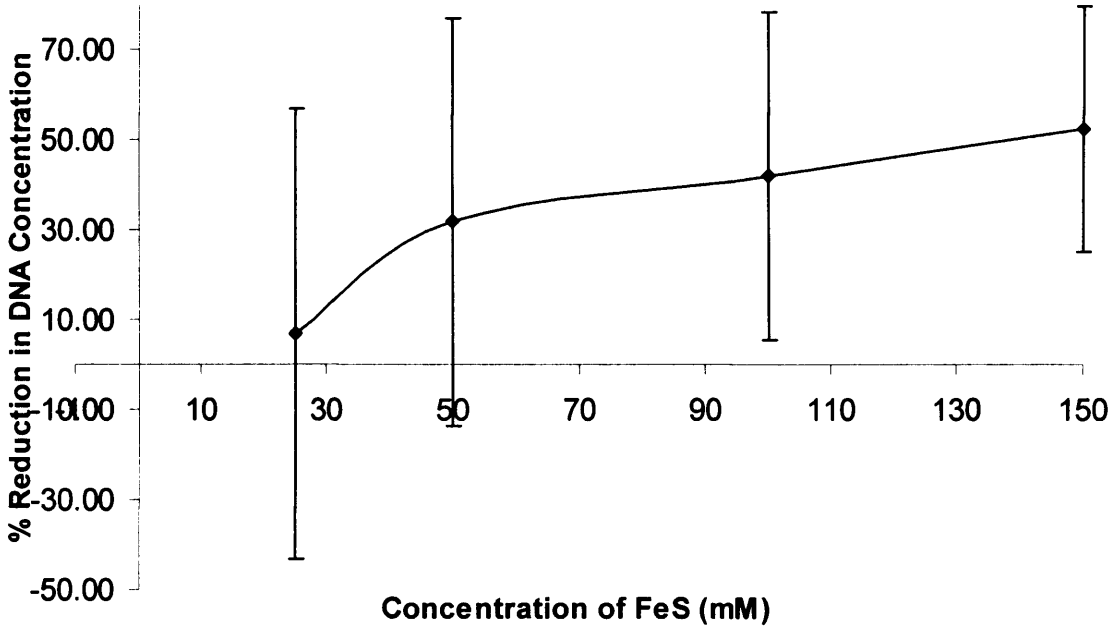


Figure 4: Graph showing percentage reduction in pDNA concentration against FeS concentration. Reduction in DNA concentration was calculated for each sample from the total of the pDNA in the relaxed and super-coiled band for each FeS + pDNA preparation subtracted from the total pDNA in the corresponding FeS-free control. Each point is the mean of 12 at pDNA at concentrations of 1.3-78.5 μg/mL against FeS concentration (25, 50, 100 and 150 mM) error bars are ± standard deviation. Raw data for this chart is provided in Appendix 3 a, b & d.

The total amount of DNA relaxed was dependent on the initial DNA concentration, the more DNA in the reaction vessel the more was relaxed across the FeS concentration range 0.1-10 mM (Fig. 5). However, the percentage pDNA relaxed was constant across initial pDNA concentration for 0.1, 1 and 10 mM FeS (Fig. 6). On average 26.5 % of pDNA was relaxed in the FeS-free control which rose to 37.7, 59.4 and 74.3 % for 0.1, 1 and 10 mM FeS respectively.

The effect of 2.8 M DMSO on the reaction at 100 and 150 mM FeS (Fig. 10, lanes 2 & 3) was to partially inhibit both the relaxation and loss of pDNA. More DNA is visible in Fig. 10, lanes 5 & 6 and more of it is in the super-coiled form than in Fig. 10, lanes 2 & 3. A DMSO control (Figs. 8, 3) showed no effect on FeS-free pDNA (Fig 10 lane 4). Fig. 10, 2&3 show the usual effects of 100 and 150 mM FeS on pDNA. At a lower concentration of FeS (1 mM) 2.8 M DMSO completely inhibited the relaxation of pDNA (Table 3).

The relaxation of pDNA induced by 0.1 mM FeS was not inhibited by 1000 units/mL catalase (Table 2). The effect of catalase was analysed at 0.1 mM FeS as at high salt concentrations enzymes suffer inhibition. This study involved 4 replicates of each treatment. With the 0.1 mM FeS control the mean percent of pDNA relaxed was 46.4 %, with the FeS + catalase sample a mean of 39.7 % of pDNA was relaxed. A one way ANOVA was performed, the F value for a $p \leq 0.05$ was calculated to be 6.0 therefore, the small reduction in percentage pDNA relaxed in the catalase samples was found to be not significant.

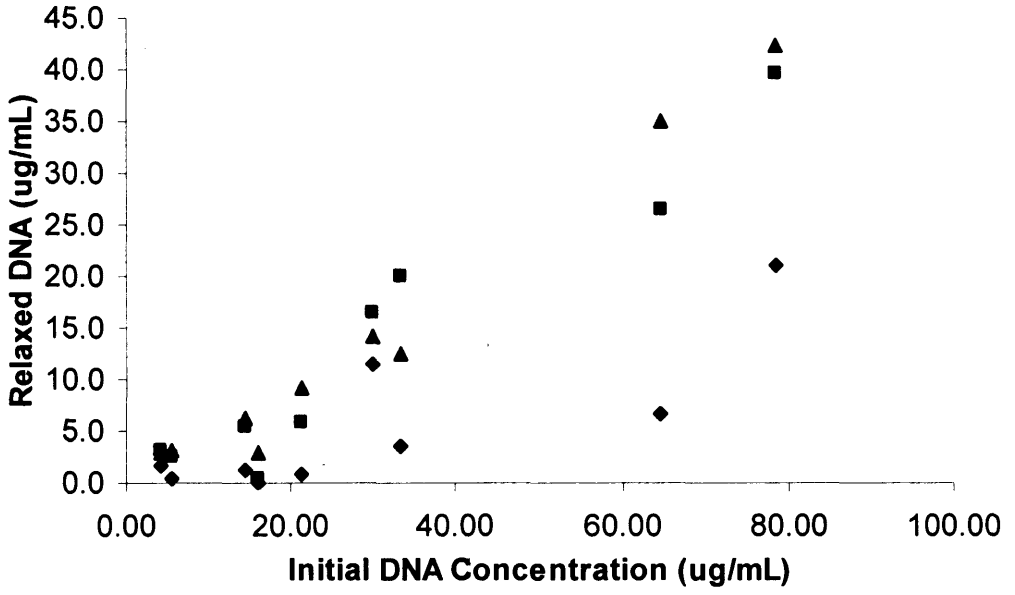


Figure 5. A chart showing the extent of relaxation of super-coiled plasmid DNA by FeS. pUC18 at varying concentrations (4.2-78.5 ug/mL) was incubated with 0.1, 1 and 10mM FeS. The graph shows the amount of pDNA in the relaxed state. The quantity of relaxed pDNA in the untreated control has been subtracted from each measurement. Raw data for this chart is provided in Appendix 3 a, b & e.

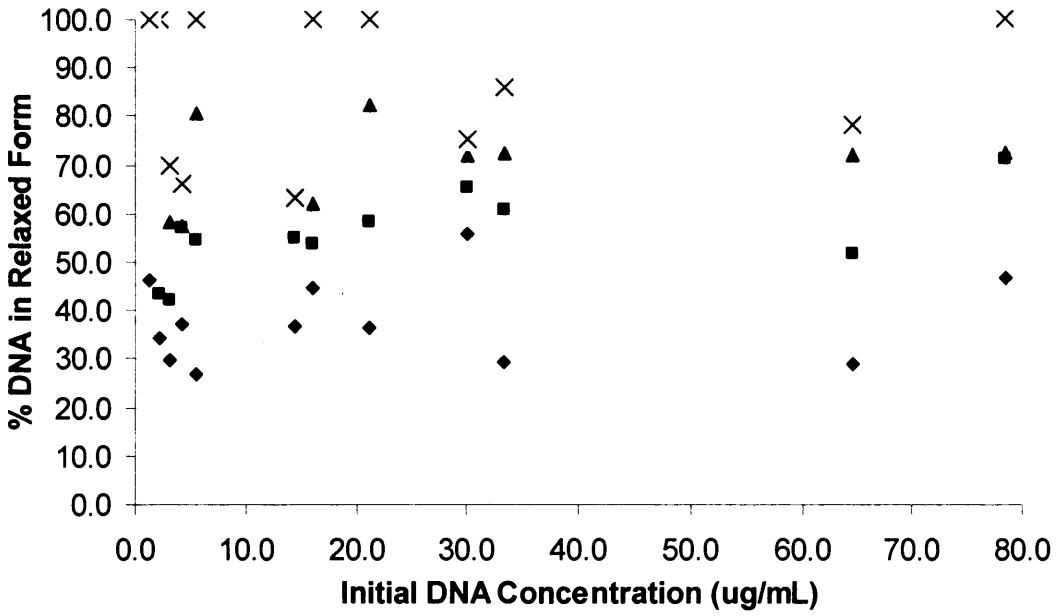


Figure 6: Graph showing FeS-induced relaxation of plasmid DNA as a function of pDNA concentration. 12 samples, with differing concentrations of DNA (1.3-78.5 μg/mL), at each FeS concentration (0.1, 1 10, 25 mM, ♦,■, ▲, x respectively) were run on 6 different gels. Each point shows the percentage relaxation for each FeS + pDNA preparation, calculated from the amount of pDNA in the relaxed state as a percentage of the total of both the relaxed and corresponding super-coiled bands. Initial DNA concentration was calculated from the total of the relaxed and super-coiled bands in the untreated positive control which contained relaxed DNA due to the extraction technique. The percentage of pDNA relaxed was independent of initial pDNA concentration. Quantification was performed using BioLine hyper ladder 1. Raw data for this chart is provided in Appendix 3 a, b & f.

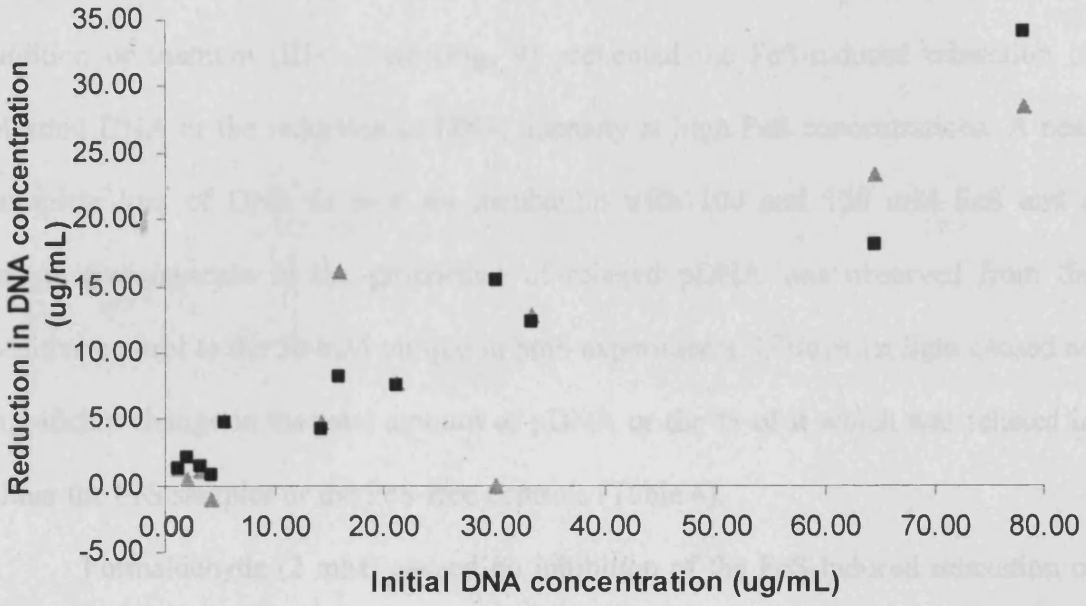


Figure 7: Graph showing total reduction in pDNA concentration against initial DNA concentration at 100 mM (▲) and 150 mM (■) FeS. Each point represents the reduction in DNA concentration, calculated for each sample from the total of the pDNA in the relaxed and super-coiled band for each FeS + pDNA preparation subtracted from the total pDNA in the corresponding FeS-free control. Raw data for this chart is provided in Appendix 3 a, b & g.

Neither conducting the electrophoretic gels under anoxia (Fig. 8) or the addition of titanium (III) citrate (Fig. 9) prevented the FeS-induced relaxation of plasmid DNA or the reduction in DNA intensity at high FeS concentrations. A near complete loss of DNA is seen on incubation with 100 and 150 mM FeS and a progressive increase in the proportion of relaxed pDNA was observed from the positive control to the 50 mM sample in both experiments. Ultraviolet light caused no significant change in the total amount of pDNA or the % of it which was relaxed in either the FeS samples or the FeS-free controls (Table 4).

Formaldehyde (2 mM) caused no inhibition of the FeS-induced relaxation of pDNA (Table 3). 47.4 % of pDNA was relaxed on incubation with 1 mM FeS and 47.4 % was relaxed when 2 mM formaldehyde was added to the solution.

Table 2. Effect of catalase on the FeS + pDNA reaction. Total pDNA and the percentage of it which is relaxed are given for a FeS + pDNA reaction in the presence and absence of 1000 µg/mL catalase. Results represent the mean of four preparations. Raw data for this table is provided in Appendix 3 h.

	0.1 mM FeS	0.1 mM FeS+catalase	F value
% pDNA relaxed	46.44	39.70	5.09

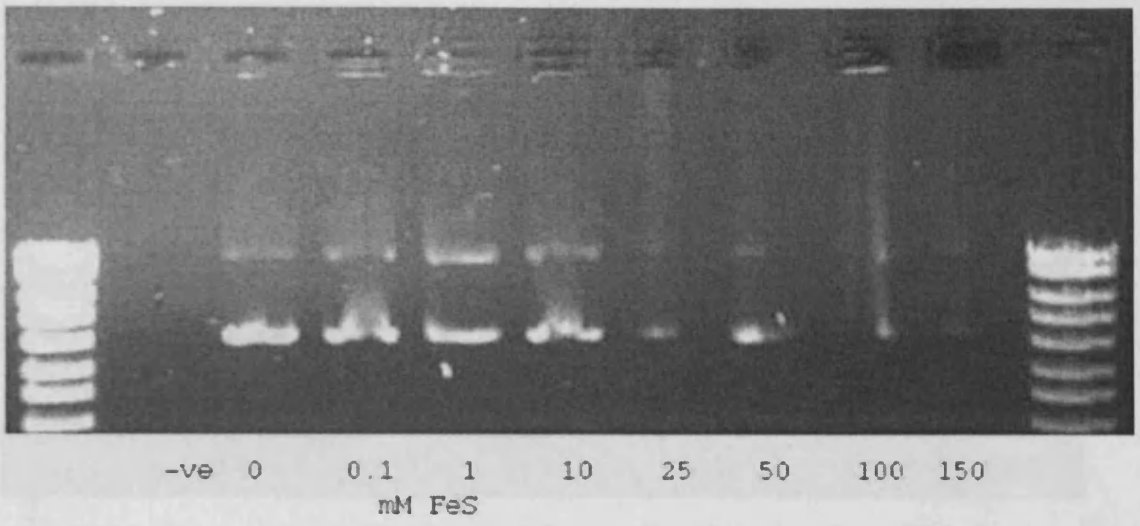


Figure 8: Image of an electrophoretic gel of FeS+pDNA conducted entirely in ≤ 10 ppm O_2 anoxic cabinet. Iron sulphide concentrations are given below the image of the gel, the positions of the relaxed and super-coiled plasmid and the size of the molecular weight markers are indicated.

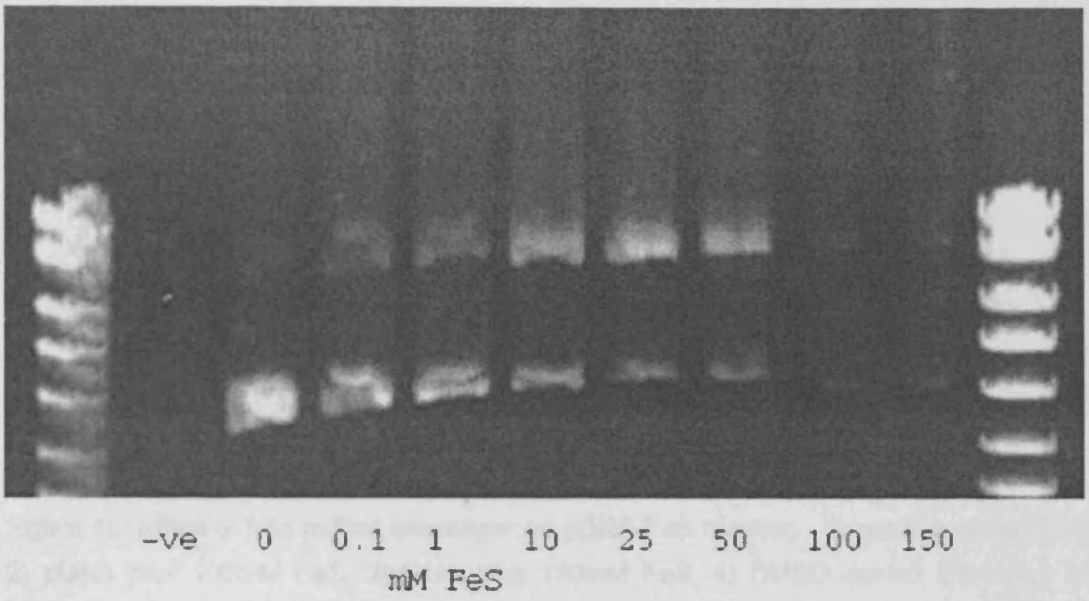


Figure 9: Image of an electrophoretic gel of FeS+pDNA+Ti(III) citrate . Iron sulphide concentrations are given below the image of the gel, the positions of the relaxed and super-coiled plasmid and the size of the molecular weight markers are indicated.

Table 3. Effects of DMSO and formaldehyde on the FeS + pDNA reaction. The means of total pDNA and the percentage of it which was relaxed are given for 4 FeS + pDNA preparations and for 4 FeS + DNA preparations with an additional 2.82M DMSO or 2 mM formaldehyde. Controls were performed with pDNA + DMSO and pDNA + formaldehyde to ensure against any effect they might have on the pDNA. Raw data for this table is provided in Appendix 3 h.

	Control	FeS	DMSO control	FeS + DMSO	CH_2O Control	FeS + CH_2O
% Relaxed	24.87	49.24	22.47	22.47	21.91	47.38

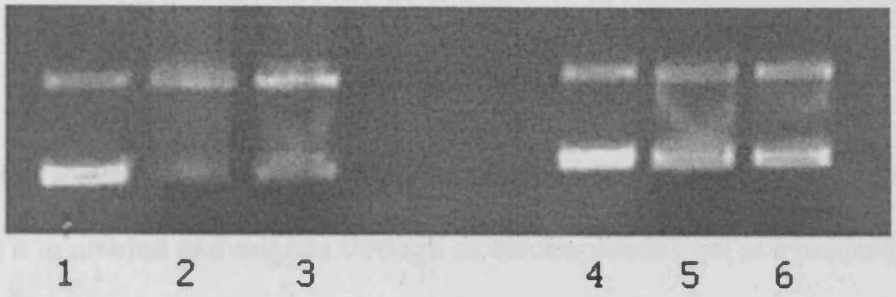


Figure 10: Effect of free radical scavenger on pDNA-FeS reaction. 1) positive control pDNA, 2) pDNA plus 100mM FeS, 3)pDNA plus 150mM FeS, 4) DMSO control DNA plus 20% DMSO, 5) pDNA plus 100mM FeS and 20% DMSO, 6) pDNA plus 150mM FeS and 20% DMSO. Raw data for this table is provided in Appendix 3 i.

Table 4. Effect of ultraviolet light on the FeS-pDNA reaction.

	Dark Control	U.V. Control	1 mM FeS	U.V. + 1 mM FeS
Total pDNA	112.9	107.7	121.2	124.0
% Relaxed	33.4	31.6	76.1	78.2

4. Discussion

On incubation with FeS solutions, plasmid DNA experienced strand breaks causing it to unwind and migrate through an electrophoretic gel at a reduced rate. FeS concentrations ≥ 0.1 mM, where no FeS_m was present, were sufficient to induce strand breaks. The degree of relaxation increased with FeS concentration over the range 0.1-25 mM. At FeS concentrations ≥ 25 mM an additional reaction occurred; intensity of both the relaxed and super-coiled pDNA bands was reduced and the bands appeared smeared and fragmented.

The FeS concentrations used in this study range over 5 magnitudes. In the pH range of these experiments, 6.7 to 8.2 for 0.1 and 150 mM respectively, the pH-independent reaction (2) controls the solubility and the aqueous FeS_{aq} clusters are the dominant dissolved species. The solubility of FeS_m in these systems is then $10^{-5.7}$ M

In the solutions with 0.1 mM and 1 mM FeS the S(-II) and Fe(II) concentrations are slightly below the measured solubility of mackinawite so no precipitate was expected or observed to form and the iron and sulphide species were in solution mostly as aqueous clusters which can be represented as the monomer FeS^0. At this concentration and pH the speciation is determined by

$$FeS^0 + H^+ = Fe^{2+} + HS^- \quad \text{..... (3)}$$

For which log K is 2.2 (Rickard, 2006). In the samples in which S(-II) and Fe(II) concentrations are equal to or above 10 mM FeS_m precipitates. Here the speciation is determined by Equation 2.

The relaxation of plasmid DNA occurred in the absence of FeS_m (0.1 & 1 mM FeS). At these concentrations the dominant species are FeS_{aq} clusters with minor

amounts of aqueous Fe(II) and S(-II) species. Aqueous FeS_{aq} clusters are not the reacting species since formaldehyde, which prevents their formation (Rickard *et al.*, 2001), had no inhibitory effect (Table 3). Despite much research on the toxicity of metals there is no known method for Fe^{2+} to directly cause DNA damage. Aqueous S(-II) does not cause relaxation of super-coiled DNA at these concentrations (Chapter 4).

4.1. Radicals

DMSO neutralises a number of free radicals including, but not only, hydroxyl radicals. DMSO did inhibit the relaxation of pDNA (Fig. 10 & Table 3). This indicates that the reaction does involve free radicals.

The Fenton reaction is the Fe^{2+}-catalysed decomposition of hydrogen peroxide into hydroxyl radicals which can react with the back-bone of DNA causing toxic effects *in vivo* (Birnboim, 1986; Imlay *et al.*, 1988) and relaxation of pDNA *in vitro* (Lloyd and Phillips, 1999; Henle *et al.*, 1999). *In vitro* studies have shown that hydrogen peroxide is produced in a solution of Fe^{2+} in the presence of oxygen and will decompose into hydroxyl radicals (Toyokuni and Sagripanti, 1992). This reaction is not expected to be occurring in this study as FeS does not induce hydrogen peroxide production (Borda *et al.*, 2001). Sulphide reacts with oxygen more readily than Fe^{2+} does scavenging it from the system preventing reaction of Fe^{2+} with oxygen which is required for H_2O_2 generation. However, the result of Borda *et al.* (2001) might be dependent on oxygen levels, FeS concentration, pH or another factor. To eliminate the possibility that the results of this study are due to the Fenton reaction, the reaction between FeS and pDNA was repeated in the presence of agents which would inhibit the Fenton reaction or the hydroxyl radicals it produces.

Catalase did not inhibit the relaxation of pDNA at 0.1 mM (Table 2); as expected hydrogen peroxide is not produced under these conditions because Fe^{2+} does not react with molecular oxygen. This is supported by the observation that titanium (III) citrate did not have an inhibitory effect either (Fig. 9). Titanium (III) citrate is a strong reducing agent whose presence makes oxidation of Fe^{2+} by O_2 even less probable. The relaxation of pDNA also occurred when the experiment was carried out completely in an anoxic cabinet in which the concentration of O_2 did not exceed 10 ppm (Fig. 8) this is further evidence against the Fenton reaction being responsible for the DNA damage.

There is indirect evidence for the generation of HS· radicals on the oxidation of sulphide (Tapley *et al.*, 1999) which requires a metal catalyst. This reaction is not thought to be involved in this study as neither the reducing agent titanium (III) citrate nor conducting the experiment in anoxic conditions inhibited the reaction.

A reaction mechanism in which radicals are generated from an FeS solution without involvement of molecular oxygen or hydrogen peroxide is proposed. Recent recalculations by Rickard and Luther (2007) of stabilities in the aqueous sulphide system, which include the Kamyshny dataset for polysulphides (Kamyshny *et al.*, 2003) show that S_2(-II) species are common and that HS_2^- is the third most abundant sulphide species (after HS^- and H_2S) in most natural waters. In free radical terms, the analogy with the oxygen system is striking. Thus the sulphur analog of the peroxide free radical ·Ö:Ö· is the persulphide free radical, ·S̈::S̈· which gives rise to the concept of a supersulphide radical S_2^- by analogy with the superoxide free radical O_2^-. The bisulphide free radical ·S̈:H is analogous to the hydroxyl radical ·OH. And, of course, the fully protonated disulphide , H_2S_2, is the sulphur analog of hydrogen

peroxide (Winnewisser *et al.*, 2003). I propose that Fe^{2+} decomposes hydrogen disulphide into sulphide radicals in a similar reaction to Fenton's;

$$Fe(II) + H_2S_2 \rightarrow Fe(III) + HS^- + HS\cdot \quad (4)$$

In this scheme disulphide is formed in an anoxic environment in a similar manner to the formation of peroxide from hydrogen peroxide in oxic systems.

Ultraviolet radiation enhances the effect of the Fenton reaction by reducing Fe(III) allowing it to react again with O_2 and/or by decomposing H_2O_2 into radicals in a non-iron dependent reaction (Ghaly *et al.*, 2001). A similar effect was not found in this study possibly because FeS strongly absorbs U.V. The abundance of FeS over Fe(III) and H_2S_2 could have prevented UV light from reducing Fe(III) and decomposing H_2S_2.

There have been a number of published descriptions of HS· radicals but they have been reported under a variety of names; for example, mercapto radicals (Norman and Storey, 1971), sulphhydral radicals (Mills *et al.*, 1987) and thiyl radicals (Baldridge *et al.*, 1987), although thiyl is also used to refer to any molecule containing a sulphur atom with an unpaired electron (RS·) especially in organic chemistry (Bonini and Augusto, 2001). Hydrogen sulphide or HS· radicals (Wang *et al.*, 1987; Stachnik and Molina, 1987) is a common and unambiguous name. HS· radicals are also reportedly produced during the thermal decomposition of alkane thiols (Baldridge *et al.*, 1987) and by the reaction between H_2S and OH· (Stachnik and Molina, 1987). The proposed decomposition of H_2S_2 by Fe^{2+} into HS· radicals would be a new reaction, although not unexpected as H_2S_2 is analogous in many ways to H_2O_2 (Winnewisser *et al.*, 2003). Other known sulphur radicals include $SO_3^-\cdot$ and $S_2O_3^-\cdot$ (Norman and Storey, 1971).

Tapley *et al.* (1999) produced evidence, using EPR, for the generation of free radicals when sulphide is oxidised. However, HS· radicals were not specifically identified or trapped and they were suggested to be involved in the production of other radicals. EPR is commonly used to investigate radical-mediated reactions but it is still difficult to identify particular radicals as a specific spin-trap is required and the radicals often have very short half-lives. Previous work in our lab (Butler and Rickard, unpublished) using 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as a spin-trap found no radical spin adduct produced by FeS in the absence or presence of hydrogen peroxide. Although when hydrogen peroxide was present there was a Fe(III) signal suggesting that the Fenton reaction was occurring. This in turn implies that the radicals present were undetectable possibly because adduct formation or signal generation were interfered with by the presence of FeS or HS- which reacts with OH·.

The quantity of pDNA which was relaxed by FeS was proportional to the initial quantity of pDNA in the reaction (Fig. 5) which was an approximately constant percentage dependent on FeS concentration (Fig.4). This is consistent with the claim of a radical-mediated reaction, the radicals can react in a number of ways other than with DNA and the probability of a reaction with DNA is dependent on the concentration of DNA.

The mechanism by which hydroxyl radicals create strand-breaks in DNA is by abstraction of one of the hydrogen atoms from the deoxyribose moiety. Balasubramian (1998) determined that OH· can react with any of the C-H bonds in the ribose or nucleobase constituent of the molecule. HS· radicals can also abstract hydrogen atoms from organic chemicals (Berberova and Shinkar, 2000) and presumably would cause DNA scission in the same manner.

$SH\cdot + R\text{-}CH\text{-}R \rightarrow H_2S + R\text{-}C\cdot + R$ .....(6)

4.2. Surface interactions

It is possible that the reduction in DNA concentration seen at ≥ 50 mM FeS is caused by the same process as the relaxation of DNA at lower FeS concentrations. If this were so then the fragments produced by continued scission of the pDNA back-bone must be too small to be retained or visualised on the gels. However, I think it is more probable that DNA is retained in the well because it interacts strongly with the surface of FeS_m (Chapter 8). The lateral streaks above the relaxed pDNA band would then be the result of FeS particles binding to pDNA, increasing its molecular weight and/or decreasing its charge creating a species with a continuum of charge/mass values lower than that of natural pDNA. The feint, thin bands formed at 150 mM (Figure 2) would then be dimers or trimers of plasmids connected to each other by FeS_m particles.

5. Conclusions

On incubation with solutions of iron (II) monosulphide pDNA experiences strand-breaks which retard its migration through an electrophoretic gel. This reaction occurs when iron and sulphur concentrations are too low for FeS_m to form. It is proposed that this is not caused by the Fenton reaction or iron-catalysed oxidation of sulphide but that it is radical-mediated. These sulphide radicals are produced through the Fe^{2+}-catalysed breakage of disulphide bonds akin to the iron-catalysed decomposition of H_2O_2 in the Fenton reaction. This reaction would occur in any

solution that contains sulphide and iron and it does not require molecular oxygen to be present.

At low FeS concentrations this may actually have assisted in genome evolution by allowing nucleic acid strands to be broken and recycled allowing novel combinations to emerge. At higher FeS concentrations an additional reaction occurs which may have been detrimental to an emerging organism but conversely, may also demonstrate that nucleic acids bind to FeS precipitates this will be explored in subsequent work (Chapter 7).

Chapter 6

The effect of transition metal redox on DNA in sulphidic systems

Abstract

Hydrothermal origin of life hypotheses involve biopolymers developing in amongst a suite of minerals, particularly sulphides. Adsorption of the polymer onto a mineral is a key requirement of these hypotheses. Iron sulphide adsorbs DNA but also induces breaks in the molecule's backbone. Plasmid DNA has been reacted with copper and zinc sulphides and sulphates and analysed with electrophoresis to ascertain if these compounds have a similar effect. Copper sulphide, but not zinc sulphide, induced strand-breaks in the DNA molecule which were attributed to sulphur-radicals produced from redox reactions between copper(I)/(II) and disulphide. Nanoparticulate copper and zinc sulphides adsorbed DNA which resulted in the reduction or prevention of DNA migration through the electrophoretic gel. Although DNA adsorbs onto all sulphide minerals studied, minerals containing redox active metals would have been less useful in this regard due to them causing depolymerisation of the molecule.

1. Introduction

Hydrothermal environments in which sulphide minerals precipitate from the exhaled sulphidic vent fluid and the iron-rich seawater are potential sites for the origin of life (Shock, 1996; Shock and Schulte, 1995; Russell and Hall, 1997; 2002; Russell *et al.*, 2005; Russell *et al.*, 1988; Russell and Martin, 2004; Russell and Turner, 1989; Wächtershäuser, 1988; 1990; 1992; 1998; 2006). Mackinawite (FeS_m) is produced in these environments and plays an important role in the hypotheses of both Russell and Wachtershauser. Mackinawite has been shown to both bind DNA and to and cause breaks in its ribo-phosphate backbone (Chapters 5 & 7). The strand breaks have been suggested to be the result of iron catalyzed decomposition of disulphide (S_2^{2-}) into

sulphur-based free radicals. The binding of nucleic acids to minerals is an important step in these hypotheses, in both of which adsorption onto a mineral surface is a requirement for nucleic acid development (Huber and Wächtershäuser, 1998; Russell and Hall, 1997; 2006) in which the mineral could potentially facilitate polymerization (Ertem and Ferris, 1996), protect molecules from hydrolysis (Shapiro, 1995; Sowerby *et al.*, 2001) and enable them to act as primitive ribosomes (Russell and Hall, 1997; 2002; Russell *et al.*, 2005) by binding and orienting the nucleic acid in such a way that it can catalyse a polymerization of amino acids. However, the finding that the same minerals can cause depolymerisation of DNA could have been a hindrance to the evolution of nucleic acids in these environments.

This paper reports an investigation into whether non-ferrous sulphide minerals, which may have been present in hydrothermal origin of life scenarios, engage in similar interactions with DNA. This is to examine if these minerals would also have affected DNA evolution in a hydrothermal scenario and also to elucidate more information about the mechanism of the reaction between FeS_m and pDNA.

Copper and zinc have been chosen for study partly due to their biological and possible pre-biological significance, but also because of their redox chemistry. Both of these metals form divalent ions in solution. Whilst Fe(II) readily oxidises to Fe(III), neither zinc nor copper readily form a +3 oxidation state. Zn(II) does not readily engage in redox reactions. Cu(II) is reduced to Cu(I) on precipitation with sulphide and thus has the potential to be oxidised. The study therefore provides key information regarding the importance of metal redox processes in metal sulphide interactions with DNA.

Precipitated CuS and ZnS are nanoparticulate equivalents to covellite and sphalerite respectively (Rickard and Luther, 2006). On precipitation with sulphide Cu(II) is reduced to Cu(I) with sulphide in the form S_2^{2-} and S^{2-} whilst zinc forms

$Zn^{2+}S^{2-}$. That is the formation of CuS from Cu(II) involves a redox reaction whereas the formation of ZnS from Zn(II) does not. Both the aqueous Cu-S and Zn-S systems also contain a number of aqueous metal sulphide complexes (Rickard and Luther, 2006) as well as potentially highly reactive aqueous clusters (Luther and Rickard, 2005).

Since the publication of the FeS-world theory some research, mostly focussing on pyrite (FeS_2), has examined interactions between metal sulphides and nucleic acids (Cohn *et al.*, 2003; Cohn *et al.*, 2006). Zinc(II) is present in and essential for many DNA-interacting proteins in the form of a zinc finger (Johnston, 1987; Berg, 1990). It has also been reported to cause the precipitation of DNA in millimolar quantities possibly due to zinc hydroxides forming and binding to the phosphate groups of DNA (Kejnovsky and Kypr, 1998). Zinc sulphide nanocrystals have been bound to DNA for applications in fluorescent DNA detection. In these studies it has been necessary for a suitable functional group to be attached to the surface of the zinc sulphide such as hydroxyl (Pathak *et al.*, 2000), thiol, carboxyl (Mitchell *et al.*, 1999) or cysteine (Li *et al.*, 2004).

Cu (I) and Cu(II) can react with hydrogen peroxide (H_2O_2) to produce oxidising species in a similar way to Fe(II) and Fe(III) respectively (Lloyd and Phillips, 1999; Oikawa and Kawanishi, 1998; Sagripanti, 1999; Yamamoto and Kawanishi, 1989; Frelon *et al.*, 2003). Copper-induced DNA damage has been reported to be site-specific due to copper ions binding to DNA chains in a site-specific manner and reacting at that locale (Frelon *et al.*, 2003). There is some debate over whether Cu(II) reacts with H_2O_2 to form hydroxyl radicals (OH·) or copper-peroxide complexes Cu(I)OOH· (Oikawa and Kawanishi, 1998; Yamamoto and Kawanishi, 1989; Frelon *et al.*, 2003).

Dittmer and Simmel (2004) synthesised CuS nanocrystals on DNA molecules. This was achieved by incubating the DNA with Cu(II), which binds to the negatively

charged phosphate back-bone, and subsequent sulphidisation of the solution. With atomic force microscopy these nanocrystals were visualised and measured. They had "heights" of 0.9 to 10 nm depending on the technique. This experiment showed that copper sulphides can be grown on Cu(II)-DNA. It is unknown, however, if preformed copper sulphide interacts with DNA.

2. Materials and methods

Plasmid DNA (pUC18) was incubated with freshly precipitated copper and zinc sulphides and copper and zinc sulphates and analysed by electrophoresis.

2.1. Plasmid DNA preparation

pUC18 plasmid DNA (pDNA) was extracted from cultures of *Escherichia coli* grown in LB broth (tryptone, yeast extract and NaCl) with 100 mg/L ampicillin using Wizard® Plus SV Minipreps DNA Purification System according to the manufacturer's instructions. pDNA was stored in nuclease free water at -20°C until required.

2.2. Sample Preparation and reactant characterization

Analytical grade $ZnSO_4.7H_20$, $CuSO_4.5H_2O$ and $Na_2S.9H_2O$ were dissolved to stock solutions of 0.5, 5, 50, 500 mM in distilled H_2O. In 0.2mL microfuge tubes the sodium sulphide solution was added to 10 mM Tris buffer, a buffer of pH 8 used commonly in DNA studies. Metal sulphate solution was then added forming the metal sulphide precipitate and pDNA was added finally to a total volume of 50μL. Metal-sulphide concentrations were varied from 0.1 mM to 150 mM; metal and sulphide ions were equimolar in all cases. In experiments with copper and zinc sulphate additional

Tris buffer was substituted for sodium sulphide, otherwise, the experimental set up was the same.

The ZnS precipitate formed by these techniques have been studied by Oldroyd and Rickard (cited in (Luther *et al.*, 1999)). They showed that the precipitate formed under these conditions possessed the sphalerite structure. Nanoparticulate sphalerite consists of ZnS particles with a size of > 1.5 nm (Banfield and Hengzhong, 2001). The nanoparticulate material cannot be viewed as merely small fragments of the bulk crystalline materials. Apart from being surface energy dominated, the first formed condensed ZnS phases are likely to contain substantial amounts of H_2O within their structures and display intrinsic structural changes (Zhang *et al.*, 2003).

The nature of the CuS precipitates depends on the pH of the solutions (Luther, 2004; Luther and Rickard, 2005) but the CuS produced by the techniques used in this investigation has been shown to have the covellite structure (Rickard, 1972). Using X-ray absorption techniques (Pattrick *et al.*, 1997) showed that these early covellite precipitates contained S_2(-II) and had a 1:1 Cu:S stoichiometry. The crystallographic structure of covellite was redefined by Evans and Konnert (Evans and Konnert, 1976) which led to covellite being a viewed as mixed Cu(I)/Cu(II) sulphide with a composition $Cu(I)_4S(-I)_4Cu(II)_2S(-II)_2$. However, X-ray absorption spectroscopic studies (Goh *et al.*, 2006; Silvester *et al.*, 1991; Van Der Laan *et al.*, 1992; Pearce *et al.*, 2006) and theoretical studies (Tossell, 1978) confirm that all the Cu in the mineral covellite is formally in the Cu(I) state. Electron spin resonance studies (Luther and Rickard, 2005) show that the reduction of Cu(II) to Cu(I) occurs in solution, prior to the formation of the solid phase, and this is related to the formation of aqueous CuS clusters.

2.3. Electrophoresis

All samples were prepared and incubated for 1 h in a Labmaster 130™ anoxic glove box filled with O_2 free N_2. The samples were removed from the glove box for electrophoretic analysis. 5 μL of the samples were mixed with 1μL of 5*loading buffer and loaded on a 0.7% agarose gel with ethidium® bromide added to a concentration of 10 μg/mL. 80 V was applied across the gel until the dye had moved sufficiently down the gel. The gels were then photographed with ultra violet light in a Gene Genius Bio Imaging System™. In some cases band intensity was measured using GeneTools™ densitometry from SynGene ™ which computes the quantity of DNA in a band from its intensity and the size of a DNA band from its rate of migration relative to a standard molecular weight marker (Bioline HyperLadder 1).

All reagents were of analytical grade. Sterile, O_2-free deionized water (dH_2O) was used throughout. MilliQ ™ 18 M Ω H_2O was sterilized through autoclaving for 2 h at 120°C and 2 Bars in a *Priorclave*™ and deoxygenated by bubbling O_2 free N_2 for at least 1 h. Studies of the resultant dissolved O_2 concentration (Butler *et al.*, 1994) shows that the solution contains measurable O_2 in the 1-10 ppmv range. All containers were sterile on purchase or sterilized through autoclaving as above except plastic bottles for metal sulphate and sodium sulphide stock solutions which were washed with 2% Decon 90® and ethanol.

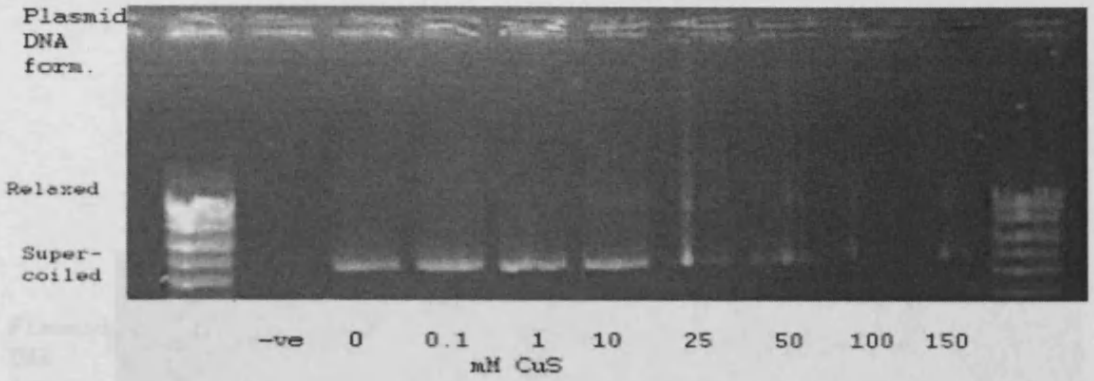
3. Results

pDNA incubated with copper sulphide experienced an increase in the prevalence of the relaxed band over the concentration range 0.1-10 mM CuS (Fig. 1). The pDNA sample used contained no or undetectable amounts of relaxed plasmid (Fig. 1, 0 mM) on reaction with 0.1-10 mM CuS a percentage of the plasmids were converted to the relaxed form (Fig. 2). The percentage of plasmid which was relaxed in each sample was

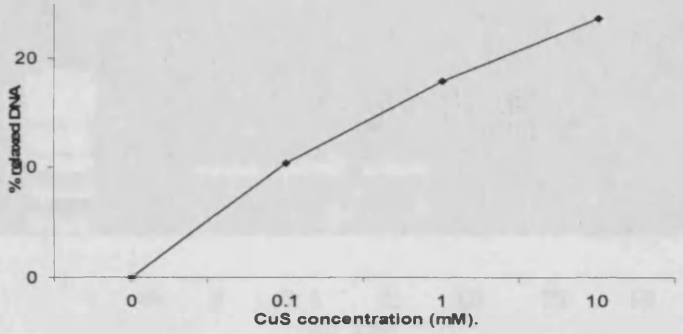
approximately proportional to the logarithm of the CuS concentration. Zinc sulphide did not cause an increase the prevalence of the relaxed upper band.

Higher concentrations of copper and zinc sulphides induced a reduction in the intensity of the bands seen on the gel. Only streaks are seen on the gel after incubation with $\geq$ 25 mM copper sulphide, this effect was not seen below this concentration. Zinc sulphide caused an almost complete loss of bands and only a wide streak from top to bottom at 10 mM. Above this concentration, consistent with that seen in some of the FeS + DNA reactions (Chapter 5), the bands come back although they are less intense. A fluorescent signal can be seen in the wells at and above 10 mM.

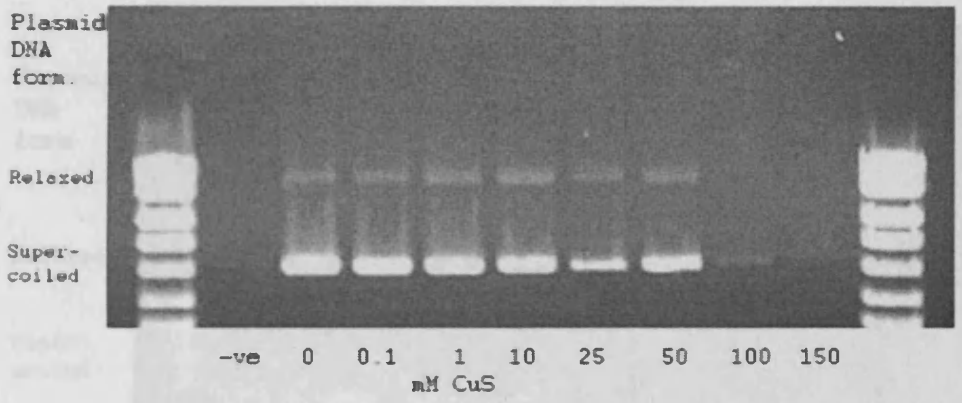
Copper (II) sulphate (Fig. 3) induced a minor increase in the prevalence of the relaxed pDNA bands over the range 0.1-150 mM. However, the effect is much less than that with equal concentrations of Fe(II) (Hatton Chapter 3). Zn (II) sulphate (Fig. 4) did not cause any increase in the proportion of relaxed pDNA bands and induced a partial loss of total pDNA at 150 mM only in which approximately 50% (38.8 ng) of the pDNA added to the reaction mixture was detected. Figure 4 also shows a slight retardation in the migration of DNA down the gel at 100 and 150 mM $ZnSO_4$. An increase in band intensity is seen at 10 mM $ZnSO_4$.



a

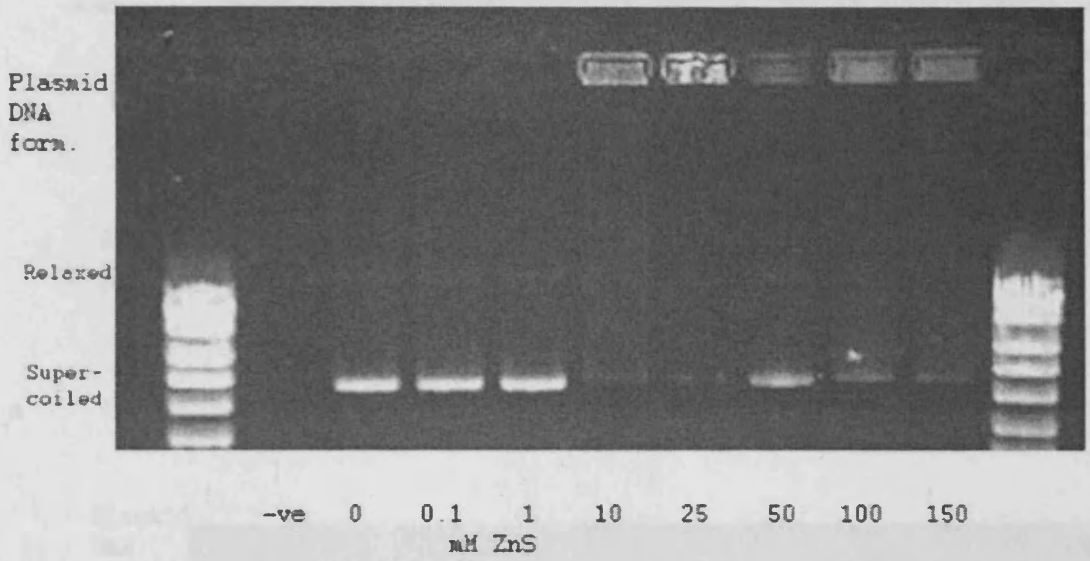


b

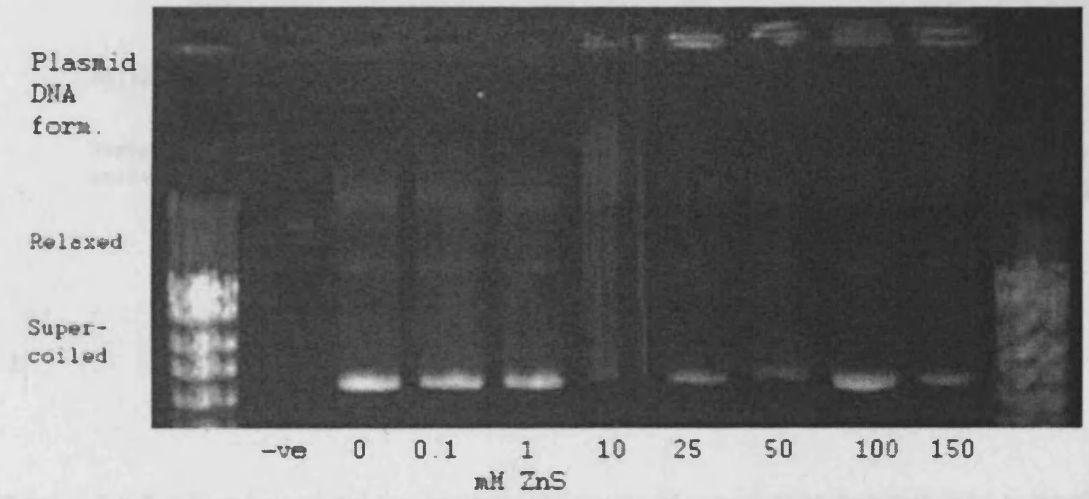


c

Figure 1. Effect of copper sulphide on plasmid DNA. (a) A typical gel electrophoresis result of the reaction between copper sulphide and pDNA. Copper sulphide concentrations are given below the image of the gel; the positions of the relaxed and super-coiled plasmid are indicated. The concentration of pDNA detected in the positive control is 12.3 ug/mL or 7.1 nMol plasmid. (b) A graph showing the percentage of pDNA in the relaxed topology against CuS concentration for the gel in figure 1a, on a logarithmic scale. The intensity of the bands in Figure 1a was measured using GeneTools from SynGene ™ and approximated to a concentration in µg/mL. Raw data is provided in appendix 4. (c) Second gel electrophoresis result of the reaction between pDNA and CuS, provided to show repeatability.

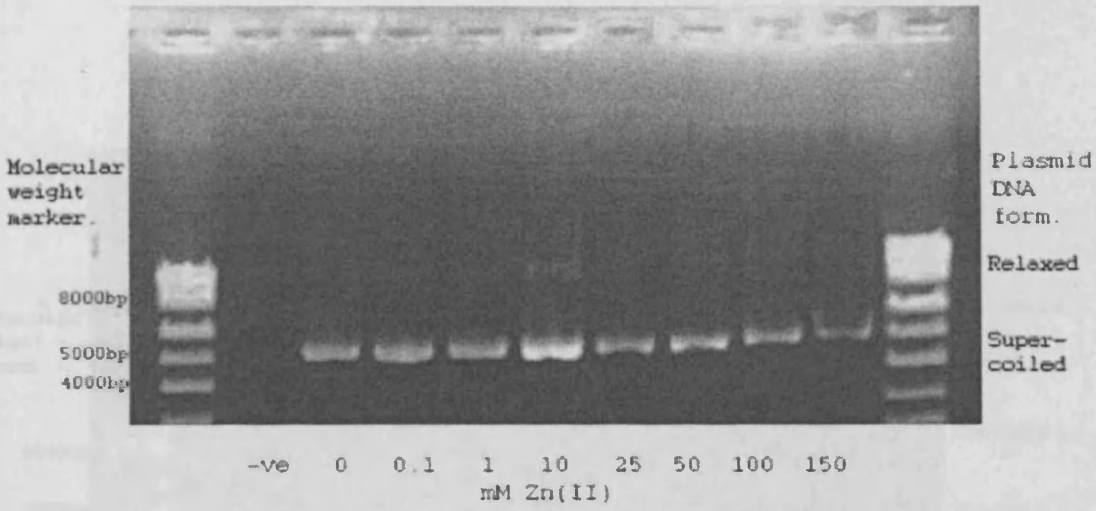


a

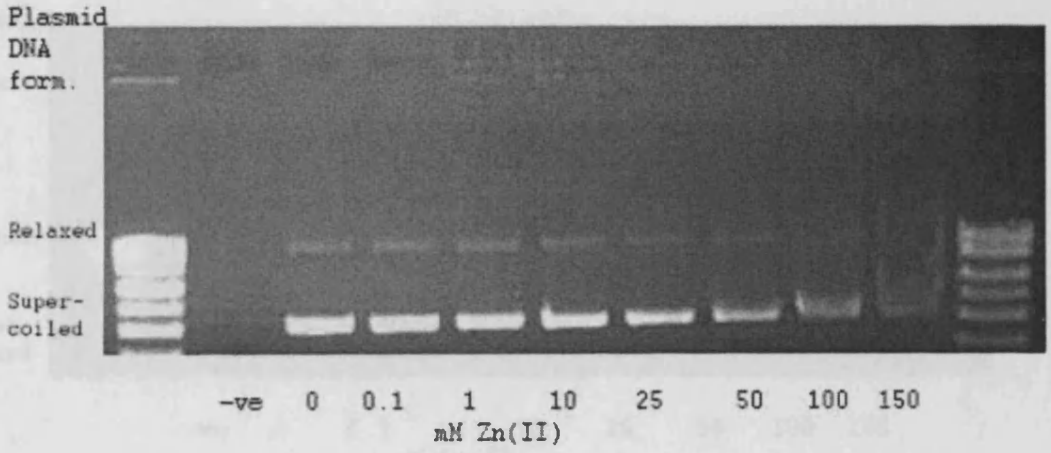


b

Figure 2. (a) Effect of zinc sulphide on pDNA. A typical gel electrophoresis of the reaction between zinc sulphide and pDNA. Zinc sulphide concentrations are given below the gel, the positions of relaxed and super-coiled plasmid are indicated. The concentration of pDNA detected in the positive control is 21.8 ug/mL or 12.5 nMol plasmid. (b) Second gel electrophoresis result of the reaction between ZnS and pDNA provided to show repeatability.



a



b

Figure 3 a & b Two typical gel electrophoresis results of the reaction between zinc sulphate and pDNA. Zinc concentrations are given beneath the gel. The positions of the relaxed and super-coiled plasmid and the size of the molecular weight markers are indicated. The images are provided to show repeatability.

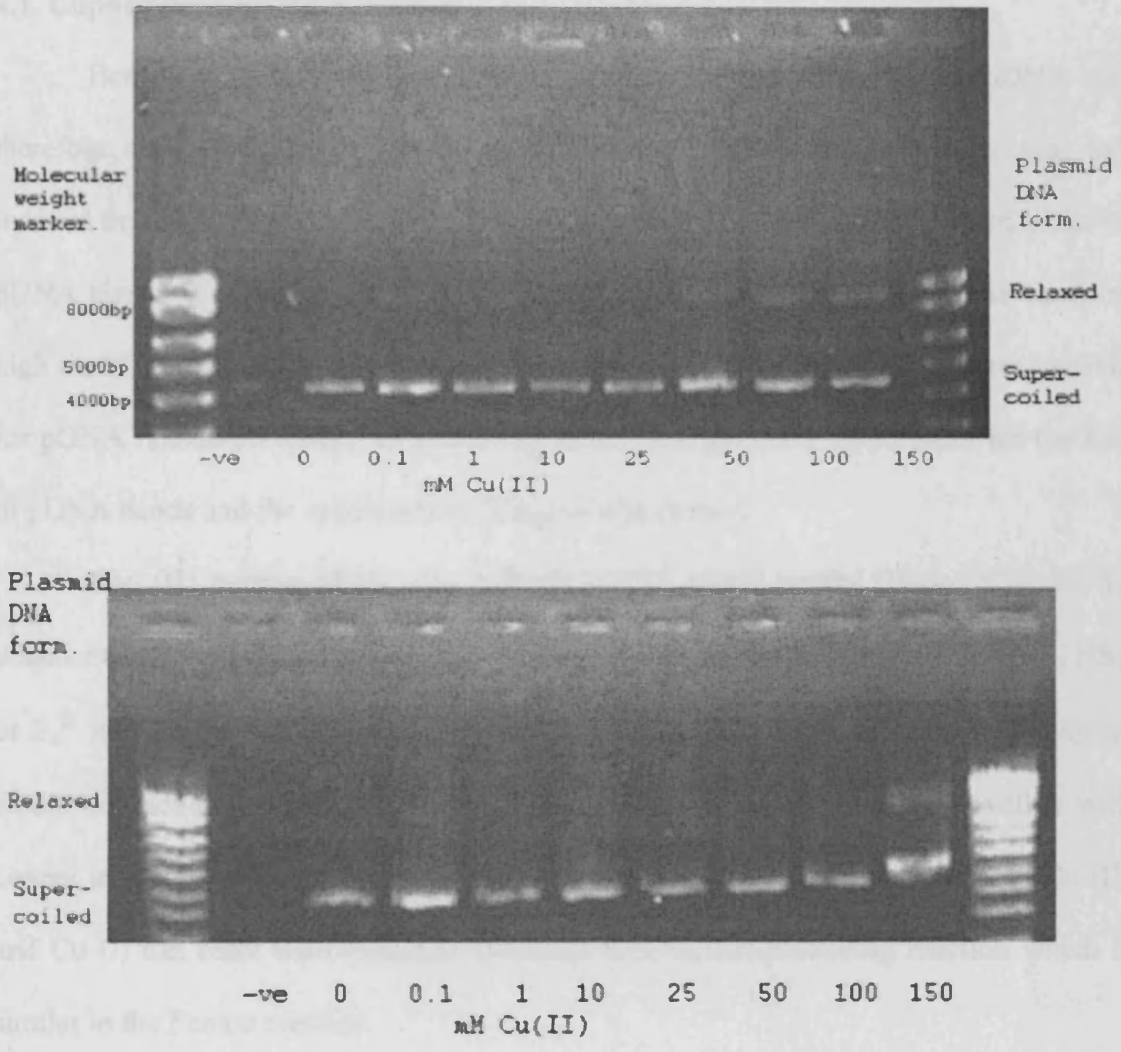


Figure 4 (a). A typical gel electrophoresis result of the reaction between copper sulphate and pDNA. The concentration of pDNA measured with densitometry in the positive control was 6.3 μg/mL which corresponds to a concentration of plasmids of 3.6 nMol. Copper concentrations are given beneath the gel. The positions of the relaxed and super-coiled plasmid and the size of the molecular weight markers are indicated. (b) Second gel electrophoresis result of the reaction between copper sulphate and pDNA provided to show repeatability.

4. Discussion

4.1. Copper-induced DNA scission

Both copper sulphate and copper sulphide caused relaxation of pDNA and therefore broke the ribo-phosphate backbone of the DNA strand. Neither zinc salt induced breaks in the DNA strand. Both of the metal sulphides studied caused a loss of pDNA signal on the gels and produced lateral streaks on the gels above the bands at high metal sulphide concentrations. This suggests that the copper (II) ion is responsible for pDNA relaxation whilst the metal sulphide precipitates are responsible for the loss of pDNA bands and the appearance of longitudinal streaks.

Iron (II) monosulphide also induced pDNA strand-breaks (Hatton Chapter 5), which was attributed to iron-catalyzed decomposition of disulphide bonds in H_2S_2, HS_2^- or S_2^{2-} into $HS\cdot$ or $S\cdot^-$ radicals. This is analogous to the Fenton reaction with sulphur atoms in place of oxygen. Although copper and sulphide precipitate as covellite with copper in the +1 redox state, a small amount of Cu(II) exists in solution. Both Cu (II) and Cu (I) can react with hydrogen peroxide in a radical producing reaction which is similar to the Fenton reaction.

$$Cu(II) + H_2O_2 \rightarrow C(I) + HOO + H^+ \quad \text{..... (1)}$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + OH\cdot + OH^- \quad \text{..... (2)}$$

It has also been proposed the reaction produces copper-peroxide complexes which react with DNA rather than free hydroxyl radicals (Pecci *et al.*, 1997; Kawanishi *et al.*, 2001; Yamamoto and Kawanishi, 1989):

$$Cu(II) + H_2O_2 \rightarrow Cu(I)OOH\cdot + H \quad \text{.....(3)}$$

$$Cu(I) + H_2O_2 \rightarrow Cu(I)OOH\cdot + H^+ \quad \text{.....(4)}$$

It is proposed here that these reactions can occur with disulphide in place of H_2O_2 as described in Chapter 5. The lack of plasmid relaxation with either zinc salt is explained as Zn(II) is not redox active and therefore can not decompose either H_2O_2 or H_2S_2.

Disulphide is not present in sulphate solutions and this reaction cannot explain the results with Cu(II) sulphate. Hydrogen peroxide should not be present either as its generation would require a reaction between Cu(I) and O_2. Cu(I) should not be present in the absence of a reducing agent acting on Cu(II) and oxygen levels are very low in this system. Cu(II) is reported to bind to phosphate and other sites on DNA (Monson and Woolley, 2003; Drouin *et al.*, 1996) and is toxic via a number of mechanisms (Stauber and Florence, 1987). However, no direct DNA-damaging reaction has been reported in the biomedical literature. This may be because the reaction seen in this study does not occur over the Cu(II) concentration ranges used in these studies which examined feasible physiological concentrations in the micromolar range, whilst this study examined millimolar concentrations of $CuSO_4$ and still only detected minimal relaxation of the plasmid.

Most nuclease enzymes which hydrolyse the phosphodiester bond in the back-bone of DNA utilise metal-ion cofactors including copper (Sreedhara and Cowan, 2001). Non-enzymatic copper complexes have been synthesised for use in biotechnology which can rapidly hydrolyse phosphodiester bonds with micro molar Cu(II) concentrations (Deck *et al.*, 2002; Gajda *et al.*, 2001). These studies do not seem

to have examined non-complexed, free, copper ions, but it is reasonable to suggest that they may have the same catalytic capacity at a reduced rate.

4.2. Metal-DNA binding

Neither of the zinc salts broke the pDNA back-bone. $ZnSO_4$ at concentrations $\geq$ 25 mM had the effect of retarding the migration of pDNA and 150 mM $ZnSO_4$ and $\geq$ 10 mM ZnS caused a loss of pDNA concentration. As it is known that Zn(II) hydroxides form in solution and bind to DNA (Kejnovsky and Kypr, 1998) it can be inferred that zinc hydroxides bound to the pDNA may have increased the mass and decreased the charge of the pDNA which slowed the electrophoretic migration, although this effect is minor. They may also have interfered with ethidium bromide's capacity to intercalate with the DNA and cause fluorescence.

The loss/reduction of pDNA band concentration observed with metal sulphides was caused by interactions between DNA and the sulphide nanoparticles. The luminescence visible in the wells at $\geq$ 10 mM ZnS is consistent with the interpretation that pDNA is prevented from migrating by being bound to the solid ZnS in the wells. As noted above sulphide nanoparticles cannot be regarded simply as fragments of the bulk minerals. The ZnS nanoparticles have been studied in more detail than any other metal sulphide forms. These studies have confirmed that the nanoparticle surface is less ordered, H_2O rich and that the surface free energies dominate the total energetics of the particles (Zhang and Banfield, 2004). This would suggest that physical surface reactions of these particles are enhanced relative to the bulk material and that the sulphide nanoparticles bind readily to DNA. DNA binds to positively charged surface sites on metal sulphides through its negatively charged phosphate groups or through covalent interactions involving an uncharged area of the molecule (Bebie and Schoonen, 2000).

The streaks between the well and the bands therefore represent pDNA with a continuum of ZnS or CuS nanoparticles bound to it retarding its migration to an extent which is variable and dependent on the number of particles bound.

5. Conclusions

Copper and zinc sulphides caused a reduction in the intensity of plasmid DNA bands on gel electrophoresis at concentrations equal to or above 25 mM and 10 mM respectively. Both sulphides caused streaks between the well and these streaks represent plasmid DNA molecules of increased mass/decreased charge due to the binding of sulphide nanoparticles to the molecule.

Similar to the results with iron, both copper sulphide and copper sulphate induced relaxation of the super-coiled pDNA at low concentrations whilst neither zinc salt did. This indicates that redox reactions of the metal ion play a crucial role in the metal sulphide/sulphate-induced scission of DNA. The oxidation probably affects DNA through radical formation.

These results show that redox-active metals in the presence of sulphide, as in the proposed hydrothermal origin of life scenarios would produce free radicals capable of causing strand-breaks in DNA molecules and other biological polymers. Additionally, sulphide precipitates in an origin of life scenario could have bound nucleic acids, potentially enabling them to act as primitive ribosomes (Russell and Hall, 1997; 2002; Russell *et al.*, 2005) as well as facilitating polymerization (Ertem and Ferris, 1996) and protecting them from hydrolysis (Shapiro, 1995; Sowerby *et al.*, 2001). Minerals which contained redox active metals would be less suitable to this purpose as they may have caused fragmentation of the nucleic acid molecule. Non-redox active metal sulphides

such as sphalerite (ZnS) would be more suitable as they bind nucleic acids without fragmenting them.

Chapter 7

Nucleic acids bind to nanoparticulate transition metal sulphides in aqueous solutions

Abstract

In the hydrothermal FeS-world origin of life scenarios nucleic acids bind to iron (II) monosulphide precipitated from the reaction between hydrothermal sulphidic vent solutions and iron-bearing oceanic water. In order to test this idea, I investigated the binding of different nucleic acids, and their constituents, to freshly precipitated, nanoparticulate iron (II) monosulphide using UV/Vis spectrometry. The degree to which the organic molecules interacted with iron sulphide was chromosomal DNA > RNA > oligomeric DNA > deoxadenosine monophosphate ≈ deoxyadenosine ≈ adenine. This work supports the hypothesis that sulphide minerals precipitated at hydrothermal vents could have assisted in the formation and polymerisation of nucleic acids.

1. Introduction

Adsorption of organic molecules onto mineral surfaces has long been considered to have been involved in the origin of life (Bernal, 1951). Adsorption on to minerals can facilitate polymerisation of nucleotides (Ertem and Ferris, 1996) and amino acids (Brack, 1993; Huber and Wächtershäuser, 1998), catalyse metabolic reactions (Cody *et al.*, 2000), discriminate between chiral enantiomers (Hazen *et al.*, 2001) protect molecules from hydrolysis (Sowerby *et al.*, 2001b; Shapiro, 1995) and provide a mechanism for concentrating molecules. The concentration of adenine in the prebiotic ocean has been estimated at 30 μM. Therefore, a mechanism to concentrate it, such as adsorption to a mineral surface, may have been required (Miller, 1987).

Adsorption is a critical process in the FeS-world origin of life hypotheses of both Wachtershauser (1988; 1990; 1992; 1994; 1997; 1998; 2003; 2006) and Russell and co-workers (Russell and Hall, 1997; 2002; 2006; Russell and Arndt, 2005; Russell *et al.*, 1988) and could have played an important part in other hypotheses such as the older prebiotic soup theory (Haldane, 1929; Miller and Urey, 1959; Oparin, 1938) by promoting reactions between molecules which then desorb from the mineral.

Considerable work has been done on the adsorption and modification of nucleic acid bases and short oligomers onto clays (Ertem and Ferris, 1993; Ferris *et al.*, 1989; Huang and Ferris, 2003; Franchi *et al.*, 2003; Winter and Zubay, 1995). Nucleic acids also adsorb onto graphite (Sowerby *et al.*, 2001a). Cohn *et al* (2001) reported the adsorption of the nucleobase adenine onto pyrite, pyrrhotite and quartz. All these studies used ground, natural minerals. This is important since natural minerals are bulk phases which have considerably different properties to their nanoparticulate equivalents. In particular, the size of the nanoparticles (e.g. 2-10 nm) overlaps the sizes of these large organic molecules. Therefore, adsorption and desorption may not be strictly relevant and the process is better described by as coupling of particles to the molecule, in the same way as has been described for quantum dots (Chan *et al.*, 1998).

In the hydrothermal FeS world hypothesis the inorganic substructure on which organic evolution takes place is a precipitate formed when sulphide-bearing hydrothermal vent fluid comes in contact with oceanic waters high in Fe(II) (Russell and Hall, 1997; Wächtershäuser, 2006). This reaction produces stoichiometric iron (II) monosulphide similar to the mineral mackinawite (FeS_m) (Ohfuji and Rickard, 2006; Russell *et al.*, 1988). No study has been reported which

has examined the binding of nucleotides or polynucleotides to natural mackinawite or precipitated FeS_m.

FeS_m may have been capable of acting as a primitive ribosome by attaching to RNA which then grips amino acids and catalyses the formation of bonds between them (Russell and Hall, 1997; 2006; Wächtershäuser, 2006). Wächtershäuser (1990; 1992) hypothesises that phosphorylation in the sugars of nucleic acids and in other biochemical roles emerged to enable these molecules to bind to positively charged surface sites on bulk mineral surfaces via electrostatic interactions. In the nanoworld, this process is more likely to be chemical bonding between the molecules and the nanoparticles. An analogous system has been described in modern natural aqueous environments where large organic molecules may act to stabilise metal sulphide clusters (Rozan *et al.*, 2000) This study examines the extent to which a variety of nucleic acids interact with freshly precipitated FeS_m. The following polymers were examined: chromosomal DNA (cDNA), oligomeric DNA (oDNA) and RNA, as well as the DNA nucleotide deoxyadenosine monophosphate (dAMP), the non-phosphorylated version of this molecule deoxyadenosine and the purine nucleobase adenine from which they are derived. Adenine has been selected for study as it is likely to have been the first nucleobase formed, through HCN polymerisation (Ferris *et al.*, 1978) and also has other key roles in biochemistry particularly in energy transfer molecules.

2. Materials and methods

Different nucleic acids were added to freshly precipitated FeS, CuS or ZnS and centrifuged to remove the solid precipitate and any adsorbed nucleic acids. The

supernatant was removed and analysed by UV-vis spectroscopy to determine the concentration of the nucleic acid remaining in solution. The initial concentration of the nucleic acid was determined by preparing a solution from the same stock, diluted to the same concentration, centrifuged and analysed by UV-vis spectroscopy.

2.1. Sample preparation

Metal sulphides were precipitated in sterile 1.5 mL Eppendorf tubes or sterile 15 mL conical centrifuge tubes from 50 mM stock solutions of sodium sulphide nonahydrate ($Na_2S.9H2O$) and ammonium iron sulphate ($Fe(NH_4)_2(SO_4)_2.6H_2O$,), copper (II) sulphate ($CuSO_4.5H_2O$) or zinc sulphate ($ZnSO_4.7H_2O$). Pre-prepared aqueous solutions of cDNA, pDNA, oDNA, RNA, adenine, deoxyadenosine or deoxyadenosine monophosphate were added after the precipitates were formed.

Adenine, 2'deoxyadenosine, 2'deoxyadenosine 5'monophosphate and RNA (R1753, E. Coli Strain W, Type XX) were purchased from *Sigma* as powders and dissolved in dH_2O. The solutions were centrifuged to remove any undissolved matter and then diluted to a concentration which on a 1:5 dilution gave an A260 nm of ~1. Oligomeric DNA (oDNA) was a 20-mer single stranded DNA molecule purchased from *MWG-biotech*. Chromosomal DNA (cDNA) was extracted from an *E. coli* culture grown in LB broth using *Bio 101 Systems* Fast DNA® Spin Kit according to manufacturer's instructions. Electrophoretic analysis determined the number of base pairs in this cDNA to vary between approximately 1000 and 10,000.

All reagents were of analytical quality. Water was of MilliQ quality with a resistance of 18 Mohms, sterilized through autoclaving and deoxygenated by bubbling oxygen free N_2 through it for at least 1h. Micro cuvettes and 15 mL centrifuge tubes were sterile on purchase, 1.5 mL microfuge tubes were sterilized through autoclaving

for 2 h at 2 Atm. Plastic bottles containing stock solutions were sterilised by washing with Decon 90® and then with ethanol.

The pH of a 10 mM, 50 mL solution of FeS in dH_2O was measured on a *Jenway 2030* ™ digital pH meter and found to be 7.4. The effect of the organic molecules on this pH is thought to be negligible so a pH of 7.4 is assumed for all the FeS samples in this study.

The nanoparticulate metal sulphides prepared as reactants in this study have been characterized in previous reports. The FeS_m used has been characterized in some detail by Ohfuji and Rickard (2006), Rickard (2006) and Rickard et al. (2006). The nature of precipitated ZnS has been discussed by Luther et al (1999), Zhang et al (2004) and Luther and Rickard (2005). CuS precipitates were originally described by Rickard (1973) and subsequently defined by Luther and Rickard (2005). FeS_m is stoichiometric nanoparticulate FeS with a mackinawite structure. The particles are between 2 and 5.7 nm thick and between 3 and 10.8 nm in length with a mean size of 3.6 x 5.8 nm. The specific surface area is 380 ± 10 $m^2 g^{-1}$. The length of a nucleic acid molecule is 0.34 nm per base. This gives lengths of 6.8 nm, ~ 27.2 nm and 340-3400 nm for the oDNA, RNA and cDNA respectively.

2.2. Analysis

Metal sulphide + nucleic acid preparations were incubated in a *Labmaster 130*™ anoxic cabinet. They were removed from the cabinet and centrifuged in an *Eppendorf* 54515D microfuge at 5000 rpm or until the supernatant had cleared. 0.5 mL of the cleared supernatant was then pipetted into a UV-permeable micro cuvette (Fisher). A *Cary 50 Probe* UV-vis spectrophotometer was used to measure the absorbance of the samples from 220 nm to 600 nm in the experiments with adenine,

adenosine, dAMP and RNA. The experiments with cDNA and oDNA were performed with a *Perkin-Elmer Lambda 2* which took a single reading at 260 nm.

1 mL samples of 10 mM FeS, CuS, ZnS or $FeSO_4$ with 44.9 μg/mL chromosomal DNA were mixed and left for one hour in an anoxic cabinet. The solutions were centrifuged at 5000 rpm for 5 minutes or until the supernatant had cleared. 0.5mL of the supernatant was pipetted into a UV cuvette and the absorbance at 260 nm was measured in the spectrophotometer. Inorganic blanks of 10 mM FeS, CuS, ZnS and $FeSO_4$ without DNA were performed simultaneously and the A260 reading of these negative controls was subtracted. In all cases A260 nm of the blanks was < 0.1. For both the DNA samples and the blanks two samples were prepared and the mean A260 nm reading was calculated. cDNA was quantified from the A260 nm reading using an extinction coefficient of 1 A260 = 50 μg/mL cDNA (Gallagher and Desjardins, 2006). In all experiments a positive control was prepared from the same nucleic acid stock solutions, diluted to the same extent as the experimental samples, to ascertain the initial concentration of the nucleic acids in the experimental samples.

Experiments with RNA, oDNA and adenine derivatives were performed over a longer time-period and with only FeS_m. Solutions of 10 mM FeS plus the nucleic acid were made up to 5 mL in 15 mL conical centrifuge tubes. FeS-free nucleic acid controls were also made up from the same stock solution and to the same concentration as in the experimental preparations. Solutions were stored in an anoxic cabinet. At the given time intervals 1 mL was subsampled into a 1.5 mL Eppendorf microfuge tube which was removed from the anoxic cabinet and centrifuged at 5000 RPM for 5 minutes or until the supernatant had cleared. Subsequently 0.5 mL of the clear supernatant was pipetted into a UV cuvette and an absorbance scan from 220 nm to 600 nm was performed.

To correct for background absorbance due to aqueous sulphide and aqueous FeS, absorbance readings were taken at 320 nm (nucleotides) or 600 nm (RNA). Nucleic acids do not absorb radiation at these wavelengths so the absorbencies are solely the result of the centrifuged FeS solutions. For the experiments with RNA 600 nm was used as it was discovered that centrifuged FeS+RNA solutions absorbed at 320 nm to a degree which was not entirely attributable to FeS. FeS blanks determined the A260/A320 of centrifuged 10 mM FeS to be 1.13 and the A260/A600 nm to be 0.95. To determine the amount of the A260 nm which was due to sulphide the A320 nm or A600 nm in each sample was multiplied by the correction factor of 1.13 or 0.95 respectively. This value was subtracted from the A260 nm of the sample and the resultant value was multiplied by its extinction coefficient (Gallagher and Desjardins, 2006) to determine the concentration of nucleic acid in solution.

3. Results

3.1. Chromosomal DNA

Mean A260 nm for the two centrifuged FeS + cDNA preparations after 1 h was 0.013 (Fig. 1) which equates to 0.65 μg/mL cDNA remaining in solution (Fig. 1), using an extinction coefficient of 50 μg/mL/A260. Therefore, a total of 44.3 μg of cDNA bound to 10 μmols (880 μg) FeS. To ensure that these results are not due to metal ions binding to the DNA and causing it to precipitate $FeSO_4$ was also incubated with cDNA centrifuged and analysed in the same manner. A cDNA concentration of 40.4 μg/mL was detected in this sample (Fig. 1), a total loss of 4.5 μg/mL, approximately 10% of the reduction in cDNA concentration observed with FeS. A

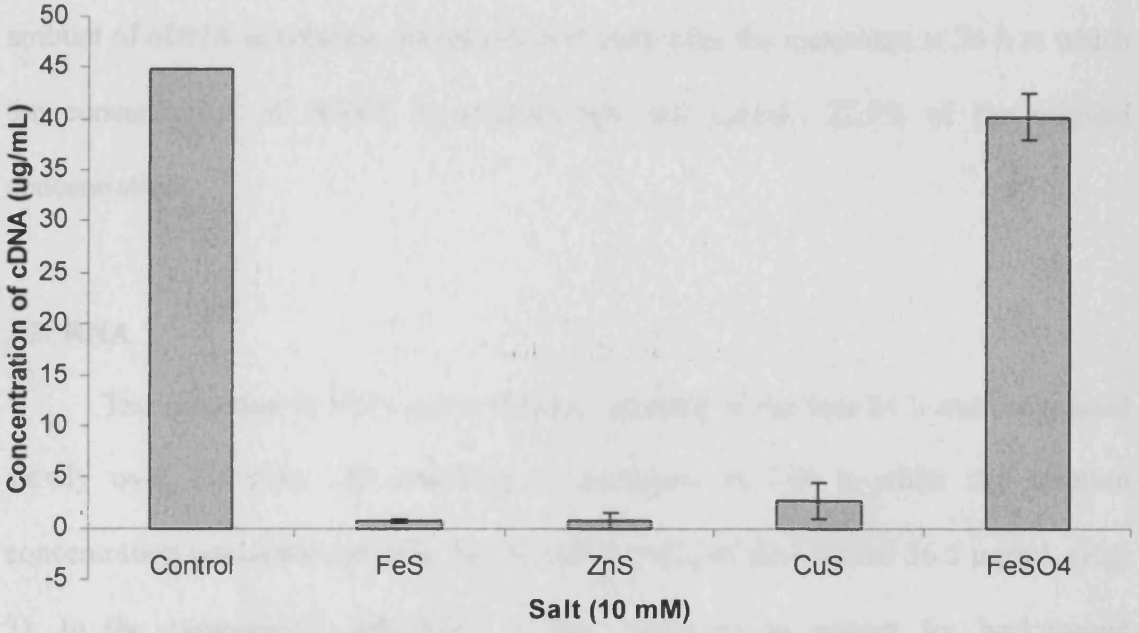


Figure 1. The amount of cDNA remaining in solution after treatment with 10 mM FeS, CuS and ZnS for 1h and centrifuged. The control shows the amount of cDNA added to each sample. In all three cases < 5% of the cDNA remained in solution. The $FeSO_4$ caused a much smaller reduction in the concentration of cDNA in solution confirming that the results are not due to precipitation of cDNA with aqueous metal ions. Chart shows the mean of two samples error bars show +/- standard deviation. Raw data is provided in appendix 5 (a).

loss in cDNA solution concentration, similar to FeS, was observed with ZnS and CuS, 44.2 μg/mL and 42.2 μg/mL respectively (Fig. 1).

3.2. Oligomeric DNA

After 1 h the reduction in oDNA solution concentration was 6.8 μg/mL from a starting concentration of 26.2 μg/mL (Fig. 2) representing a reduction of 26.0%. The amount of oDNA in solution decreased over time with the maximum at 24 h at which the concentration of oDNA in solution was 6.0 μg/mL; 22.9% of the original concentration.

3.3. RNA

The reduction in RNA concentration occurred in the first 24 h and progressed slowly over the next 120 reaching a maximum at 144 h when the solution concentration was approximately 24.1% (8.8 μg/mL) of the original 36.5 μg/mL (Fig. 3). In the experiments with RNA it was necessary to correct for background absorbance at 600 nm rather than 320 nm due to an unexpected absorption at ~ 300 – 400 nm (Fig 4). This absorbance is greater than the combined absorbance of RNA and an FeS blank which are both low and approximately constant over this region whilst the absorbance of the FeS + RNA supernatant decreases from 300 nm to 400 nm by ~ 0.2 Abs.

3.4. Adenine and derivatives

The DNA monomers adenine, adenosine and dAMP all adsorbed onto FeS_m to a much lower extent than the three polymers in this study. The reduction in concentration of the monomers occurred in the first 24 h to 67.9%, 66.5% and 84.7% of their original

concentrations for adenine, deoxyadenosine and dAMP respectively (Fig 5). The concentration of adenine and adenosine in solution rose slightly over time whilst the concentration of dAMP dropped slightly between 24 and 48 h. After 360 h 82.9% of the dAMP remained in solution compared to 74.3% and 76.2% for adenine and adenosine respectively.

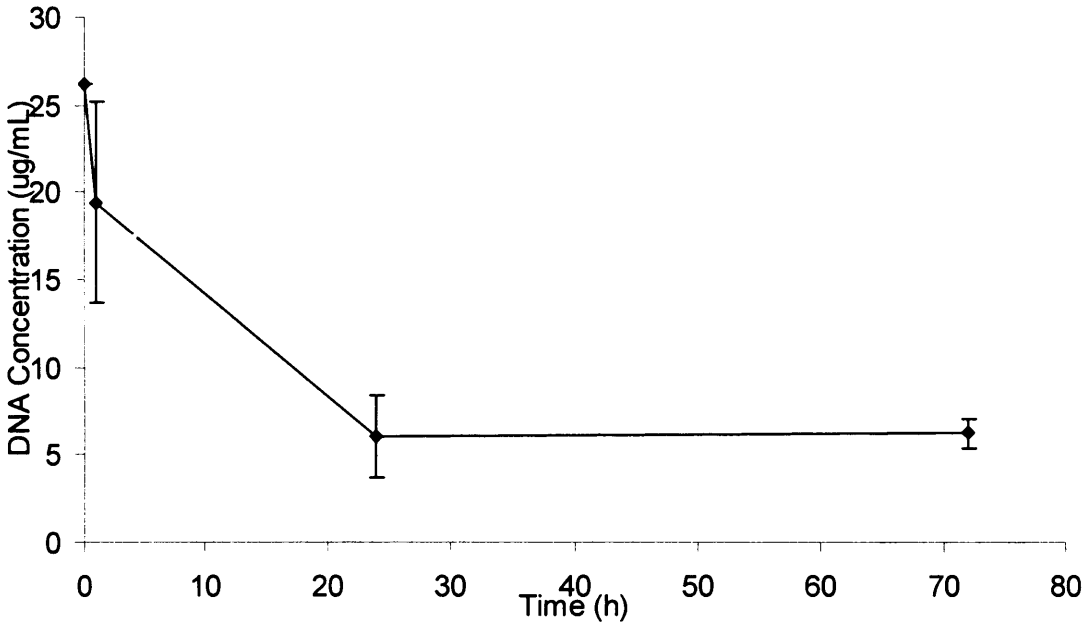


Figure 2. Decrease in oDNA concentration on treatment with 10 mM FeS over a 72 h period. The amount of oDNA in solution decreased over time, reaching a minimum at 24 h. The concentration of oligomeric DNA remaining in solution was determined using an extinction coefficient of 37 µg/mL/A260 nm (Gallagher and Desjardins, 2006). The results represent the mean of two oDNA samples corrected for sulphide absorption at A260 nm through the use of 10 mM FeS blanks. 4 10 mM blanks were prepared, centrifuged and analysed, the average A260 nm x 37 of these samples was 2.92 with a standard deviation of 1.64. 2.92 was subtracted from both of the A260 nm readings for each time interval. The graph displays the mean and the error bars show the standard deviation of these two figures. Raw data is presented in Appendix 5(b).

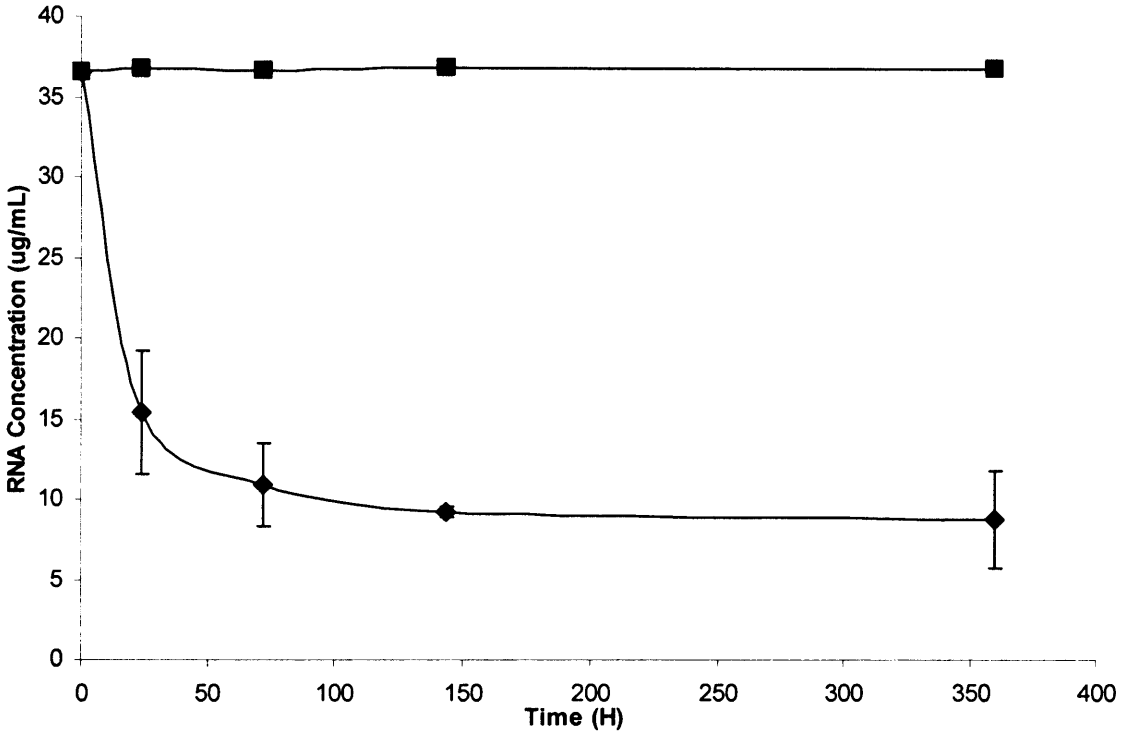


Figure 3. Decrease in RNA concentration on treatment with 10 mM FeS onto over a 144 h period. The amount of RNA in solution decreased over time in a logarithmic fashion, A260 nm was reduced to less than 50% within 1 h. Very little reduction in RNA concentration occurred between 72 and 144 h. The results represent the mean of two RNA + 10 mM FeS samples corrected for sulphide absorbance by subtracting background absorbance at 600 nm multiplied by a correction factor of 0.95. The concentration of RNA was determined using an extinction coefficient of 1 A260 nm = 40 μg/mL RNA (Gallagher and Desjardins, 2006). Raw data is provided in Appendix 5(c).

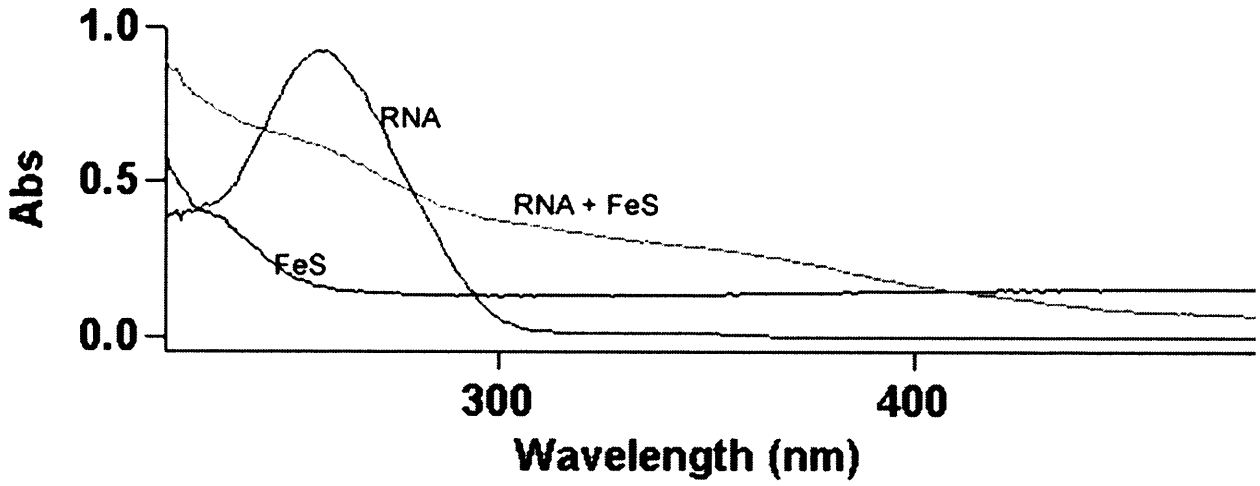


Figure 4. Absorption of RNA + FeS samples from 220 nm – 550 nm with RNA and FeS controls after 24 h incubation. The absorption between ~ 300 nm and 380 nm is greater than the combined absorption of FeS and RNA.

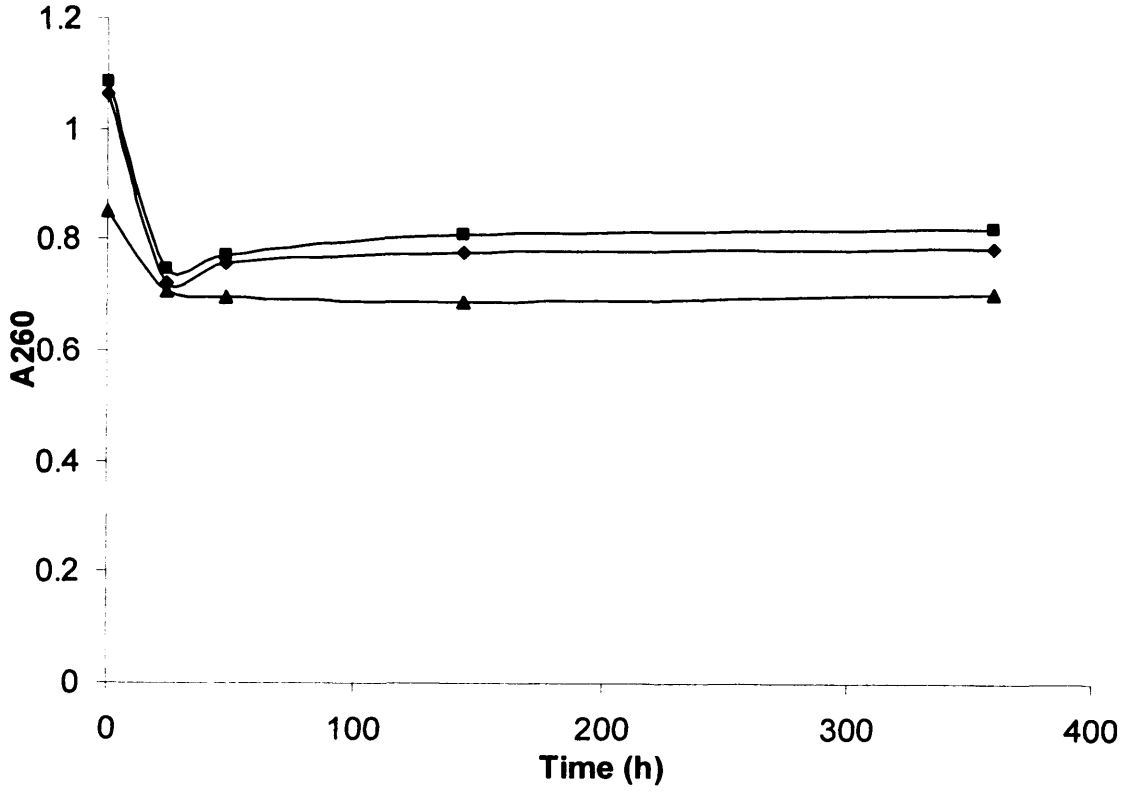


Figure 5. The decrease in adenine (♦), deoxyadenosine (■) and deoxyadenosine monophosphate (▲) concentration on treatment with 10 mM FeS. Only a small fraction of the molecules were attached, in all three cases. Maximum attachment occurred within the first 24 h and did not significantly change after this time. Deoxyadenosine monophosphate experienced the smallest reduction in A260 nm. Each result represents the mean of two RNA + FeS samples corrected for background absorbance at 320 nm multiplied by a correction factor of 1.13. Error bars are included and show the standard deviation of the two samples but lie within the symbol size in most cases. Raw data is provided in Appendix 5 (d).

4. Discussion

All the nucleic acids examined in this study interacted the surface of FeS_m to some extent. To determine if other metal sulphides interacted with DNA, CuS and ZnS were also mixed with cDNA, centrifuged and analysed. The results were similar to those with FeS. However, further investigation into these minerals was not performed because Zn^{2+} causes DNA sedimentation (Kejnovsky and Kypr, 1998) and because CuS did not consistently centrifuge to a clear supernatant but occasionally left a suspension which absorbed at 260 nm substantially.

Chromosomal DNA exhibited a greater tendency to bind with FeS_m than either RNA or oDNA which both bound substantially more than any of the monomers. This suggests that length is a significant factor in determining the interactions between nucleic acids and FeS_m. However, even small polymers (oDNA, 20 base pairs) experienced a reduction in concentration of more than 50% given sufficient time which did not occur with the monomers. The reduction in RNA concentration (~80 base pairs) was slightly greater than that of oDNA which could be attributed to the difference in length or the differences in chemistry of the two molecules.

Adenine, deoxyadenosine and deoxyadenosine monophosphate all exhibited similar binding capacity to FeS_m. This indicates that adenine itself is capable of interacting with FeS_m and this is not enhanced by the presence of the ribose-sugar group or, critically, the charged phosphate group. This is compatible with the view that RNA adsorbed more than oDNA due to its greater length and not differences in its sugar because the sugar does not seem to be involved in binding. This finding is contrary to the adsorption of nucleic acids onto clays which was found to require the

phosphate group (Franchi *et al.*, 2003) and that double stranded DNA had a lower affinity for the mineral than single stranded DNA. In this case divalent cations promoted adsorption by intercalating between the DNA and the clay, neutralising the negative charges on both.

FeS nanoparticles have a mean size of 3.6 x 5.8 nm (Ohfuji and Rickard, 2006), substantially larger than nucleic acid monomers but small compared to the lengths of some of the polymers used in this study: 27.2 and 340 – 3400 for RNA and cDNA respectively. Interactions between bulk minerals and small organic molecules are usually discussed in terms of adsorption onto the mineral. Although the adenine derivatives are small enough to have adsorbed onto a single nanoparticle, the polymers are not. It is envisaged that the FeS nanoparticles, and possibly aqueous clusters, attached to the nucleic acid polymers at multiple locations. The resultant FeS-nucleic acid complexes were of sufficient weight that they centrifuged out of the solution. It should be noted that at the concentrations used in this study the FeS_m flocculated; the majority of the FeS_m would not pass through a 0.45 μm filter. These loosely aggregated flocs may provide larger surfaces for adsorption although they are still comprised of nanoparticles.

The pKa of adenine is about 3.7 (Dawson *et al.*, 1986) therefore it is uncharged at pH 7.4. FeS_m has no net charge at this pH either: Wolthers *et al* (2005) reported that the point of zero charge (PZC) of disordered mackinawite is reported to be ~ 7.5. Bebie *et al.* (1998) reported that all metal sulphides have an isoelectric point between pH 0.6 and 3.3 (1998) although they did not examine FeS. More alkaline PZC's have been reported which are thought to be the result of slight oxidation (Bebie *et al.*, 1998). Wolthers *et al* (2005) dismisses the effect of oxygen because of careful anoxic conditions and because continuous dissolution of the FeS_m surface increases

aqueous S(-II) concentration which is more reactive towards oxygen. Although it should be noted that in this experiment, as in Wolthers (2005) the FeS_m is formed by precipitation of iron sulphate and sodium sulphide and no attempt was made to remove the sodium or sulphate ions. Sorption of these onto the surface can affect the surface charges (Bebie *et al.*, 1998).

Adsorption of adenine onto FeS_m may be a physisorption process arising through induced inter-molecular forces. Uncharged adenine can bind to graphite (Cohn *et al.*, 2001), and other uncharged mineral surfaces through van der Waals forces. Adenine has a planar shape which allows it maximum contact with the mineral surface which enhances the van der Waals forces.

The interactions between pyrite and organic molecules were found to be independent of overall surface charges and governed by interaction with specific surface sites (Bebie and Schoonen, 2000). Plekan *et al.* (2007) found that adenine adsorbs onto pyrite through both physisorption and chemisorption. Unlike the flat monolayer which adenine forms on the surface of graphite, it adsorbs at an angle on pyrite, suggested to be the result of a chemical bond formed between Fe atoms in pyrite and N atoms in adenine (Plekan *et al.*, 2007).

Although the pH in this study is approximately equal to the PZC of FeS_m this does not mean that there are no surface charges; mackinawite has a variety of both negatively and positively charged surface sites. Wolthers *et al.* (2005) described a surface complexation model for disordered mackinawite. Two major surface functional groups are described; a hydroxylated iron group $FeOH^0$ and an acidic sulphide group SH^0. The acidic sulphur group is dominant in conditions where FeS is saturated and pH < ~10 as is the case in the samples in this study. Thus, the Fe-SH bond is expected to be favoured over the Fe-OH bond so the surface is described

through monocoordinated and tricoordinated sulphur sites: FeSH and Fe_3SH respectively. These can produce charged species through protonation or deprotonation. At pH 7.4 the most abundant groups are FeS^- and $Fe_3SH_2^+$ which both occur at a concentration of ~ 1.5×10^{-3} g mol^{-1}.

The abundance of thiol sites provides both positive and negative sites for potential reaction. However, surface sulphur atoms in pyrite did not interact with adenine and the iron atoms which did are less prevalent on the surface of FeS_m (Plekan *et al.*, 2007). The dominance of thiol groups might explain why adenosine monophosphate did not bind more readily than adenosine. Negatively charged phosphate would be more likely to bind with the Fe-OH group. Ertem and Ferris (1998) hypothesised that adenine adsorption onto clays occurs at negatively charged sites through the protonation of adenine. Although adenine was not protonated in bulk solution on entering the acidic interlayer protons could be donated to the molecule, probably at one of the NH_2 groups, giving it a positive charge. A similar reaction could be occurring here in which adenine, deoxyadenosine and dAMP are protonated through interaction with the acidic sulphur sites.

FeS is considerably more soluble than pyrite. Substantial Fe^{2+}, S(-II) and $FeS_{(aq)}$ exist in solution (Rickard and Luther, 2007). This is relevant because aqueous ions can mediate the adsorption of nucleic acids onto mineral surfaces by intercalating between them. Franchi *et al.* (2003) found that divalent cations were able to enhance the adsorption of nucleic acids onto negative sites on clays (Franchi *et al.*, 2003) and the adsorption of 5'AMP onto pyrite (Pontes-Buarques *et al.*, 2001). Aqueous iron or iron sulphide complexes interact with the organic molecules in solution, forming nucleic acid-Fe(S) complexes which, via the iron group could form a bond with a surface thiol group. This suggestion is supported by the results with RNA. RNA not

only adsorbed onto the FeS_m surface but as this absorbance between ~300 nm and 400 nm is greater than the combined absorbance of RNA and an FeS blank it suggests the existence of an aqueous RNA-Fe(S) complex. This might arise from RNA interacting with aqueous FeS clusters. These clusters form spontaneously in the presence of iron and sulphide (Luther and Rickard, 2005) and are also key electron-transfer agents in some enzymes including the ancient ferredoxins.

The nucleobases of double stranded cDNA are orientated within the double helix and the phosphates are orientated externally. The extent of the interaction between cDNA and FeS is then unexpected given the theory that it is the nucleobase which is responsible for the interaction. At greater lengths van der Waals forces may be the dominating factor which is enhanced with larger molecules. Alternatively, the molecule's greater length may have resulted in more FeS particles binding to it resulting in a greater increase in mass and a greater tendency to centrifuge out of solution.

5. Conclusions

All the nucleic acids studied interacted with FeS_m. Adenine couples with FeS_m as rapidly as or faster than dAMP so it can be inferred that the moiety which allows for interaction with FeS is not the phosphate as would be expected nor the ribose sugar but the nucleobase itself. This is interesting as it has been speculated that phosphorylation of sugars emerged to facilitate binding to pyrite/FeS minerals (Wächtershäuser, 1988; 1990). Purine nucleobases (such as adenine) have been noted to bind to pyrite without phosphorylation and this study demonstrates that the same is true for nanoparticulate FeS.

Although a number of mechanisms have been proposed, this study has not determined the nature of the interaction between sulphide precipitates and nucleic acids. Spectroscopic work is required to determine this, particularly the sites of interaction and the orientation of the molecule. This would be a challenge because of the nanoparticulate nature of the material. A flat orientation with adenine in parallel sheets would be favourable for origin of life hypotheses as this would more readily promote polymerisation between neighbouring molecules. This was the case with graphite (Cohn *et al.*, 2001), however, graphite is not thought to be a feasible prebiotic mineral.

It is not possible at this stage to determine if the increased attachment of FeS_m to cDNA over RNA or oDNA was because of its larger size or its double stranded structure. However, since it appears that it is the nucleobases which bind to FeS_m which are orientated within the double helix it would be expected that, if the double stranded nature had any effect, it would be to reduce interaction. Therefore, it would appear that length of the molecule is a significant factor. This could promote elongation of nucleic acids on an FeS_m surface.

This work provides evidence that an FeS mineral could have been a site of sequestration, protection and polymerisation of nucleic acid monomers. It also supports the hypothesis that RNA could have acted as a bridge between hydrothermally precipitated FeS and amino acids (Russell and Hall, 2002; 2006) thus, potentially, originating the translation and transcription system and provides a possible mechanism for ribozymes to gain electron transfer capabilities by binding with aqueous FeS clusters or nanoparticles.

Chapter 8

Discussion and conclusions

1. DNA Scission by Metal-Promoted Hydroxyl and Sulphur Radical Production

Scission of pDNA molecules occurs on reaction with solutions of FeS (Chapter 5), CuS (Chapter 6), $FeSO_4$ (Chapter 3) and $CuSO_4$ (Chapter 6) through the production of free radicals. The radicals were produced by the iron or copper-catalysed decomposition of the single covalent bond between oxygen atoms in peroxide or sulphide atoms in disulphide.

In the case of $FeSO_4$ solutions, hydroxyl radicals are produced from hydrogen peroxide generation initiated by the reaction between O_2 and $Fe^{2+}_{(aq)}$. In the presence of Fe^{2+}, $OH\cdot$ radicals are produced through the Fenton reaction. The mechanism of DNA damage by $CuSO_4$ is presently unknown but is not thought to be a Fenton–like process due to the lack of Cu(I) in aqueous solutions to react with O_2.

A sulphide-analogue of the Fenton reaction is proposed (Chapter 5) in which disulphide (S_2(-II)) is decomposed into $HS\cdot$ or $S^{-}\cdot$ radicals by Fe^{2+}, Fe^{3+}, Cu^{+} or Cu^{2+} as hydrogen peroxide is in the Fenton reaction. Whilst hydrogen peroxide is generated through the reaction between Fe(II) and O_2, disulphide is present in sulphide solutions regardless of the presence of oxygen (Luther *et al.*, 1985). Iron and copper ions have reduced and oxidised states (+2 and +3 for Fe, +1 and +2 for Cu). In either redox state both metals can react with hydrogen peroxide to produce radicals but the reduced ion is needed to generate hydrogen peroxide from O_2. Cu(I) does not exist in the sulphate solutions in significant concentrations. However, it is formed from Cu(II) on precipitation with S(-II), these solutions also contain S_2^{2-}. In the presence of sulphide the metal ions can induce radical production in either redox state.

Zinc sulphide, zinc sulphate and sodium and ammonium sulphide solutions did not cause scission of DNA strands. Aqueous Zn (II) and Na (II) species do not readily donate electrons and therefore cannot reduce dioxygen or decompose hydrogen peroxide or disulphide. The absence of a reaction with ZnS is further confirmation of the suggested radical reaction as Zn(II) in contrast to Cu(I) and Fe(II) does not have a more oxidised state.

1.1. Implications for the origin of life

This study indicates that in any environment with < 0.1 mM S(-II) and/or 10 ppm O_2 in the presence of Cu(II) and/or Fe(II), free radicals will be produced which react with the ribose-phosphate backbone of DNA causing scission. The environments proposed in the iron-sulphur origin of life hypotheses meet these conditions through the meeting of sulphurous vent water with iron-bearing oceanic water. The Hadean ocean may have met these conditions as well. The Hadean ocean is proposed to have contained 0.1 to 0.5 mM Fe(II) (Walker and Brimblecombe, 1985), estimates of O_2 concentration vary from 10^{-12} of the present atmospheric level (PAL) (Kasting *et al.*, 1979) to 50% more than the PAL (Ohmoto, 1997). The evidence for the higher concentration has been criticised (Holland, 1999) and most calculations estimate the oxygen level to be closer to Kasting's result but not as low. Carver (1981) estimated an O_2 concentration of 10^{-3} to 10^{-1} PAL (200 – 20000 ppm) which is supported by Canfield's (2005) estimation of 10^{-3} PAL. The result is that the O_2 concentrations in this study, were lower (< 10 ppm) than those proposed for the Hadean ocean. This means that oxidative free radical production may have been even more prevalent in that ocean than in the experimental systems.

Double-stranded plasmid DNA was used in this study partly because of its usefulness in DNA-damage assays. It is expected that the same reaction would occur with any DNA molecule or with RNA as they are all susceptible to radical attack. RNA may react more readily with iron sulphide than DNA. DNA forms a very stable double helix structure that makes it is resistant to any kind of breakdown. RNA exists as a single strand that folds in upon itself forming hair-pin loops, coils and other tertiary structures. Although this provides some additional stability, it does not provide nearly as much stability as the helical structure of DNA. Also, the presence of an additional hydroxyl group on the carbon-2 position of the ribose ring allows molecule to engage in a greater range of reactions than DNA can. The reasons for these differences are that the role of RNA is transient unlike DNA, which is used for long-term storage of hereditary information. This is a problem for the RNA world hypothesis, as it would require long term storage of hereditary information in RNA, a task that is difficult in the relative safety of a living cell and almost unfeasible outside of one.

The effect that the scission of nucleic acids would have had on an emerging genetic system is difficult to predict as it is dependent on many unknown variables related to the radical generating mechanism, the nature of the genetic system and its chemical environment. If the reaction was sufficiently severe that any DNA polymers that formed were rapidly depolymerised then the origin of life could not occur in this locality. In this study DNA was not depolymerised to this extent, the non-appearance of pDNA on the electrophoretic gels was attributed to adsorption onto FeS_m (Chapter 5) or green rusts (Chapter 3) rather than extensive fragmentation. However, temperature and pH could have significant effects. Although the temperatures the proposed in the FeS worlds are always hydrothermal, there is little consensus on the

exact temperature and temperature gradients may have existed. A pH gradient of 5 to 8 is a feature of Russell's hypothesis.

Extensive fragmentation of DNA, or another polymer, could be inhibited by the presence of other organic chemicals. For example, Cohn (2003a; 2006) found that lipids inhibited destruction of RNA by pyrite-induced free radicals, lipids may have appeared early in the FeS world through abiotic processes (Wächtershäuser, 1988; 1992). These mechanisms may not be as efficacious if the radicals in this study were produced at DNA-metal ion binding sites and react immediately. A less intense reaction, in which scission occurs at a rate which is lower than the rate of polymerisation, could be said to have retarded the growth of nucleic acids and been a hindrance to nucleic acid development without preventing it entirely. Conversely it could have conferred benefits to the emerging system.

Huber et al (2003) reported that peptide bonds, between two phenylanine amino acids, were both formed and hydrolysed in a hot aqueous environment containing carbon monoxide and coprecipitated iron and nickel sulphides. This gave rise to the idea of a cyclic turnover of amino acids to peptides and back to amino acids, something which happens in extant metabolism. This cycle is said to provide a "dynamic chemical library" and prevents functionless peptides being futile sinks for valuable amino acids. A similar scenario can be imagined with a nucleic acid cycle. Although nucleic acid polymerisation on FeS has not been demonstrated yet, it has been proposed to occur. Russell's hypothesis asserts that polymerisation is driven through the electrochemical gradient across the membrane.

The genetics of Russell's system is most fully discussed in Koonin and Martin (2005). Their proposal involves a cycle of DNA-RNA hybrid replication in which most sequences are entirely selfish replicators that do not code for anything other than

their own replication. The selfless replicators are fewer and code for the production of RNA or peptide based catalysts which facilitate the replication of themselves and other sequences. As in Huber's peptide cycle, scission of the selfish replicators would free up nucleotides and oligomers from functionless molecules allowing them to be assimilated into other sequences, which could develop the ability to code for catalysts. Scission of selfless replicators by radicals would be detrimental to the system by causing them to loose their coding ability.

Although this study has focussed on the reactions of DNA in a prebiotic environment its findings have implications to other areas of origin of life research and to other fields. Hydroxyl radicals react readily with a variety of organic chemicals including the low molecular weight organics produced through the carbon fixation reactions and autotrophic metabolism proposed by Wächtershäuser and Russell such as 1-5 C alkane thiols (Heinen and Lauwers, 1996) and 1-2 C carboxylic acids (Huber and Wächtershäuser, 1997; Kanemoto *et al.*, 1992). As with the reaction with DNA, it is difficult to predict the effect this would have as the reaction can be both constructive and destructive. A common mode of reaction is abstraction of a hydrogen atom from the organic molecule

$$R\text{-}H + OH\cdot \rightarrow R\cdot + H_2O \quad \text{...} \quad (1)$$

The fate of the resultant, highly reactive, organic radical will be dependent on conditions. It could decompose further or react with other organic radicals, combining in novel ways. This is a potential source of new pathways and reactants which is required for the metabolic evolution described in the FeS-world hypotheses. Synthetic organic chemists employ metal-promoted radicals in the production of complex

chemicals and they are particularly useful in the formation of carbon-carbon bonds (Giese, 1986; Iqbal *et al.*, 1994). Around 50% of industrial polymers are produced using free radical processes (Moad and Solomon, 1995). Carbon-carbon bond formation is obviously an essential process in the development of metabolism for which few viable mechanisms have been proposed and only small carbon chains have so far been produced in experiments. Radical-promoted organic synthesis is an interesting possibility in the origin of life, especially in an FeS world, which seems to have been neglected by researchers.

1.2. Other effects

The proposed discovery of iron-promoted decomposition of bisulphide into sulphur radicals has implications outside origin of life research. The genotoxicity of sulphide (Attene-Ramos *et al.*, 2006) has been attributed to its reaction with intracellular Fe^{2+} as FeS is reported to decompose hydrogen peroxide more rapidly than iron alone (Berglin and Carlsson, 1985). The evidence for this is that FeS and H_2O_2 decomposed deoxyribose more rapidly than Fe^{2+} and H_2O_2. The experiment was not set up to detect hydroxyl radicals or the decomposition of H_2O_2 and no mechanism is proposed for the capability of FeS to decompose hydrogen peroxide. An alternative proposal would be that the enhanced deoxyribose break down was due to $HS\cdot$ radicals.

Rickard and Luther (2007) reported that greigite forms spontaneously from mackinawite in anoxic systems. This reaction involves the oxidation of two thirds of the Fe(II) in mackinawite but the oxidising agent in these conditions is not known. The reaction proposed here would suggest that bisulphide radicals could be the missing electron acceptors.

It is suspected that for an emerging living system in an FeS rich environment the evolution of methods of protection against these reactions would have been strongly selected for from an early stage. In both eukaryotes and prokaryotes, excess iron is stored as an iron-sulphur complex along with proteins which can be assembled or disassembled depending on iron excess or deficiency (Cairo *et al.*, 2002; Rodriguez and Smith, 2003). In bacteria, iron exists mostly as insoluble ferric complexes. Bacteria regulate iron levels by sensing intracellular iron concentration and modulating the transcription of a number of proteins (called iron regulatory proteins or IRP's) involved in the uptake and storage of iron. Iron dependent enzymes, whose main function may be nothing to do with iron regulation may also be transcribed to a greater degree and used to sequester excess iron (Cairo *et al.*, 2002; Rodriguez and Smith, 2003).

The interaction between nucleic acids and FeS has implications for the evolution of sulphate reducing bacteria which produce intraceullalar FeS_m. Compartmentalisation of this FeS away from DNA/RNA (or compartmentalisation of DNA/RNA) is a strategy which could prevent this form of DNA damage. Cohn (Cohn *et al.*, 2003b) found that lipids protected RNA from pyrite-induced hydroxyl radicals possibly through compartmentalisation.

The presence or absence of FeS could have an effect on ecologies and also on the use of molecular tools to examine them. Luther et al (Luther *et al.*, 2001) found that the presence or absence of FeS affected the distribution of the tubeworm: *Riftia pachyptila*. This was attributed to the removal of sulphide by iron from the solution as the tubeworms obtain organic carbon from symbiotic, sulphur-consuming, chemosynthetic bacteria. An organism's capacity to withstand attack by free radicals

may also affect its distribution at hydrothermal sites. Molecular ecology studies on organisms in anoxic sulphidic sediments could be affected by this reaction.

2. Adsorption of nucleic acids onto sulphides and hydroxides

This study demonstrates that double and single stranded nucleic acid polymers as well as adenine and the nucleoside and nucleotide derived from it bind to copper, iron and zinc sulphides (Chapter 7). It is thought that the monomers adsorbed onto the surface of single nanoparticles whilst multiple nanoparticles attached onto the longer polymers: cDNA and RNA. It also provides evidence that plasmid DNA bound to Fe(II)/Fe(III) green rusts (Chapter 3). All of these minerals could be present in a hydrothermally precipitated mineral assemblage (Russell *et al.*, 2005). Wächtershäuser's hypothesis focuses entirely on mackinawite and pyrite as the mineral substrate for organic evolution and proposes that once organic chemicals detach from these minerals they are irretrievably lost (Russell and Hall, 1997; Wächtershäuser, 1992; 1994). A hypothesis that realises the potential for a greater variety of minerals to promote a greater range of reactions would be preferable especially if the organic chemicals can reattach to a mineral after detaching.

In Russell's hypothesis the incubator for life is an assemblage of mostly iron, but also copper, zinc and nickel sulphides, oxides and hydroxides and magnesium-rich clays (Russell *et al.*, 2005). Due to the proposed compartmentalised structure of the mineral, detached organics are not necessarily lost but are able to react in solution, reattach to other sites or diffuse into other compartments. It has been proposed that the prebiotic soup could have been supplied by compounds which had formed on

mineral surfaces (Bada and Lazcano, 2002). This theory also makes use of a range of minerals as does the proposal of Cockell (2006) that life may have originated in a hydrothermal system within a meteoritic impact crater.

Adsorption of nucleotides onto these minerals could have facilitated their polymerisation (Ertem and Ferris, 1996) and protected these polymers from hydrolysis (Sowerby *et al.*, 2001; Shapiro, 1995). Iron sulphide may have simultaneously promoted the polymerisation and depolymerisation of nucleic acids. Also, the binding of RNA to FeS has been proposed to have been instrumental in the evolution of the genetic coding for peptides. Mackinawite acted in a similar manner to ribosomes in extant cells. Rows of RNA triplets are said to have bound to the mineral and oriented in a manner that allowed them to grip amino acid monomers, with some specificity, and promote peptide bonds between them (Russell and Hall, 1997; 2006; Wächtershäuser, 2006). However, this relies on the assumption that the phosphate group on the nucleotides would bind to the mineral orientating the nucleobase away from the mineral. This study indicates that nucleotides do not bind to mackinawite through the phosphate group, although conditions such as pH may affect this.

The role of nucleotides in extant biochemistry is not limited to the storage and transmission of information and it should not be assumed that this was their first role in biology. Wächtershäuser (1988; Wächtershäuser, 2006) hypothesises that nucleotides and sugars, long before the advent of base pairing, first served as ligands for metal catalysts. Adenine, thought to be the most ancient nucleobase (Orgel, 2000; Oro, 1961), is life's universal molecule of energy transport. Adenosine diphosphate is phosphorylised using energy derived from metabolism, the resultant triphosphate dephosphorylises with a concomitant release of energy. Nucleotides may have operated as energy transduction molecules by phosphorylising on an FeS surface. This

would be driven by either the oxidative conversion of mackinawite to pyrite in Wächtershäuser's hypothesis or the electrochemical gradient that Russell compares to the proton motive force which generates ATP in extant metabolism. The energised nucleotide would have been able to detach and diffuse to another location such as the cytoplasm or lipid membrane of Wächtershäuser's semi-cellular structures or the interior of Russell's FeS cells.

Not only would nucleic acids bind to bulk FeS minerals they would also interact with aqueous FeS clusters and FeS nanoparticles in solution. Again the extent of this latter effect is crucial. Nucleic acid polymers entirely coated in FeS particles would not be able to interact with other nucleic acids or amino acids. Alternatively DNA molecules combined with moderate quantities of sulphide particles could possess some novel quantities.

Catalytic RNA molecules were discovered in the ciliated protozoan *Tetrahymena thermophila* by Kruger (1982) who found an RNA molecule with regions capable of self-splicing by breaking and forming phosphodiester bonds. These catalytic RNA molecules have been subsequently called ribozymes. Ribozymes which catalyse the cleavage and polymerisation of RNA have been found in nature and synthesised (Scott *et al.*, 1995; Doudna and Cech, 2002). Ribozymes capable of redox reactions have not been developed, possibly because research in this area has concentrated on catalysis of RNA replication. Iron sulphur clusters are used in electron transfer reactions in biology today in the ancient class of enzymes: ferredoxins. RNA-FeS ribozymes could have been capable of fulfilling this role prior to the existence of protein enzymes.

As well as potentially fulfilling the catalytic role that proteins later took over nucleic acids could have had a structural role. This is particularly relevant in Russell's

theories in which FeS-cells would be subject to collapse and dissolution. Nucleic acids, and possibly abiotically generated lipids, could have coated the interior surfaces offering some protection against destruction of the cell. Nucleic acids in the vicinity of pores between the cells could also have had a role in controlling diffusion between the cells. It is essential that diffusion between the cells is fast enough to allow rapid colonisation of newly formed cells but not so rapid as to cause homogenisation between cells (Koonin and Martin, 2005).

3. Further work

Only indirect evidence for the sulphur-based radicals has been presented. Spin-trapping experiments could potentially be used to detect them and identify them from OH·. EPR is commonly used to investigate radical-mediated reactions but it is still difficult to identify particular radicals as a specific spin-trap is required and the radicals often have very short half-lives. Previous work in our lab (Butler and Rickard, unpublished) using 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as a spin-trap found no radical spin adduct produced by FeS in the absence or presence of hydrogen peroxide. Although when hydrogen peroxide was present there was a Fe(III) signal suggesting that the Fenton reaction was occurring. This in turn implies that the radicals present were undetectable possibly because adduct formation or signal generation were interfered with by the presence of FeS or HS- which reacts with OH·. At high concentrations of $FeSO_4$ and FeS, the concentration of pDNA on the gels was reduced and in some cases completely absent. This has been attributed to adsorption onto the solid in the wells of the gels as pDNA was found to bind strongly to FeS. However, the state of this bound DNA has not been resolved. Several questions

remain which would require further research. It is not known if the reaction is at a random site or site specific. It would not be expected that the reaction with HS· radicals would be site specific but their production could occur at sites where FeS had bound to DNA, therefore they would not migrate far in solution. DNA has specific sites for metal interaction which the Fe^{2+} or FeS may be bound to. After the initial radical attack which causes relaxation of the plasmid the next reaction could take place at this site producing monomers from the ends of the relaxed plasmid. Alternatively, the adsorbed DNA may remain as relatively intact plasmids. The extent to which aqueous FeS nanoparticles and clusters are bound to the DNA is also unknown.

This study attempted to resolve some of these unknowns. Gel electrophoresis experiments involving 20-nucleotide oligomers did not find fragmentation of the molecule, this would be evidence for a site specific reaction. Analytical techniques employed to analyse the product of the reaction and determine the state of the DNA missing from the gels suffered interference due to the presence of FeS. FeS would clog and damage a HPLC column and therefore must be removed prior to this form of analysis. When samples of 150 mM FeS + pDNA were filtered or centrifuged no ultraviolet absorption was detected by reverse phase HPLC or UV-vis spectroscopy above that of FeS blanks. FeS is soluble in acids. However, the amount of HCL required to dissolve the FeS produced a solution too acidic to be injected into a HPLC column and electrophoretic gels showed it also destroyed the DNA.

FeS also interfered with mass spectrometric attempts to analyse the product. Because of the high FeS to DNA ratio and the range of masses that FeS produces during ionisation a high signal to noise ratio was produced. Mass spectrometry on large polymers is in its infancy and no way of analysing the 1.75 M atomic mass units

plasmid could be found. I used MALDI-TOF MS to analyse 150 mM FeS + DNA samples, although peaks possibly corresponding to nucleotides were detected, further work would be required to distinguish these from FeS particles. Any DNA fragments present did not show up possibly due to its low concentration compared to the FeS. This technique could be used to analyse oligomeric DNA treated with FeS but not large plasmids. I tried electrospray mass spectrometry on samples of dAMP treated with FeS. This was to determine if FeS particles could bind to nucleotides. FeS concentration was 0.1 or 1 mM to minimise background measurements due to it. Peaks were detected which corresponded in terms of mass and isotopic pattern to dAMP+Fe and dAMP+Fe_xS_x, were x = 1-5. Suggesting that iron ions and iron sulphur cluster were binding to the DNA molecule and were responsible for some of the observed results. However, it was not possible to determine if these compounds were actually formed in solution or if they were formed through the electrospray ionisation technique. If further research could confirm that these compounds were formed in solution it would indicate that nucleotides and FeS clusters interact on a 1:1 basis with, therefore, no site specificity. By treating adenosine and deoxyadenosine with FeS, this technique could also be used to determine the site of interaction, the adsorption experiments (Chapter 7) would imply that adducts would form between FeS and adenine.

4. List of Conclusions

1. Treatment of pDNA with FeS, CuS, Fe(II)SO_4 and Cu(II)SO_4 broke the ribo-phosphate backbone of the DNA molecule. This effect occurs at the lowest

concentration of FeS tested; 0.1mM where there is no FeS_m present and is thought to occur through free radicals.

2. Reduction in electrophoretic intensity of DNA was caused by Fe(III)Cl, $Fe(II)SO_4$, $Cu(II)SO_4$, FeS, ZnS and CuS at concentrations around 25 – 50 mM.

3. Migration of DNA in electrophoresis was retarded by FeS, CuS and ZnS which is attributed to the binding of aqueous sulphide nanoparticles and clusters to the DNA.

4. DNA, RNA, adenine, deoxyadenosine and deoxyadenosine monophosphate bound to FeS, CuS, ZnS and Fe(II)/Fe(III) hydroxides. This seemed to occur through the nucleobase rather than the phosphate. Longer DNA molecules adsorbed more readily than oligomers, RNA or monomers.

5. The hypothesis that hydrothermally precipitated sulphide minerals could adsorb and concentrate nucleic acid constituents promoting nucleic acid formation and then polymerisation is supported although the hypothesis that the phosphate group emerged to allow this binding is not necessary. Although, aqueous redox active metals and sulphide would have resulted in a simultaneous depolymerisation of nucleic acids. This may have been a hindrance or a benefit to nucleic acid evolution.

6. Non-redox active metal sulphides, such as zinc, could have provided some of the functionality of iron sulphide especially with regards to nucleic acid formation, but without producing free radicals and therefore, simultaneous nucleic acid destruction. However, iron is essential to the theories of Wächtershäuser and Russell.

7. Further research, into the extent and nature of DNA-FeS interactions and the nature of a genetic system emerging within or on a hydrothermal iron sulphide mineral, would be required to determine if these results are a challenge to the hypothesis that a proto-organism with DNA replication, translation and transcription is capable of emerging in these environments.

References

Allen, D. E. and Seyfried, W. E. (2003) Compositional controls on vent fluids from ultramafic-hosted hydrothermal systems at mid-ocean ridges: An experimental study at 400°C, 500 bars. *Geochimica et Cosmochimica Acta,* **67,** 1531-1542.

Attene-Ramos, M. S., Wagner, E. D., Plewa, M. J. and Gaskins, H. R. (2006) Evidence that hydrogen sulfide is a genotoxic agent. *Molecular Cancer Research,* **4,** 9-14.

Bada, J. L. and Lazcano, A. (2002) Origin of life. Some like it hot, but not the first biomolecules. *Science,* **296,** 1982-1983.

Bahcall, J. N., Pinsonneault, M. H. and Basu, S. (2001) Solar models: Current epoch and time dependences, neutrinos, and helioseismological properties. *Astrophysics Journal,* **555,** 990-1012.

Balasubramian, B., Pogozelksi, W. K. and Tullius, T. D. (1998) DNA strand breaking by the hydroxyl radical is governed by the accessible surface areas of the hydrogen atoms of the DNA backbone. *Proceedings of the national academy of science,* **95,** 9738–9743.

Baldridge, K. M., Gordon, M. S. and Johnson, D. E. (1987) Thermal decomposition of methanethiol and ethanethiol. *Journal of Physical Chemistry,* **91,** 4145-4155.

Banfield, J. F. and Hengzhong, Z. (2001) Nanoparticles in the environment. *Reviews in Mineralogy and Geochemistry,* **44,** 1-58

Banks, D. A. (1985) A fossil hydrothermal worm assemblage from the Tynagh lead-zinc deposit in Ireland. *Nature,* **313,** 128-131.

Bebie, J. and Schoonen, M. A. A. (2000) Pyrite surface interaction with selected organic aqueous species under anoxic conditions. *Geochemical Transactions,* **1,** 47.

Bebie, J., Schoonen, M. A. A., Fuhrmann, M. and Strongin, D. R. (1998) Surface charge development on transition metal sulfides: An electrokinetic study *Geochimica et Cosmochimica Acta,* **62,** 633-642.

Beinert, H. (2000) Iron-sulfur proteins: Ancient structures, still full of surprises. *Journal of Biological Inorganic Chemistry,* **5,** 2-15.

Berberova, N. T. and Shinkar, E. V. (2000) The radical cation of hydrogen sulfide and related organic reactions. *Russian Chemical Bulletin International Edition,* **49,** 1178-1184.

Berg, J. M. (1990) Zinc fingers and other metal-binding domains. *The Journal Of Biological Chemistry,* **265,** 6513-6516.

Berglin, E. H. and Carlsson, J. (1985) Potentiation by sulfide of hydrogen peroxide-induced killing of *Escherichia coli*. *Infection and Immunology,* **49,** 538-543.

Berglin, E. H. and Carlsson, J. (1986) Effect of hydrogen sulfide on the mutagenicity of hydrogen peroxide in *Salmonella typhimurium* strain ta102. *Mutation Research,* **175,** 5-9.

Bernal, J. D. (1951) *The physical basis for life,* London, Routledge and Kegan Paul

Berner, R. A. (1962) Tetragonal FeS, a new iron sulfide. *Science,* **137,** 669.

Birnboim, H. C. (1986) DNA strand breaks in human leukocytes induced by superoxide anion, hydrogen peroxide and tumor promoters are repaired slowly compared to breaks induced by ionizing radiation. *Carcinogenesis,* **7,** 1511-1517.

Birnboim, H. C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research,* **7,** 1515-1523.

Bonini, M. G. and Augusto, O. (2001) Carbon dioxide stimulates the production of thiyl, sulfinyl, and disulfide radical anion from thiol oxidation by peroxynitrite. *Journal of Biological Chemistry,* **276,** 9749-9754.

Borda, M. J., Elsetinw, A. R., Schoonen, M. A. and Strongen, D. R. (2001) Pyrite-induced peroxide formation as a driving force in the evolution of photosynthetic organisms on an early Earth. *Eleventh Annual V.M. Goldschmidt Conference,* May 20-24, 2001, Hot Springs, Virginia, abstract no.3195

Boyce, A. J. C., M.L. and Russell, M.J. 1983 (1983) Formation of fossil hydrothermal chimneys and mounds from silvermines, ireland. *Nature,* **306,** 545-550.

Brack, A. (1993) From amino acids to prebiotic active peptides: A chemical reconstitution. *Pure and applied chemistry,* **65,** 1141-1151.

Butler, I. B., Schoonen, M. A. A. and Rickard, D. T. (1994) Removal of dissolved oxygen from water: A comparison of four common techniques. *Talanta,* **41,** 211-215

Cairo, G., Recalcati, S., Pietrangelo, A. and Minotti, G. (2002) The iron regulatory proteins: Targets and modulators of free radical reactions and oxidative damage. *Free Radical Biology and Medicine,* **15,** 1237- 1243.

Canfield, D. E. (2005) The early history of atmospheric oxygen: Homage to Robert M. Garrels. *Annual Review of Earth and Planetary Sciences,* **33,** 1-36.

Carver, J. H. (1981) Prebiotic atmospheric oxygen levels *Nature,* **292,** 136-138.

Chan, W. C., Nbsp, W & Nie, S. (1998) Quantum dot bioconjugates for ultrasensitive nonisotopic detection. *Science,* **281,** 2016-2018.

Cockell, C. S. (2006) The origin and emergence of life under impact bombardment. *Philosophical Transactions of the Royal Society B: Biological Sciences,* **361,** 1845-1856.

Cody, G. D. (2004) Transition metal sulfides and the origins of metabolism. *Annual Review of Earth and Planetary Sciences,* **32,** 569-599.

Cody, G. D., Boctor, N. Z., Brandes, J. A., Filley, T. R., Hazen, R. M. and Yoder, H. S. (2004) Assaying the catalytic potential of transition metal sulfides for abiotic carbon fixation. *Geochimica et Cosmochimica Acta,* **68,** 2185–2196.

Cody, G. D., Boctor, N. Z., Filley, T. R., Hazen, R. M., Scott, J. H., Sharma, A. and Yoder, H. S., Jr. (2000) Primordial carbonylated iron-sulfur compounds and the synthesis of pyruvate. *Science,* **289,** 1337-1340.

Cohn, C., Borda, M. and Schoonen, A. (2003a) Fate of RNA in the presence of pyrite: Relevance to the origin of life. *225th ACS National Meeting*, New Orleans, LA, March 23-27, 2003.

Cohn, C., Mueller, S., Wimmer, E., Leifer, N., Greenbaum, S., Strongin, D. R. and Scoonen, M. (2006) Pyrite-induced hydroxyl radical formation and its effect on nucleic acids. . *Geochemical transactions.*, **7,** (article #3).

Cohn, C. A., Borda, M. J. and Schoonen, M. A. A. (2003b) Lipid protection of organics from pyrite-induced hydroxyl radical damage. *Northeast Regional Meeting of the American Chemical Society, Saratoga Springs, NY.*

Cohn, C. A., Hansson, T. K., Larsson, H. S., Sowerby, S. J. and Holm, N. G. (2001) Fate of prebiotic adenine. *Astrobiology,* **1,** 477-480.

Corliss, J. B., Baross, J. A. and Hoffman, S. E. (1981) An hypothesis concerning the relationship between submarine hot springs and the origin of life on Earth. *Oceanologica Acta*, 59–69.

Corliss, J. B., Dymond, J., Gordon, L., Edmond, J. M., Von Herzen, R. P., Ballard, R. D., Green, K., D, W., Bainbridge, A., Crane, K. and Van Andel, T. H. (1979) Submarine thermal springs on the Galapagos rift. *Science,* **203**, 1073-1083.

Da Silva, J. J. R. F. and Williams, R. J. P. (1991) *The biological chemistry of the elements,* Oxford, Clarendon Press.

Darwin, F. (1888) *The life and letters of Charles Darwin,* London, John Murray.

Dawson, R. M. C., Elliott, D. C., Elliott, W. H. and Jones, K. M. (1986) *Data for biochemical research,* Oxford: Oxford University Press.

De Duve, C. (2005) The onset of selection. *Nature,* **433,** 581-582.

De Duve, C. and Miller, S. L. (1991) Two-dimensional life? *Proceedings of the National Academy of Science,* **88,** 10014-10017.

Deck, K. M., Tseng, T. A. and Burstyn, J. N. (2002) Triisopropyltriazacyclononane copper(ii): An efficient phosphodiester hydrolysis catalyst and DNA cleavage agent. *Inorganic Chemistry,* **41,** 669-677.

Delacour, A., Frueh-Green, G. L., Bernasconi, S. M. and Kelley, D. S. (2005) The influence of high seawater fluxes on sulfur compositions of the serpentinized peridotites at the Lost City Hydrothermal Field. *American Geophysical Union, Fall Meeting,* abstract #V51B-1488.

Dittmer, W. U. and Simmel, F. C. (2004) Chains of semiconductor nanoparticles templated on DNA. *Applied Physics Letters,* **85,** 633-635.

Dore, E., Frontali, C. and Gratton, E. (1972) The role of ions in the acid melting of DNA. *Biopolymers,* **11,** 2033-2041.

Doudna, J. A. and Cech, T. R. (2002) The chemical repertoire of natural ribozymes. *Nature,* **418,** 222-228.

Douville, E., Charlou, J. L., Oelkers, E. H., Bienvenu, P., Jove Colon, C. F., Donval, J. P., Fouquet, Y., Prieur, D. and Appriou, P. (2002) The rainbow vent fluids (36°14'N, mar): The influence of ultramafic rocks and phase separation on trace metal content in mid-Atlantic ridge hydrothermal fluids. *Chemical Geology,* **184,** 37-48.

Drouin, R., Rodriguez, H., Gao, S.-W., Gebreyes, Z., O'connor, T. R., Holmquist, G. P. and Akman, S. A. (1996) Cupric ion/ascorbate/hydrogen peroxide-induced DNA damage: DNA-bound copper ion primarily induces base modifications. *Free Radical Biology and Medicine,* **21,** 261-273.

Duguid, J., Bloomfield, V. A., Benevides, J. and Thomas, G. J., Jr. (1993) Raman spectroscopy of DNA-metal complexes. I. Interactions and conformational effects of the divalent cations: Mg, Ca, Sr, Ba, Mn, Co, Ni, Cu, Pd, and Cd. *Biophysics Journal,* **65,** 1916-1928.

Ertem, G. and Ferris, J. P. (1993) Montmorillonite catalysis of RNA oligomer formation in aqueous solution. A model for the prebiotic formation of RNA. *Journal of the American Chemistry Society,* **115,** 12270-12275.

Ertem, G. and Ferris, J. P. (1996) Synthesis of RNA oligomers on heterogeneous templates. *Nature,* **379,** 238-240.

Ertem, G. and Ferris, J. P. (1998) Formation of RNA oligomers on montmorillonite: Site of catalysis. *Origins of Life and Evolution of Biospheres,* **28,** 485-499.

Evans, H. T. and Konnert, J. A. (1976) Crystal structure refinement of covellite. *American Mineralogist,* **61,** 996-1000.

Evans, J., H.T., Milton, C., Chao, E. C. T., Adler, I., Mead, C., Ingram, B. and Berner, R. A. (1964) Vallerite and the new iron sulphide, mackinawite. *U.S. Geological Survey Professional Paper,* **475-D**.

Fallick, A. E., Ilich, M. and Russell, M. J. (1991) A stable isotope study of the magnesite deposits associated with the alpine-type ultramafic rocks of Yugoslavia. *Economic Geology,* **86,** 847-861.

Ferris, J. P. (1992) Chemical markers of prebiotic chemistry in hydrothermal systems. *Origins of life and Evolution of the Biosphere,* **22,** 109-134.

Ferris, J. P. (1999) Prebiotic synthesis on minerals: Bridging the prebiotic and RNA worlds. *Biological Bulletin,* **196,** 311-4.

Ferris, J. P., Ertem, G. and Agarwak, V. K. (1989) The adsorption of nucleotides and polynucleotides on montmorillonite clay. *Origins of Life and Evolution of the Biosphere,* **19,** 153-64.

Ferris, J. P., Joshi, P. C., Edelson, E. H. and Lawless, J. G. (1978) HCN: A plausible source of purines, pyrmidines and amino acids on the primitive Earth. *Journal of molecular evolution,* **11** 293-311.

Filtness, M., Butler, I. and Rickard, D. (2003) The origin of life: The properties of iron sulphide membranes. *Applied Earth Science, Transactions Institution Mining Metallurgy,* **112B,** 171-172.

Floyd, R. A. and Lewis, C. A. (1983) Hydroxyl free radical formation from hydrogen peroxide by ferrous iron-nucleotide complexes. *Biochemistry,* **22,** 2645-2649.

Franchi, M., Ferris, J. P. and Gallori, E. (2003) Cations as mediators of the adsorption of nucleic acids on clay surfaces in prebiotic environments. *Origins of life and Evolution of the Biosphere,* **33,** 1-16.

Frelon, S., Douki, T., Favier, A. and Cadet, J. (2003) Hydroxyl radical is not the main reactive species involved in the degradation of DNA bases by copper in the presence of hydrogen peroxide. *Chemical Research in Toxicology,* **16,** 191-197.

Gajda, T., Dupre, Y., Torok, I., Harmer, J., Schweiger, A., Sander, J., Kuppert, D. and Hegetschweiler, K. (2001) Highly efficient phosphodiester hydrolysis promoted by a dinuclear copper(II) complex. *Inorganic Chemistry,* **40,** 4918-4927.

Galaris, D., Zhu, B.-Z. and Frei, B. (2002) On the role of iron and copper ions in hydrogen peroxide-induced cellular DNA damage. *Free Radical Biology and Medicine,* **32,** 198-199.

Gallagher, S. R. and Desjardins, P. R. (2006) Quantitation of DNA and RNA with absorption and fluorescence spectroscopy. *Current Protocols in Molecular Biology,* Appendix 3D.

Ghaly, M. Y., Hartel, G., Mayer, R. and Haseneder, R. (2001) Photochemical oxidation of p-chlorophenol by UV/H2O2 and photo-fenton process. A comparative study. *Waste Management,* **21,** 41-47.

Giese, B. (1986) *Radicals in organic synthesis: Formation of carbon-carbon bonds,* Oxford, Pergamon Press.

Gilbert, W. (1986) Origin of life: The RNA world. *Nature,* **319,** 618.

Goh, S. W., Buckley, A. N. and Lamb, R. N. (2006) The oxidation states of copper and iron in mineral sulfides, and the oxides formed on initial exposure of chalcopyrite and bornite to air *Geochimica et Cosmochimica Acta,* **70,** 2210-2228.

Haldane, J. B. S. (1929) The origin of life. *Rationalist Annual,* **3,** 148-153.

Hall, A. J. (1986) Pyrite-pyrrhotine reactions in nature. *Mineralogical Magazine,* **50,** 223-229.

Hazen, R. M., Filley, T. R. and Goodfriend, G. A. (2001) Selective adsorption of L- and D-amino acids on calcite: Implications for biochemical homochirality. *Proceedings of the National Academy of Sciences,* **98,** 5487-5490.

Heinen, W. and Lauwers, A. M. (1996) Organic sulfur compounds resulting from the interaction of iron sulfide, hydrogen sulfide and carbon dioxide in an anaerobic aqueous environment. *Origins of Life and Evolution of Biospheres (Formerly Origins of Life and Evolution of the Biosphere),* **26,** 131-150.

Henle, S. E., Han, Z., Tang, N., Rai, P., Luo, Y. and Linn, S. (1999) Sequence-specific DNA cleavage by Fe^{2+}-mediated Fenton reactions has possible biological implications. *Journal of Biological Chemistry,* **274,** 962-971.

Henry, S. (2005) New studies on the Murchison meteorite shed light on the pre-RNA world. *ChemBioChem,* **6,** 801-803.

Holland, H. D. (1999) When did the Earth's atmosphere become oxic? A reply. *Geochemical News,* **100,** 20-22.

Huang, W. and Ferris, J. P. (2003) Synthesis of 35-40 mers of RNA oligomers from unblocked monomers. A simple approach to the RNA world. *Chemical Communications (Cambridge),* **21,** 1458-9.

Huber, C., Eisenreich, W., Hecht, S. and Wächtershäuser, G. (2003) A possible primordial peptide cycle. *Science,* **301,** 938 - 940.

Huber, C. and Wächtershäuser, G. (1997) Activated acetic acid by carbon fixation on (Fe,Ni)S under primordial conditions. *Science,* **276,** 245-47.

Huber, C. and Wächtershäuser, G. (1998) Peptides by activation of amino acids with CO on (Ni,Fe)S surfaces: Implications for the origin of life. *Science,* **281,** 670.

Hughes, T. J., Sparacino, C. and Frazier, S. (1984) Validation of chemical and biological techniques for evaluation of vapors in ambient air/mutagenicity testing of twelve (12) vapor-phase compounds. *Report of EPA (No. EPA-600/1-84-005).*

Imlay, J. A., Chin, S. M. and Linn, S. (1988) Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science,* **240,** 640-642.

Iqbal, J., Bhatia, B. and Nayyar, N. K. (1994) Transition-metal promoted radical reactions in organic-synthesis - the formation of carbon-carbon bonds. *Chemical Reviews,* **9,** 2549-2549.

Jakob, B. and Heber, U. (1996) Photoproduction and detoxification of hydroxyl radicals in chloroplasts and leaves and relation to photoinactivation of photosystems I and II. *Plant Cell Physiology,* **37,** 629-635.

Jezowska-Bojczuk, M., Szczepanik, W., Lesniak, W., Ciesiolka, J., Wrzesinski, J. and Bal, W. (2002) DNA and RNA damage by Cu(II)-amikacin complex. *European Journal of Biochemistry,* **269,** 5547-5556.

Johnston, M. (1987) Genetic evidence that zinc is an essential co-factor in the DNA binding domain of gal4 protein. *Nature,* **328,** 3-355.

Kamyshny, A., Goifman, A., Rizkov, D. and Lev, O. (2003) Kinetics of disproportionation of inorganic polysulfides in undersaturated aqueous solutions at environmentally relevant conditions. *Aquatic Geochemistry,* **9,** 291-304.

Kandler, O. (1994) Cell wall biochemistry and three-domain concept of life. *Systematic and applied microbiology,* **16,** 501-509

Kandler, O. (1995) Cell wall biochemistry in archaea and its phylogenetic implications. *Journal of Biological Physics,* **20,** 165-169.

Kandler, O. (1998) The early diversification of life and the origin of the three domains: A proposal. In Wiegel, J. and Adams, M. W. W. (Eds.) *Thermophiles: The keys to molecular evolution and the origin of life.* London, Taylor and Francis.

Kanemoto, M., Shiragami, T., Pac, C. and Yangida, S. (1992) Semiconductor photocatalysis. Effective photoreduction of carbon dioxide catalyzed by ZnS

quantum crystallites with low density of surface defects. *Journal of Physical Chemistry,* **96,** 3521-3526.

Kasting, J. F. (1990) Bolide impacts and the oxidation state of carbon in the Earth's early atmosphere. *Origins of Life and Evolution of Biospheres,* **20,** 199-231.

Kasting, J. F. (1993) Earth's early atmosphere. *Science,* **259,** 920-927.

Kasting, J. F., Liu, S. C. and Donahue, T. M. (1979) Oxygen levels in the prebiological atmosphere. *Journal of Geophysical Research,* **84,** 3097-3107

Kawanishi, S., Hiraku, Y., Murata, M. and Oikawa, S. (2001a) The role of metals in site-specific DNA damage with reference to carcinogenesis. *Free Radical Biology and Medicine,* **32,** 822-832.

Kawanishi, S., Hiraku, Y. and Oikawa, S. (2001b) Mechanism of guanine-specific DNA damage by oxidative stress and its role in carcinogenesis and aging. *Mutation Research/Reviews in Mutation Research,* **488,** 65-76.

Kawanishi, S., Oikawa, S., Inoue, S. and Nishino, K. (2002) Distinct mechanisms of oxidative DNA damage induced by carcinogenic nickel subsulfide and nickel oxides *Environmental Health Perspectives,* **110,** 789–791.

Kejnovsky, E. and Kypr, J. (1998) Millimolar concentrations of zinc and other metal ions cause sedimentation of DNA. *Nucleic Acids Research,* **26,** 5295-5292.

Kimura, J. E. and Szent-Gyorgyi, A. (1969) Donor-acceptor interactions of nitrogen. *Proceedings of the National Academy of Sciences,* **62,** 286-288.

Koonin, E. V. and Martin, W. (2005) On the origin of genomes and cells within inorganic compartments. *Trends in Genetics,* **21,** 647-654.

Kruger, K., Grabowski, P. J., Zaug, A. J., Sands, J., Gottschling, D. E. and Cech, T. R. (1982) Self-splicing RNA: Autoexcision and autocyclization of the ribosomal RNA intervening sequence of tetrahymena. *Cell,* **31,** 147-57.

Krupp, R. E. (1994) Phase relations and phase transformations between the low-temperature iron sulfides mackinawite, greigite, and smythite. *European Journal of Mineralogy,* **6,** 265-278.

Lazcano, A. and Miller, S. L. (1996) The origin and early evolution of life: Prebiotic chemistry, the pre-RNA world, and time. *Cell Press,* **85,** 793-798.

Li, Y., Chen, J., Zhuo, S., Wu, Y., Zhu, C. and Wang, L. (2004) Application of l-cysteine-capped ZnS nanoparticles in the determination of nucleic acids using the resonance light scattering method. *Microchimica Acta,* **146,** 13-19.

Lloyd, D. R. and Phillips, D. H. (1999) Oxidative DNA damage mediated by copper(II), iron(II) and nickel(II) Fenton reactions: Evidence for site-specific

mechanisms in the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intrastrand cross-links. *Free Radical Biology and Medicine,* **32,** 822-832.

Luther, G. W. (2004) Kinetics of the reactions of water, hydroxide ion and sulfide species with CO_2, OCS and CS_2: Frontier molecular orbital considerations. *Aquatic Geochemistry,* **10,** 81-97.

Luther, G. W., Giblin, A. E. and Varsolona, R. (1985) Polarographic analysis of sulfur species in marine porewaters. *Limnology and Oceanography,* **30,** 727-736

Luther, G. W. and Rickard, D. (2005) Metal sulfide cluster complexes and their biogeochemical importance in the environment. *Journal of Nanoparticle Research,* **7,** 389–407.

Luther, G. W., Rozan, T. F., Taillefert, M., Nuzzio, D. B., Di Meo, C., Shank, T. M., Lutz, R. A. and Cary, S. C. (2001) Chemical speciation drives hydrothermal vent ecology. *Nature,* **410,** 813-816.

Luther, G. W., Theberge, S. M. and Rickard, D. T. (1999) Evidence for aqueous clusters as intermediates during zinc sulfide formation. *Geochimica et Cosmochimica Acta,* **63,** 3159-3169.

Macleod, G., Mckeown, C., Hall, A. J. and Russell, M. J. (1994) Hydrothermal and oceanic pH conditions of possible relevance to the origin of life. *Origins of Life and Evolution of the Biosphere,* **24,** 19-41.

Maisonneuve, J. (1982) The composition of the Precambrian ocean waters. *Sedimentary Geology,* **31,** 1-11.

Martin, W. and Russell, M. J. (2003) On the origins of cells: A hypothesis for the evolutionary transitions from abiotic geochemistry to chemoautotrophic prokaryotes, and from prokaryotes to nucleated cells. *Philosophical Transactions of the Royal Society, B: Biological Sciences,* **358,** 59 - 85.

Miller, S. L. (1953) A production of amino acids under possible primitive Earth conditions. *Science,* **117**, 528-529.

Miller, S. L. (1987) Which organic compounds could have occurred on the prebiotic Earth? *Cold Spring Harbour Symposium on Quantitative Biology.*

Miller, S. L. and Urey, H. C. (1959) Organic compound synthesis on the primitive Earth. *Science,* **130**, 245-251.

Mills, G., Schmidt, K. H., Matheson, M. S. and Meisel, D. (1987) Thermal and photochemical reactions of sulfhydryl radicals. Implications for colloid photocorrosion. *Journal of Physical Chemistry,* **91,** 1590-1596.

Mitchell, G. P., Mirkin, C. A. and Letsinger, R. L. (1999) Programmed assembly of DNA functionalized quantum dots. *Journal of American Chemical Society,* **121,** 8122-8123.

Miyakawa, S., Yamanashi, H., Kobayashi, K., Cleaves, H. J. and Miller, S. L. (2002) Prebiotic synthesis from CO atmospheres: Implications for the origins of life. *Proceedings of the National Academy of Sciences,* **99,** 14628-14631.

Moad, G. and Solomon, D. H. (1995) *The chemistry of free radical polymerisation,* Oxford, Pergamon Press.

Monson, C. F. and Woolley, A. T. (2003) DNA-templated construction of copper nanowires. *Nano Letters,* **3.**

Morse, J. W. and Mackenzie, F. T. (1998) Hadean ocean carbonate geochemistry. *Aquatic Geochemistry,* **4,** 301-319.

Mozgova, N. N., Krasnov, S. G., Batuyev, B. N., Borodaev, Y. S., Efimov, V. E., Markov, F. V. and Stepanova, T. V. (1996) The first report of cobalt pentlandite from a mid-Atlantic ridge hydrothermal deposit. *The Canadian Mineralogist,* **34,** 23-28.

Nagel, S., Genius, J., Heiland, S., Horstmann, S., Gardner, H. and Wagner, S. (2006) Diphenyleneiodonium and dimethylsulfoxide for treatment of reperfusion injury in cerebral ischemia of the rat. *Brain Research,* **1132,** 210-217.

Nelson, K. E., Levy, M. and Miller, S. L. (2000) Peptide nucleic acids rather than RNA may have been the first genetic molecule. *Proceedings of the National Academy of Science,* **97,** 3868-3871.

Nguyenduy, T., Morel, D. R., Collee, G., Eberhard, M., Melvin, C., Robinson, D. R., Repine, J. E., Lowenstein, E. and Zapol, W. M. (1986) Hydroxyl radicals mediate acute pulmonary vasoconstriction and thromboxane release during protamine reversal of heparin in awake sheep. *Annual meeting of the Federation of American Society for Experimental Biology,* **45,** 70.

Norman, R. O. C. and Storey, P. M. (1971) Electron spin resonance studies. Part XXXI. The generation, and some reactions, of the radicals $SO^{3-}\cdot$, $S_2O^{3-}\cdot$, $S^{-}\cdot$, and SH in aqueous solution. *Journal of the Chemical Society B: Physical Organic,* 1009 - 1013.

O'loughlin, E. J. and Burris, D. R. (2004) Reduction of halogenated ethanes by green rust. *Environmental Toxicology and Chemistry,* **23,** 41–48.

Ohfuji, H. and Rickard, D. (2006) High resolution transmission electron microscopic study of synthetic nanocrystalline mackinawite. *Earth and Planetary Sciences Letters,* **241,** 227-233.

Ohmoto, H. (1997) When did the Earth's atmosphere become oxic. *Geochemical News,* **93,** 12-13.

Oikawa, S. and Kawanishi, S. (1998) Distinct mechanisms of site-specific DNA damage induced by endogenous reductants in the presence of iron(III) and copper(II). *Biochimica et Biophysica Acta - Gene Structure and Expression,* **1399,** 19-30.

Oparin, A. I. (1938) *The origin of life,* New York, Dover.

Orgel, L. E. (2000) Self-organizing biochemical cycles. *Proceedings of the National Academy of Sciences,* **97,** 12503-12507.

Oro, J. (1961) Mechanism of synthesis of adenine from hydrogen cyanide under possible primitive Earth conditions. *Nature,* **191,** 1193-1194.

Österberg, R. (1997) On the prebiotic role of iron and sulfur. *Origins of Life and Evolution of Biospheres,* **27,** 481-484.

Papineau, D., Mojzsis, S. J. and Schmitt, A. K. (2007) Multiple sulfur isotopes from Paleoproterozoic Huronian interglacial sediments and the rise of atmospheric oxygen. *Earth and Planetary Science Letters,* **255,** 188-212.

Pathak, S., Choi, S.-K., Arnheim, N. and Thompson, M. E. (2000) Biosynthesis and immobilization of nanaoparticles and their applications. *Journal of American Chemical Society,* **123,** 4103-4104.

Pattrick, R. A. D., Mosselmans, J. F. W., Charnock, J. M., England, K. E. R., Helz, G. R., Garner, C. D. and Vaughan, D. J. (1997) An XAS study of the semi-conducting sulfides M_2S_3(m = As, Sb, Bi). *Geochima et Cosmochima Acta,* **61,** 2023-2036.

Pavlov, A. A. and Kasting, J. F. (2002) Mass-independent fractionation of sulfur isotopes in archean sediments: Strong evidence for an anoxic archean atmosphere. *Astrobiology,* **2,** 27-41.

Pecci, L., Montefoschi, G. and Cavallini, D. (1997) Some new details of the copper-hydrogen peroxide interaction. *Biochemical and Biophysical Research Communications,* **235,** 264-267.

Plekan, O., Feyer, V., Sutara, F., Skala, T., Svec, M., Chab, V., Matolin, V. and Prince, K. C. (2007) The adsorption of adenine on mineral surfaces: Iron pyrite and silicon dioxide. *Surface Science,* **601,** 1973-1980.

Pontes-Buarques, M., Tessis, A. C., Bonapace, J. A. P., Monte, M. B. M., Cortés-Lopez, G., De Souza-Barros, F. and Vieyra, A. (2001) Modulation of adenosine 5'-monophosphate adsorption onto aqueous resident pyrite: Potential mechanisms for prebiotic reactions. *Origins of Life and Evolution of Biospheres,* **31,** 343-362.

Rickard, D. (2006a) Characteristics of mackinawite, tetragonal FeS. *Geochimica et Cosmochimica Acta Supplement,* **70,** 533.

Rickard, D. (2006b) The solubility of FeS. *Geochimica et Cosmochimica Acta,* **70,** 5779-5789.

Rickard, D., Butler, I. B. and Olroyd, A. (2001) A novel iron sulphide switch and its implications for Earth and planetary science. *Earth Planetary Science Letters,* **189.**

Rickard, D., Griffith, A., Oldroyd, A., Butler, I. B., Lopez-Capel, E., Manning, D. A. C. and Apperley, D. C. (2006) The composition of nanoparticulate mackinawite, tetragonal iron(II) monosulfide. *Chemical Geology,* **235,** 286-298.

Rickard, D. and Luther, G., W. (2006) Metal sulfide complexes and clusters. *Reviews in Mineralogy and Geochemistry,* **61,** 421-504.

Rickard, D. and Luther, G. W. (1997) Kinetics of pyrite formation by the H_2S oxidation of iron (II) monosulfide in aqueous solutions between 25 and 125°C: The mechanism. *Geochimica et Cosmochimica Acta,* **61,** 135-147.

Rickard, D. and Luther, G. W. (2007) Chemistry of iron sulphides. *Chemical Reviews.*

Rickard, D. and Morse, J. W. (2005) Acid volatile sulphide (AVS). *Marine Chemistry,* **97,** 141.

Rickard, D. T. (1972) Covellite formation in low temperature aqueous solutions. *Mineralium Deposita,* **7,** 180-188.

Rickard, D. T. (1973) Copper sulphide formation chemistry at low temperatures. *Mineralogy and Petrology,* **19,** 60-76.

Rodriguez, H., Holmquist, G. P., D'agostino, R. J., Keller, J. and Akman, S. A. (1997) Metal ion-dependent hydrogen peroxide-induced DNA damage is more sequence specific than metal specific. *Cancer Res,* **57,** 2394-2403.

Rodriguez, S. and Smith, I. (2003) Mechanisms of iron regulation in mycobacteria: Role in physiology and virulence. *Molecular Microbiology,* **47,** 1485 - 1494.

Rozan, T. F., Lassman, M. E., Ridge, D. P. & Luther, G. W. (2000) Evidence for fe, cu and zn complexation as multinuclear sulfide clusters in oxic river waters. *Nature,* **406,** 879-882.

Rozenberg-Arska, M., Asbeck, V. S., J, M. T. F. and Verhoef, J. (1985) Damage to chromosomal and plasmid DNA by toxic oxygen species. *Journal of General Microbiology,* **131,** 3325-3330.

Russell, M. J. and Arndt, T. (2005) Geodynamic and metabolic cycles in the Hadean. *Biogeosciences,* **2,** 97-111.

Russell, M. J. and Hall, A. J. (1997) The emergence of life from iron monosulphide bubbles at a submarine redox and pH front. *Journal of the Geological Society, London,* **154,** 377-402.

Russell, M. J. and Hall, A. J. (2002) From geochemistry to biochemistry. *The Geochemical News,* **113,** 6-12.

Russell, M. J. and Hall, A. J. (2006) The onset and early evolution of life. *The Geological Society of America,* **198,** 1-32.

Russell, M. J., Hall, A. J., Boyce, A. J. and Fallick, A. E. (2005) On hydrothermal convection systems and the emergence of life. *Economic Geology,* **100,** 419-438.

Russell, M. J., Hall, A. J., Cairns-Smith, A. G. and Braterman, P. S. (1988) Submarine hot springs and the origin of life. *Nature,* **336,** 117-117.

Russell, M. J., Hall, A. J. and Gize, A. P. (1990) Pyrite and the origin of life. *Nature,* **344**.

Russell, M. J. and Martin, W. (2004) The rocky roots of the acetyl CoA pathway. *Trends in Biochemical Sciences,* **29,** 358-63.

Russell, M. J. and Turner, D. (1989) In vitro growth of iron sulphide chimneys: Possible chambers for origin-of-life experiments. *Terra Nova,* **1,** 238-241.

Sagripanti, J. L. (1999) DNA damage mediated by metal ions with special reference to copper and iron. *Metal Ions in Biological Systems,* **36,** 179-209.

Schoonen, M. A. A., Xu, Y. and Bebie, J. (1999) Energetics and kinetics of the prebiotic synthesis of simple organic acids with the $FeS\text{-}H_2S/FeS_2$ redox couple as reductant. . *Origins Life and Evolution of the Biosphere,* **29,** 5-32

Scott, W. G., Finch, J. T. and Klug, A. (1995) The crystal structure of an AII-RNA hammerhead ribozyme: A proposed mechanism for RNA catalytic cleavage. *Cell,* **81,** 991-1002.

Sedwick, P. N. and Mcmurtry, G. M. (1994) Carbon dioxide and helium in hydrothermal fluids from Loihi seamount, Hawaii, USA: Temporal variability and implications for the release of mantle volatiles. *Geochimica et Cosmochimica Acta,* **58,** 1219-1227.

Shapiro, R. (1995) The prebiotic role of adenine: A critical analysis. *Origins of Life and Evolution of Biospheres,* **25,** 83-98.

Shock, E. L. (1990) Geochemical constraints on the origin of organic compounds in hydrothermal systems *Origins of life and Evolution of the Biosphere,* **20,** 331-367.

Shock, E. L. (1992) Chemical environments of submarine hydrothermal systems. *Origins of Life and Evolution of the Biosphere,* **22,** 67-107.

Shock, E. L. (1996) Hydrothermal systems as environments for the emergence of life. In Bock, G. R. and Goode, J. A. (Eds.) *Evolution of hydrothermal ecosystems on Earth (and Mars?), ciba foundation symposium.*

Shock, E. L., McCollom, T. and Schulte, M. D. (1998) The emergence of metabolism from within hydrothermal systems. In Wiegel, J. and Adams, M. W. W. (Eds.) *Thermophiles: The keys to molecular evolution and the origin of life?* Taylor and Francis.

Shock, E. L. and Schulte, M. D. (1995) Hydrothermal systems as locations of organic synthesis on the early Earth and Mars. *Transactions of the American Geophysical Union,* 76/46 Supplement, (P21A-6), F335.

Silvester, E., Grieser, F., Sexton , B. A. and Healy, T. W. (1991) Spectroscopic studies on copper sulfide sols. *Langmuir,* **7,** 2917-2922.

Sowerby, S. J., Cohn, C. A., Heckl, W. M. and Holm, N. G. (2001a) Special feature: Differential adsorption of nucleic acid bases: Relevance to the origin of life. *Proceedings of the National Academy of Sciences,* **98,** 820-822.

Sowerby, S. J., Mörth, C. and Holm, N. G. (2001b) Effect of temperature on the adsorption of adenine. *Astrobiology,* **1,** 481 -487

Sreedhara, A. and Cowan, J. A. (2001) Catalytic hydrolysis of DNA by metal ions and complexes, *Journal of Biological Inorganic chemistry,* **6,** 337-347.

Stachnik, R. A. and Molina, M. J. (1987) Kinetics of the reactions of SH radicals with NO_2 and O_2. *Journal of Physical Chemistry,* **91,** 4603-4606.

Stauber, J. L. and Florence, T. M. (1987) Mechanism of toxicity of ionic copper and copper complexes to algae *Marine Biology,* **94,** 511-519.

Suleimenov, O. M. and Seward, T. M. (1997) A spectrophotometric study of hydrogen sulphide ionisation in aqueous solutions to 350°C, *Geochimica et Cosmochima Acta,* **61,** 5187-5198.

Tapley, D. W., Buettner, G. R. and Shick, J. M. (1999) Free radicals and chemiluminescence as products of the spontaneous oxidation of sulfide in seawater, and their biological implications. *Biological Bulletin,* **196,** 52-56.

Tessis, A. C. and Vieyra, A. (1996) Divalent cations modify adsorption of 5'-AMP onto precipitated calcium phosphate: A model for cation modulation of adsorptive processes in primitive aqueous environments. *Journal of Molecular Evolution,* **43,** 425-430.

Thatcher, D. R., Hitchcock, A., Hanak, J. A. J. and Varley, D. L. (1997) Method of plasmid DNA production and purification. US Patent 5,981,735.

Theberge, S. M. and Luther, G. W. (1997) Determination of the electrochemical properties of a soluble aqueous FeS species present in sulfidic solutions. *Aquatic Geochemistry,* **3,** 191-211.

Tossell, J. A. (1978) Theoretical studies of the electronic structure of copper in tetrahedral and triangular coordination with sulfur. *Physics and Chemistry of Minerals,* **2,** 225-236.

Toyokuni, S. and Sagripanti, J. (1992) Iron-mediated DNA damage: Sensitive detection of DNA strand breakage catalyzed by iron. *Journal of Inorganic Biochemistry,* **47,** 241-248.

Toyokuni, S. and Sagripanti, J. (1993) Induction of oxidative single- and double-strand breaks in DNA by ferric citrate. *Free Radical Biology and Medicine,* **15,** 117-123.

Van Der Laan, G., Patrick, R. A. D., Henderson, C. M. B. and Vaughan, D. J. (1992) Oxidation state variations in copper minerals studied with Cu 2 p X-ray absorption spectroscopy. *The Journal of Physics and Chemistry of Solids,* **53,** 1185-1190

Vaughan, D. J. and Craig, J. R. (1978) *Mineral chemistry of metal sulfides,* Cambridge, Cambridge University Press.

Von Damm, K. L. (2000) Chemistry of hydrothermal vent fluids from 9-10 deg N, east Pacific rise - 'time zero,' the immediate posteruptive period. *Journal of Geophysical Research,* **105,** 203-211.

Wächtershäuser, G. (1988) Before enzymes and templates: Theory of surface metabolism. *Microbiological Reviews,* **52,** 452-484.

Wächtershäuser, G. (1990) Evolution of the first metabolic cycles. *Proceedings of the National Academy of Science,* **87,** 200-204.

Wächtershäuser, G. (1992) Groundworks for an evolutionary biochemistry: The iron-sulphur world. *Progress in Biophysics and Molecular Biology,* **58,** 85-201.

Wächtershäuser, G. (1994) Life in a ligand sphere. *Proceedings of the National Academy of Sciences,* **91,** 4283-4287.

Wächtershäuser, G. (1997) The origin of life and its methodological challenge. *Journal of Theoretical Biology,* **187,** 483-494.

Wächtershäuser, G. (1998) Origin of life in an iron sulphur world. In Brack, A. (Ed.) *The molecular origins of life: Assembling pieces of the puzzle.* Cambridge, Cambridge University Press.

Wächtershäuser, G. (2003) From pre-cells to Eukarya - a tale of two lipids. *Molecular Microbiology,* **47,** 13-22.

Wächtershäuser, G. (2006) From volcanic origins of chemoautotrophic life to bacteria, Archaea and Eukarya. *Philosophical Transactions of the Royal Society B: Biological Sciences,* **361,** 1787-1808.

Walker, J. C. and Brimblecombe, P. (1985) Iron and sulfur in the pre-biologic ocean. *Precambrian Research,* **28,** 205-222.

Wang, N. S., Lovejoy, E. R. and Howard, C. J. (1987) Temperature dependence of the rate constant for the reaction HS + NO_2. *Journal of Physical Chemistry,* **91,** 5743-5749.

Wilde, S. A., Valley, J. W., Peck, W. H. and Graham, C. M. (2001) Evidence from detrital zircons for the existence of continental crust and oceans on the Earth 4.4 Gyr ago. *Nature,* **11,** 175-188.

Winnewisser, G., Lewen, F., Thorwirth, S., Behnke, M., Hahn, J., Gauss, J. and Herbst, E. (2003) Gas-phase detection of HSOH: Synthesis by flash vacuum pyrolysis of di-tert-butyl sulfoxide and rotational-torsional spectrum. *Chemistry - A European Journal,* **9,** 5501-5510.

Winter, D. and Zubay, G. (1995) Binding of adenine and adenine-related compounds to the clay monmorillonite and the mineral hydroxylapitite. *Origins of Life and Evolution of the Biosphere,* **25,** 61.

Woese, C. (1998) The universal ancestor. *Proceedings of the National Academy of Scences,* **95,** 6854-6859.

Woese, C. R. (1987) Bacterial evolution. *Microbiological Reviews,* **51,** 221-271.

Woese, C. R. and Fox, G. E. (1977) The concept of cellular evolution. *Journal of Molecular Evolution,* **10,** 1-6.

Wolthers, M., Charlet, L., Van Der Linde, P. R., Rickard, D. and Van Der Weijden, C. H. (2005) Surface chemistry of disordered mackinawite (FeS). *Geochimica et Cosmochimica Acta,* **69,** 3469-3481.

Yaffee, M., Walter, P., Richter, C. and Müller, M. (1996) Direct observation of iron-induced conformational changes of mitochondrial DNA by high-resolution

field-emission in-lens scanning electron microscopy. *Proceedings of the National Academy of Scences,* **93** 5341-5346.

Yamamoto, K. and Kawanishi, S. (1989) Hydroxyl free radical is not the main active species in site-specific DNA damage induced by copper (II) ion and hydrogen peroxide. *Journal of Biological Chemistry,* **264,** 15435-15440.

Zedef, V., Russell, M. J., Fallick, A. E. and Hall, A. J. (2000) Genesis of vein stockwork and sedimentary magnesite and hydromagnesite deposits in the ultramafic terranes of southwestern Turkey: A stable isotope study. *Economic Geology,* **95,** 429-445.

Zehnder, A. J. and Wuhrmann, K. (1976) Titanium (iii) citrate as a nontoxic oxidation-reduction buffering system for the culture of obligate anaerobes. *Science,* **194,** 1165-1166.

Zhang, H. and Banfield, J. F. (2004) Aggregation, coarsening, and phase transformation in ZnS nanoparticles studied by molecular dynamics simulations. *American Chemical Society,* **4,** 713-718

Zhang, H., Gilbert, B., Huang, F. and Banfield, J. F. (2003) Water-driven structure transformation in nanoparticles at room temperature. *Nature,* **424,** 1025-1029.

Zubay, G. (2003) *Origins of life on Earth and in the cosmos*, Harcourt Academic Press.

Appendix 1

Gel densitometry readings for Chapter 3, Figure 4

	DMSO free controls							
mM Fe(II)	0.1		1		10		100	
Relaxed Band	25.7	18	9.3		22.2	24.1	0	0
Supercoiled Band	41	42.9	10.5				0	0
Total DNA	63.8		19.8		23.15		0	
Total relaxed	21.85		9.3		23.15		0	
% relaxed	34.25		46.97		100.00		#DIV/0!	

	20% DMSO.							
mM Fe(II)	0.1		1		10		100	
Relaxed Band	19.3	51.7	19.8	29.1	48.2	24	0	0
Supercoiled Band	68.4	15.2	14	19.7	14.8	10.2	0	0
Total DNA	77.3		41.3		48.6		0	
Total relaxed	35.5		24.45		36.1		0	
% relaxed	45.92		59.20		74.28		#DIV/0!	

mM Fe(II)	0.1		1		10		100	
	Control	DMSO	Control	DMSO	Control	DMSO	Control	DMSO
Relaxed	4.37	7.1	1.86	4.89	4.63	7.22	0	0
Supercoiled	8.39	8.36	2.1	3.37	0	2.5	0	0

Appendix 2

Gel densitometry readings for Chapter 4, Figs 2 and 3

Sample 1								
mM S-(II)	0	0.1	1	10	25	50	100	150
Relaxed	8.0	9.0	8.4	8.5	8.5	0.0	0.0	0.0
Super-coiled	34.1	32.4	31.8	34.1	33.3	30.8	0.0	3.2
Band 1	0.0	0.0	0.0	0.0	0.0	4.2	11.0	10.8
Band 2	0.0	0.0	0.0	0.0	0.0	0.0	5.1	4.5
Total (ug/ml)	42.1	41.4	40.2	42.6	41.8	35.0	16.1	18.5

Sample 2								
mM S-(II)	0	0.1	1	10	25	50	100	150
Relaxed	6.8	6.4	7.5	8.9	6.5	0.0	0.0	0.0
Super-coiled	12.8	15.0	15.8	15.9	14.5	14.0	0.0	0.0
Fragment 1	0.0	0.0	0.0	0.0	0.0	0.0	8.1	8.4
Total (ug/ml)	19.6	21.4	23.3	24.8	21.0	14.0	8.1	8.4

mM S(-II)	0	0.1	1	10	25	50	100	150
Sample 1 (42.06 ug/ml	42.06	41.4	40.2	42.56	41.76	35.02	16.12	18.46
Sample 2 (19.56 ug/ml	19.56	21.42	23.34	24.78	21.02	14.04	8.14	8.44

Appendix 3

Appendix 3 (a): Gel Densitometry Readings for Chapter 5, Figs 3, 4, 5, 6 & 7

Band readings, no conversion								
Initial DNA concentration: 78.5 ug/ml			Loading volume: 2ul					
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	49.3	91.6	128.5	133.9	39.2	115.7	69	61.4
Coiled	107.6	104.5	52.4	51.6	0	37.2	31	27.5
Total	156.9	196.1	180.9	185.5	39.2	152.9	100	88.9
Initial DNA concentration: 64.6 ug/ml			Loading volume 2ul					
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	30.9	44.4	84	101	113.6	122.8	63.5	62.6
Coiled	98.2	110	78.1	39.6	32.1	0	19	30.5
Total	129.1	154.4	162.1	140.6	145.7	122.8	82.5	93.1
Initial DNA concentration: 33.3 ug/ml			Loading volume: 2 ul					
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	24.4	47.3	57.2	52.6	55.6	55.6	44.9	29.1
Coiled	35.6	37.3	30.4	20.4	18.2	18.2	15.1	0
Total	60	84.6	87.6	73	73.8	73.8	60	29.1
Initial DNA concentration: 30.0 ug/ml			Loading volume: 2 ul					
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	13.8	20.8	53.7	38.6	78.2	0	35.8	35.4
Coiled	52.8	50.3	35	14.7	12.8	0	5.3	6.5
Total	66.6	71.1	88.7	53.3	91	0	41.1	41.9
Initial DNA concentration: 21.2 ug/ml			Loading volume: 5ul					
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	20.4	23.6	40.7	52.5	72.7	39	47.2	47.9
Coiled	53.5	41.1	29	11.4	0	0	0	0
Total	73.9	64.7	69.7	63.9	72.7	39	47.2	47.9
Initial DNA concentration: 16.1 ug/ml			Loading volume: 5 ul					
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	21.2	27.2	48.2	52.1	41.9	33	28.3	30.5
Coiled	51	46.7	39.4	31.6	24.5	20.4	20.3	20.9
Total	72.2	73.9	87.6	83.7	66.4	53.4	48.6	51.4
Initial DNA concentration 14.4 ug/ml			Loading volume: 5ul					
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	19.9	19.9	21.1	29.9	26.3	7.8	0	27.2
Coiled	36	24.8	18.2	18.2	0	0	0	0
Total	55.9	44.7	39.3	48.1	26.3	7.8	0	27.2
Initial DNA concentration: 20.9 ug/ml			Loading volume: 10 ul					
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	5.7	13.4	21	19.6	25.4	17.7	13.3	17.3
Coiled	15.2	22.5	15.8	14.5	13	12.6	12.7	0
Total	20.9	35.9	36.8	34.1	38.4	30.3	26	17.3
Initial DNA concentration 4.1 ug/ml			Loading volume: 5ul					

mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	10.1	9.4	12.7	22.4	31.8	24.9	20.4	16.5
Coiled	21.3	22.4	17.4	16	13.8	0	0	0
Total	31.4	31.8	30.1	38.4	45.6	24.9	20.4	16.5
Initial DNA concentration 3.1 ug/ml			Loading volume: 10ul					
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	10.9	14.4	34.6	41.3	40.2	22.7	5.2	6.6
Coiled	42.1	39.4	29	9.9	0	0	0	0
Total	53	53.8	63.6	51.2	40.2	22.7	5.2	6.6
Initial DNA concentration: 2.1 ug/ml			Loading volume: 10 ul					
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	6.2	9.5	11.6	18.4	16.9	20.4	16.7	0
Coiled	15	18.1	15.1	0	0	0	0	0
Total	21.2	27.6	26.7	18.4	16.9	20.4	16.7	0
Initial DNA concentration 1.3 ug/ml			Loading volume: 10ul					
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	0	7.4	11.3	9.6	0	0	0	0
Coiled	12.3	8.6	0	0	0	0	0	0
Total	12.3	16	11.3	9.6	0	0	0	0

Appendix 3 (b): DNA Concentrations Calculated from Gel Densitometry Readings for Chapter 5 Figs 3, 4, 5, 6 & 7

Concentration of DNA (ug/ml)

Initial DNA concentration: 78.5 ug/ml				Loading volume: 2ul		Standardisation factor: 1		
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	24.7	45.8	64.3	67.0	19.6	57.9	34.5	30.7
Coiled	53.8	52.3	26.2	25.8	0.0	18.6	15.5	13.8
Total	78.5	98.1	90.5	92.8	19.6	76.5	50.0	44.5

Initial DNA concentration: 64.6 ug/ml				Loading volume: 2ul		Standardisation factor: 1.0005		
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	15.5	22.2	42.0	50.5	56.8	61.4	31.8	31.3
Coiled	49.1	55.0	39.1	19.8	16.1	0.0	9.5	15.3
Total	64.6	77.2	81.1	70.3	72.9	61.4	41.3	46.6

Initial DNA concentration: 33.3 ug/ml				Loading volume: 2ul		Standardisation factor: 1.0005		
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	6.9	10.4	26.9	19.3	39.1	0.0	17.9	17.7
Coiled	26.4	25.2	17.5	7.4	6.4	0.0	2.7	3.3
Total	33.3	35.6	44.4	26.7	45.5	0.0	20.6	21.0

Initial DNA concentration: 30.0 ug/ml				Loading volume: 2ul		Standardisation factor: 1		
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	12.2	23.7	28.6	26.3	27.8	27.8	22.5	14.6
Coiled	17.8	18.7	15.2	10.2	9.1	9.1	7.6	0.0
Total	30	42.3	43.8	36.5	36.9	36.9	30	14.55

Initial DNA concentration: 21.2 ug/ml				Loading volume: 5ul		Standardisation factor: 1.436		
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	5.9	6.8	11.7	15.1	20.9	11.2	13.6	13.8
Coiled	15.4	11.8	8.3	3.3	0.0	0.0	0.0	0.0
Total	21.2	18.6	20.0	18.4	20.9	11.2	13.6	13.8

Initial DNA concentration: 16.1 ug/ml				Loading volume: 5ul		Standardisation factor: 1.436		
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	5.7	5.7	6.1	8.6	7.6	2.2	0.0	7.8
Coiled	10.3	7.1	5.2	5.2	0.0	0.0	0.0	0.0
Total	16.1	12.8	11.3	13.8	7.6	2.2	0.0	7.8

Initial DNA concentration: 14.4 ug/ml				Loading volume: 5ul		Standardisation factor: 1		
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	4.2	5.4	9.6	10.4	8.4	6.6	5.7	6.1
Coiled	10.2	9.3	7.9	6.3	4.9	4.1	4.1	4.2
Total	14.4	14.8	17.5	16.7	13.3	10.7	9.7	10.3

Initial DNA concentration: 5.4 ug/ml				Loading volume: 10ul		Standardisation factor: 1.019		
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	1.1	1.5	3.5	4.2	4.1	2.3	0.5	0.7
Coiled	4.3	4.0	3.0	1.0	0.0	0.0	0.0	0.0
Total	5.4	5.5	6.5	5.2	4.1	2.3	0.5	0.7

Initial DNA concentration: 4.2 ug/ml				Loading volume: 5ul		Standardisation factor: 1		
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	1.1	2.7	4.2	3.9	5.1	3.5	2.7	3.5
Coiled	3.0	4.5	3.2	2.9	2.6	2.5	2.5	0.0
Total	4.2	7.2	7.4	6.8	7.7	6.1	5.2	3.5

Initial DNA concentration: 3.1 ug/ml				Loading volume: 10ul		Standardisation factor: 1		
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	1.0	0.9	1.3	2.2	3.2	2.5	2.0	1.7
Coiled	2.1	2.2	1.7	1.6	1.4	0.0	0.0	0.0
Total	3.1	3.2	3.0	3.8	4.6	2.5	2.0	1.7

Initial DNA concentration: 2.1 ug/ml				Loading volume: 10ul		Standardisation factor: 1		
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	0.6	1.0	1.2	1.8	1.7	2.0	1.7	0.0
Coiled	1.5	1.8	1.5	0.0	0.0	0.0	0.0	0.0
Total	2.1	2.8	2.7	1.8	1.7	2.0	1.7	0.0

Initial DNA concentration: 1.3 ug/ml				Loading volume: 10ul		Standardisation factor: 1.019		
mM FeS	0	0.1	1	10	25	50	100	150
Relaxed	0.0	0.8	1.2	1.0	0.0	0.0	0.0	0.0
Coiled	1.3	0.9	0.0	0.0	0.0	0.0	0.0	0.0
Total	1.3	1.6	1.2	1.0	0.0	0.0	0.0	0.0

Appendix 3(c): Data for Figure 3

Initial DNA concentration (ug/ml)		% DNA in relaxed form			
	0	0.1	1	10	25
78.45	31.4	46.7	71.0	72.2	100.0
64.58	23.9	28.8	51.8	71.8	78.0
33.32	20.7	29.3	60.5	72.4	85.9
30.00	40.7	55.9	65.3	72.1	75.3
21.22	27.6	36.5	58.4	82.2	100.0
16.05	35.6	44.5	53.7	62.2	100.0
14.44	29.4	36.8	55.0	62.2	63.1
5.40	20.6	26.8	54.4	80.7	100.0
4.18	27.3	37.3	57.1	57.5	66.1
3.14	32.2	29.6	42.2	58.3	69.7
2.12	29.2	34.4	43.4	100.0	100.0
1.25	0.0	46.3	100.0	100.0	100.0
m	26.5	37.7	59.4	74.3	86.5
SD	13.7	19.2	25.5	26.5	27.7

Appendix 3(d): Data for figure 4

Initial DNA concentration (ug/ml)	% Reduction in DNA concentration			
	25.00	50.00	100.00	150.00
78.5	75.0	2.5	36.3	43.3
64.6	-12.8	4.9	36.1	27.9
33.3	-36.7	100.0	38.3	37.1
30.0	-23.0	-23.0	0.0	51.5
21.2	1.6	47.3	36.2	35.2
16.1	52.8	85.8	99.7	51.2
14.4	8.0	26.0	32.7	28.8
5.4	24.2	57.2	90.2	87.5
4.2	-83.7	-45.0	-24.4	17.2
3.1	-45.2	20.7	35.0	47.5
2.1	20.3	3.8	21.2	100.0
1.3	100.0	100.0	100.0	100.0
m	6.7	31.7	41.8	52.3
StDev	52.25236	47.21045	37.98271	28.2854

Appendix 3(e): Data for figure 5

Initial DNA concentration (ug/ml)	Total DNA in relaxed state			
	0.0	0.1	1.0	10.0
78.5	0.0	21.1	39.6	42.3
64.6	0.0	6.7	26.5	35.0
33.3	0.0	3.5	20.0	12.4
30.0	0.0	11.5	16.4	14.1
21.2	0.0	0.9	5.8	9.2
16.1	0.0	0.0	0.4	2.9
14.4	0.0	1.2	5.4	6.2
5.4	0.0	0.4	2.4	3.1
4.2	0.0	1.6	3.1	2.8

Appendix 3(f): Data for figure 6

Initial DNA Concentration (ug/ml)	% DNA in relaxed state.				
		mM FeS			
	0	0.1	1	10	25
78.45	31.4	46.7	71.0	72.2	100.0
64.58	23.9	28.8	51.8	71.8	78.0
33.32	20.7	29.3	60.5	72.4	85.9
30.00	40.7	55.9	65.3	72.1	75.3
21.22	27.6	36.5	58.4	82.2	100.0
16.05	35.6	44.5	53.7	62.2	100.0
14.44	29.4	36.8	55.0	62.2	63.1
5.40	20.6	26.8	54.4	80.7	100.0
4.18	27.3	37.3	57.1	57.5	66.1
3.14	32.2	29.6	42.2	58.3	69.7
2.12	29.2	34.4	43.4	100.0	100.0
1.25	0.0	46.3	100.0	100.0	100.0

Appendix 3(g): Data for figure 7

Initial DNA Concentration (ug/mL)	Reduction in DNA concentration(ug/mL) mM FeS	
	100	150
78.45	28.45	34.00
64.58	23.31	18.01
33.32	12.76	12.36
30.00	0.00	15.45
21.22	7.66	7.46
16.05	16.05	8.24
14.44	4.72	4.16
5.40	4.87	4.73
4.18	-1.02	0.72

Appendix 3 (h): Data for table 2

	0.1 mM FeS				0.1 mM FeS+catalase			
	1	2	3	4	1	2	3	4
Relaxed	11.0	11.0	11.0	15.3	11.1	10.6	7.2	6.8
Supercoiled	13.2	14.5	13.0	14.5	14.1	13.7	12.3	13.1
Total (ug/mL	24.2	25.6	24.0	29.9	25.3	24.3	19.5	20.0
%relaxed	45.5	43.2	45.8	51.3	44.0	43.6	36.9	34.2

Appendix 3 (i): Data for table 3

	Positive contol				
Rep No.	1	2	3	4	M
Supercoiled	8.78	6.82	5.78	6.72	7.03
Relaxed	23.30	21.40	18.02	21.70	21.11
% relaxed	5.47	4.83	4.86	4.73	4.97
total (ug/ml)	32.08	28.22	23.80	28.42	28.13

	FeS control				
Rep No.	1	2	3	4	M
Supercoiled	13.88	15.64	18.48	14.34	15.59
Relaxed	19.48	16.10	13.76	15.06	16.10
% relaxed	8.32	9.86	11.46	9.76	9.85
total (ug/ml)	33.36	31.74	32.24	29.40	31.69

	Formaldehyde control				
Rep No.	1	2	3	4	M
Supercoiled	5.58	6.20	4.00	5.34	5.28
Relaxed	18.38	21.14	16.66	18.58	18.69
% relaxed	4.66	4.54	3.87	4.46	4.38
total (ug/ml)	23.96	27.34	20.66	23.92	23.97

	Formaldehyde + 1mMFeS				
Rep No.	1	2	3	4	M
Supercoiled	13.20	16.82	12.62	7.64	12.57
Relaxed	14.68	13.02	13.98	12.28	13.49
% relaxed	9.47	11.27	9.49	7.67	9.48
total (ug/ml)	27.88	29.84	26.60	19.92	26.06

	DMSO control				
Rep No.	1	2	3	4	M
Supercoiled	5.76	3.66	3.94	5.52	4.72
Relaxed	20.18	17.20	16.86	19.82	18.52
% relaxed	4.44	3.51	3.79	4.36	4.02
total (ug/ml)	25.94	20.86	20.80	25.34	23.24

	DMSO + Fes				
Rep No.	1	2	3	4	M
Supercoiled	3.74	4.38	6.22	7.82	5.54
Relaxed	16.96	18.96	19.30	19.46	18.67
% relaxed	3.61	3.75	4.87	5.73	4.49
total (ug/ml)	20.70	23.34	25.52	27.28	24.21

Appendix 3 (j): Data for table 4

	Dark Positive				
	1	2	3	4	m
Relaxed	42.4	38.5	35.0	34.9	37.7
Supercoiled	75.1	80.7	72.4	72.4	75.2
Total (ug/mL	117.5	119.2	107.4	107.3	112.9
% relaxed	36.1	32.3	32.6	32.5	33.4

	Dark FeS				
	1	2	3	4	m
Relaxed	97.4	90.2	88.0	93.6	92.3
Supercoiled	28.5	27.7	31.0	28.5	28.9
Total (ug/mL	125.9	117.9	119.0	122.1	121.2
% relaxed	77.4	76.5	73.9	76.7	76.1

	UV Positive				
	1	2	3	4	m
Relaxed	30.8	36.3	34.0	35.0	34.0
Supercoiled	68.6	72.9	76.4	76.9	73.7
Total (ug/mL	99.4	109.2	110.4	111.9	107.7
% relaxed	31.0	33.2	30.8	31.3	31.6

	UV FeS				
	1	2	3	4	m
Relaxed	88.4	95.2	94.7	109.7	97.0
Supercoiled	30.5	24.5	23.2	29.9	27.0
Total (ug/mL	118.9	119.7	117.9	139.6	124.0
% relaxed	74.3	79.5	80.3	78.6	78.2

Appendix 4

Gel densitometry readings for Chapter 6, Figure 1b

	mM CuS			
	0	0.1	1	10
relaxed	0.0	9.1	15.9	18.2
closed	61.5	77.5	72.7	58.5
total (ug/mL)	61.5	86.6	88.6	76.6
%relaxed	0.0	10.5	18.0	23.7

Appendix 5

Appendix 5 (a): Raw data for Figure 1

	A260nm 1	A260nm 2	m	ug/mL DNA
cDNA	0.896	0.9	0.898	44.9
FeS	0.0766	0.0983	0.08745	4.3725
ZnS	0.0107	0.0113	0.011	0.55
CuS	0.0006	0.0694	0.035	1.75
FeSO4	0	0	0	0
FeS+cDNA	0.1035	0.0983	0.1009	5.045
ZnS+ cDNA	0.0134	0.0362	0.0248	1.24
CuS+ cDNA	0.1146	0.0404	0.0775	3.875
FeSO4+cDNA	0.7753	0.8409	0.8081	40.405

Appendix 5(b): Raw data for Figure 2

Time	A260nm 1	A260nm 2	m	ug/mL DN
0		0.7090	0.7090	23.3330
1	0.4925	0.6909	0.5917	18.9929
24	0.1969	0.2640	0.2305	5.6267
72	0.2314	0.2417	0.2366	5.8524

Appendix 5(c): Raw data for Figure 3

		A260nm	A320nm	A600nm	modifiedA	modifiedA	ug/ml RN	m
0hrs	RNA	0.9120	0.0000	0.0000	0.0000	0.0000	36.4800	36.4800
24hrs	RNA	0.9120	0.0140	0.0000	0.9120	0.0140	36.4800	36.4800
	RNA+FeS1	0.4840	0.2490	0.0340	0.4516	0.2106	18.0626	
	RNA+FeS2	0.3440	0.1430	0.0280	0.3173	0.1114	12.6915	15.3770
72hrs	RNA	0.9190	0.0120	0.0000	0.9190	0.0120	36.7600	36.7600
	RNA+FeS1	0.4000	0.2150	0.0870	0.3170	0.1167	12.6801	
	RNA+FeS2	0.2870	0.1130	0.0640	0.2259	0.0407	9.0378	10.8589
144hrs	RNA	0.9170	0.0112	0.0000	0.9170	0.0112	36.6800	36.6800
	RNA+FeS1	0.2770	0.1290	0.0552	0.2243	0.0666	8.9736	
	RNA+FeS2	0.2880	0.1300	0.0554	0.2351	0.0674	9.4059	9.1898
360hrs	RNA	0.9210	0.0160	0.0000	0.9210	0.0160	36.8400	36.8400
	RNA+FeS1	0.3410	0.1840	0.0710	0.2733	0.1038	10.9306	
	RNA+FeS2	0.2550	0.1130	0.0930	0.1663	0.0079	6.6511	8.7909

Appendix 5(d): Raw data for Figure 5

	24hr		
	A260nm	A320nm	modified 260
Adenine	1.0640	0.0000	1.0640
FeS 1	0.7820	0.0490	0.7266
FeS 2	0.7450	0.0240	0.7179
Adenosine	1.0860	0.0000	1.0860
FeS 1	0.7830	0.0300	0.7491
FeS 2	0.7840	0.0340	0.7456
dAMP	0.8530	0.0000	0.8530

	48hr		
	A260nm	A320nm	modified26
Adenine	1.0650	0.0000	1.0650
FeS 1	0.7800	0.0180	0.7597
FeS 2	0.7830	0.0260	0.7536
Adenosine	1.0870	0.0000	1.0870
FeS 1	0.8140	0.0410	0.7677
FeS 2	0.8240	0.0430	0.7754
dAMP	0.8610	0.0070	0.8531

FeS 1	0.7410	0.0260	0.7116
FeS 2	0.7220	0.0190	0.7005

FeS 1	0.7570	0.0500	0.7005
FeS 2	0.7360	0.0350	0.6965

	144hrs		
	260	320	modified 260
Adenine	1.0670	0.0000	1.0670
FeS 1	0.8860	0.0919	0.7822
FeS 2	0.8730	0.0827	0.7795
Adenosine	1.0930	0.0000	1.0930
FeS 1	0.9530	0.1190	0.8185
FeS 2	0.9490	0.1300	0.8021
dAMP	0.8570	0.0000	0.8570
FeS 1	0.7550	0.0589	0.6884
FeS 2	0.7540	0.0529	0.6942

	360hrs		
	260	320	modified2
Adenine	1.0870	0.0000	1.0870
FeS 1	0.9590	0.1480	0.7918
FeS 2	0.9250	0.1200	0.7894
Adenosine	1.0900	0.0030	1.0866
FeS 1	0.9850	0.1250	0.8438
FeS 2	1.0300	0.1930	0.8119
dAMP	0.8600	0.0070	0.8521
FeS 1	0.7670	0.0600	0.6992
FeS 2	0.7780	0.0560	0.7147

A260	0	24	48	144	360
Adenine	1.0640	0.7223	0.7566	0.7809	0.7906
Adenosine	1.0860	0.7473	0.7715	0.8103	0.8278
dAMP	0.8530	0.7061	0.6985	0.6913	0.7070