Dioxygen Free Radical Reactions

João Pedro Cabaço Moniz Barreto

A Thesis submited in partial fulfilment of the
requirements of Oxford Brookes University
for the degree of Doctor of Philosophy.

October 1997

Abstract

This thesis is concerned with the problems relating to the creation and simulation of models of dioxygen free radical reactions under biologically relevant conditions. The wide range of values of rate constants, concentrations and time scales concerned poses great problems for the choice and efficiency of the mathematical methods. The problems were particularly severe when assessing mechanisms leading to the production of $OH^{\cdot}$.

Firstly, existing computer programs for deterministic simulation of kinetic models were compared. Two that met criteria for suitability for the task were used to study existing models of dioxygen free radicals and then to develop new ones. It will be shown that neither of the two contributed to understanding the mechanisms leading to the production of $OH^{\cdot}$. Although the models could be simulated, the results obtained raised serious questions about the validity of the approach. The main problem being the interpretation of the very low concentrations of an intermediate such as $OH^{\cdot}$ with an extremely short half-life.

A new computer program implementing a Monte Carlo method not previously applied in biochemistry was developed and used to study the Haber-Weiss reaction and ultimately to investigate the conditions necessary for the production of $OH^{\cdot}$, its lifetime, and its efficacy in starting lipid peroxidation. Using this Monte Carlo method, I have demonstrated that: the number of molecules of hydroxyl radical in a volume of cellular dimensions fluctuates between zero and one, reactions involving $OH^{\cdot}$ obviously only taking place in the latter case (deterministic simulation wrongly represents this number as a concentration corresponding to the non-integral time average of these two states and hence implies continous low level occurrence of reactions involving $OH^{\cdot}$); when the reactants of a processes are not available this will not occur (a situation which is not correctly represented by a deterministic approach); the production of $OH^{\cdot}$ is always possible, however if there is no catalyst available it is highly improbable; one hydroxyl radical is enough to initiate biologically damaging oxidative processes, even if it has to diffuse into a membrane.

This research resolves these apparently irreconcilable differences by showing that they are the product of inadequate modelling — resulting principally from the tendency of the deterministic approach to average a small number of highly significant events into an undetectably low background level. The modelling framework proposed here, however,

presents a much truer picture, allowing the study of those catastrophic events which are otherwise hidden because of their extremely low frequency. Only through adopting this approach can scientists hope to study the mechanisms by which extremely rare events, such as the generation of hydroxyl radical, initiate processes whose effects are so profoundly deleterious to biological systems.

Acknowledgements

There is a large number of people to whom I will always be in debt for the varying degrees of help and support they provided in these last six years. It would take pages if I were to mention all of those who have been directly or indirectly involved in keeping my life in research ticking during these years.

First of all, I would like to thank David Fell, my supervisor and friend, who was not only responsible for sparking my move into a new area of research during those chats in the woods of the old Eastern Germany in the far winter of' 89, but also for always being there, sometimes ready to help just by encouraging a line of thought, at other times by sharing his great analytical powers.

I also would like to thank Allen Hill for all the suggestions he made about the directions that could be taken with such research.

For allowing me to question all the time what they were doing, sharing their knowledge, and listening patiently to all my long monologues on how and why dioxygen free radicals are involved in all the interesting problems, I would also like to thank my colleagues Simon Thomas, John Woods and Mark Pullman.

I thank Pedro Mendes, Alex and Xana Carmo, Ana Furtado and Antonio Duarte for all those long stimulating discussions about the state of so many things (science included).

To Helen Woods I owe a very special debt of gratitude, first for being such a different friend, then for all the help in forcing me to organize all my thoughts when I started to get lost. I also would like to thank Charles Jackson for all the questions he had and never asked.

I am grateful to the Junta Nacional de Investigaçcão Científica e Técnologica (Portugal) for the financial support (grant BD-384-90-ID) given to me during a three year period.

I would like to dedicate this thesis to all my family, and especially to my mother and father who never lost their hope...

Foreword

My interest in the area of free radicals in biology was stimulated while studying for my degree in biochemistry in the early eighties when I was asked to write a dissertation with two other colleagues on the subject of the metabolism of oxygen sulphur and seleniun. It was decided that, in order for us to be able to cover such wide field, each of us would concentrate on one topic and subsequently we would endeavour to integrate the data collected. The aspect I concentrated on was the metabolism of oxygen.

The normal practice was to give the student an initial list of references relevant to the area chosen which they were to explore and pursue the direction that would suit them better. Two items from my list made a big impression: the paper that is responsible for my addiction to the field of oxygen free radicals, and a book that for a long time was considered the "bible" by researchers in the field of free radicals.

The paper "Biochemical effects of excited state molecular oxygen" (J. Bland, *J. Chem. Edu.* **53** 274-279 (1976)) introduced me to the world of oxygen free radicals. One of its main points was to demonstrate the large range of biochemical roles molecular oxygen has in:

- dye sensitized photooxidations;
- blood diseases;
- cancer inducing mechanisms;
- possible radical-like ageing mechanisms;
- the role of the bactericidal activities of phagocytes and
- metabolic hydroxylations.

The other was a textbook edited by Pryor. Unfortunately its reputation was inversely related to its availability and I only managed to get my hands on a copy long after the deadline for the monograph. The book was entitled "Free radicals in biology" and was the first collection of all the available knowledge about free radicals and their implications for biology. Lost in all that information, I rediscovered the multidisciplinary implications of this field.

As a result of the wealth of information available the editor had written an introductory chapter demonstrating the extensive involvement of free radicals in many processes. As well as the topics mentioned above other biological mechanisms known to involve oxygen free radicals were mentioned. These included:

- radical production by enzymes,
- photosynthesis,
- radiation damage,
- the chemistry of oxygen at high pressures,
- the chemistry of ozone, NO, NO_2, singlet oxygen, and other components of smog,
- the chemistry of hydrogen peroxide and the superoxide anion radical, and
- the autoxidation of lipids.

One other fact that struck me at the time was the source of the references (interestingly this was one of the reasons behind the publication of the book). Although the term radical had been well known for a long time in chemistry[1], there were only scattered publications on the implications of free radicals in biology. For example their involvement in radiation damage and food preservation was well documented, and there were some publications theorising on the mechanisms of oxygen toxicity, but it was only during the 70s that interest in the field grew. As the number of publications increased, new specialised journals were created, resulting in books and review papers which compiled the wealth of new data being produced.

During the 70s, when the boom occurred, different groups were proposing various mechanisms and theories for the oxygen toxicity which were not always compatible. One

[1] especially organic chemistry, albeit with a slightly different meaning

of the main examples is the controversy that surrounded one of the species, the *hydroxyl radical*. Agreement could not be reached concerning the possibility of its biological production, nor was there consensus about how damaging this species was. All this controversy is the origin of my contribution to the field.

I had done some theoretical modelling and computer simulation of simple systems (more specifically investigating the kinetics of simultaneous reactions) and realised that a great deal could be learnt using this approach. The selection of reactions and all the necessary parameters is a process that greatly contributes to a general understanding of how models work. As a result I thought that this approach could aid the understanding of the mechanisms involved in free radical biology. My degree project was therefore devoted to the modelling and computer simulation of systems of free radical reactions, thereby introducing a novel method (albeit theoretical) to the study of these problems, though, of course, both modelling and computer simulation have been used to study other types of problems in biology and chemistry. It was the belief that there were further applications for modelling in free radical reactions that led to the following thesis.

Contents

1 Introduction: oxygen free radicals **1**

1.1 Oxygen . . . 2

1.2 Free radicals . . . 5

1.3 Dioxygen free radicals . . . 8

1.3.1 Singlet dioxygen $^1\triangle_g O_2$ and $^1\sum_g^+ O_2$ . . . 10

1.3.2 Radical anion superoxide (O_2^-) . . . 11

1.3.3 Hydrogen peroxide . . . 15

1.3.4 Hydroxyl radical $OH^{\cdot}$ . . . 17

1.4 Layout of this Thesis . . . 21

2 Modelling in biochemistry **23**

2.1 What is a model? . . . 24

2.2 Model creation . . . 25

2.3 Simulation: . . . 28

2.4 Differential equations . . . 29

2.4.1 Numerical methods . . . 31

2.5 Stochastic Methods . . . 32

3 Introduction: modelling of free radical reactions **35**

3.1 My previous models . . . 35

3.2 Other models of oxygen free radical reactions . . . 39

3.2.1 The Single Reaction Approach . . . 40

3.2.2 An integrated approach (deterministic) . . . 42

3.3 Plan of this work . . . 63

3.3.1 The search for simulation tools . . . 63

3.3.2 The deterministic simulation 64
3.3.3 The stochastic simulation 65

4 Simulation tools **66**
4.1 Requirements and strategy 66
4.2 Review of the available programs 69
4.2.1 Mathematical modelling tools 71
4.2.2 Metabolic modelling tools 72
4.2.3 SCAMP and GEPASI 74
4.2.4 Discussion 79
4.3 Conclusion 87

5 Deterministic simulation **89**
5.1 Deterministic simulation of known models 90
5.1.1 Further investigation of my models 92
5.1.2 The study of the model proposed by Tappel *et al* 101
5.1.3 The study of the model proposed by Babbs and Steiner 103
5.1.4 The study of the model proposed by Remacle *et al* 104
5.1.5 The study of the model proposed by Suzuki and Ford 106
5.1.6 The study of the model proposed by Volkov and Lebedev 109
5.1.7 Interim Conclusion 109
5.2 The study of hydroxyl radical production 111
5.2.1 The Haber-Weiss Reaction 112
5.2.2 The Haber Weiss cycle 115
5.2.3 Fenton-type reactions 119
5.2.4 The sulphenic acid hypothesis 121
5.3 Discussion 124

6 Monte Carlo modelling **126**
6.1 Choice of a suitable method 127
6.1.1 Simple Monte Carlo simulator 127
6.1.2 The importance of random number generation 129
6.1.3 Bunker and Gillespie's simulation method 131
6.2 Application to dioxygen free radical simulations 140

6.2.1 The Haber-Weiss reaction 141
6.2.2 The Haber-Weiss cycle and the production of $OH^{\cdot}$ 142
6.2.3 The interconversion block of dioxygen free radicals 145
6.2.4 Lipid peroxidation 147
6.2.5 The two compartment model 150
6.3 Discussion 151

7 Conclusion **153**

Chapter 1

Introduction: oxygen free radicals

Why oxygen free radicals? Increasingly, nowadays, these terms which previously belonged to the scientific arena have crossed over into the public domain. It seems to have become fashionable for the cosmetic industry to use these terms — invoking them as harmful agents which have to be neutralised. As public awareness of such things as carcinogenic agents, the role of vitamins, diet, and their impact upon our health increases, alongside society's increasing sophistication, it has become possible for an assimilation of more and more scientific notions into our common lexicon. The conditions are ripe for the exploitation of such phrases as "oxygen free radicals" by advertisers — especially those promoting health supplements and cosmetics (*e.g.* Earl Mindel, (1979), "The Vitamin bible", Arlington Books, London).

Despite the overexploitation of the phrase "oxygen free radicals" in certain quarters, the resulting familiarity has contributed to at least a partial understanding of their role and importance by the general public. This fact makes possible more precise communications by scientists concerning the role of oxygen free radicals in such diverse fields as: carcinogenesis; the role of certain vitamins and oligoelements like selenium in cancers and in age related diseases such as Parkinsons and premature ageing; artherosclerosis [11, 68, 71, 72, 156, 159, 158, 176]; pre-eclampsia (work done by Prof. Chris Redman, Silver Star Team, John Radcliffe Hospital, Oxford) etc.

There remains a need for a detailed definition of what oxygen free radicals are, as well as a precise elucidation of their function within biological systems. To this end I propose to break the phrase "oxygen free radicals" into two parts — "oxygen" and "free radicals" — which I will examine in turn before proceeding to an exposition of oxygen free radicals

themselves.

1.1 Oxygen

> " About 500 years ago, Leonardo da Vinci observed that only a part of air is consumed during combustion and respiration ... however he was ahead of his time because the concept of elements ... had not yet been formulated. About 300 years later Lavoisier recognized oxygen as an element and established its necessity for combustion and respiration."
>
> Cook and Lauer (1968)

Oxygen was first produced by C.W. Scheell in 1772 from heating metal oxides (HgO_2) and was described as "empyreal air". Independently Joseph Priestley (1774) obtained it, also by heating HgO, and he named it "dephlogisticated air" to denote that it had been separated from "phlogiston", the imaginary substance that was supposed to be the raw material of fire [182].

As mentioned above oxygen was first recognized as a chemical element by A.L. Lavoisier, in his $1775 - 77$ experiments. He named it "oxygine", later "oxygene", from the greek for "acid maker", explaining in his "Elements of Chemistry" that "one of the most general properties of this base was to form acids by combining with many different substances" [169, 182]. Lavoisier, however, subscribed to another scientific fiction of the era — "caloric", an intangible and weightless substance that was supposedly the cause of heat. He wrote that the union of oxygen and "caloric" produced oxygen gas, which is the same as was formerly called pure air or vital air [169].

So what is oxygen? Oxygen is a molecule made up of two atoms of oxygen! Because oxygen never occurs in the atomic form (it is always in a molecule — either paired with another oxygen atom (O_2) or bound to some other element) it has become acceptable for molecular oxygen (O_2, strictly speaking dioxygen) to be referred to by the generic name of oxygen (similarly — to be totally correct — oxygen free radicals are actually dioxygen free radicals).

So why dioxygen? The chemical and physical properties of oxygen made this element a prime candidate for the biochemical development of biological structures/organizations. According to Szent-Gyorgy [187, 188] there have been two phases in the development of

life — the first, that he calls α, is pre-oxidative and made use of the earth's environmental characteristics, specifically of the fact that there was a very high source of photochemical energy that contributed to a very reductive atmosphere. This led to the development of biological structures which evolved photosynthetic systems. These organisms contributed to the appearance of molecular oxygen about 2×10^9 years ago. He then postulates that the resulting accumulation of oxygen contributed to the development of an ozone layer (O_3) which then allowed the possible evolution of other biological structures (he calls this the β phase). This evolution made dioxygen an essential compound for most living organisms: the formation of reactive dioxygen intermediates appears to be commonplace in aerobically metabolizing cells and the free radicals that are produced are damaging to biological materials. The reason why the evolving biological systems used dioxygen is due to the following characteristics:

- it is a powerful oxidising agent (i.e. it has high electron affinity);
- it has two unpaired electrons, and
- it has low reactivity,

resulting in the production of a biologically two edged sword: on one side dioxygen is vital for its role in electron transfer reactions; on the other it is very dangerous because it leads to the production of certain chemical species that are extremely reactive, and therefore damaging to biological structures.

Knowing these characteristics one might still ask at this point why the biological structures evolved to utilise dioxygen in preference to any other element. The reasons for this choice are due to the fact that even though dioxygen contains the potential for very high reactivity it is in fact relatively kinetically inert.

This kinetic inertness in solution can be explained by the electronic structure of dioxygen. Although molecular oxygen contains an even number of electrons, it has two unpaired electrons in its two HOMO (Highest Occupied Molecular Orbital), Figure 1.1. These give dioxygen a spin quantum number of one ($S = \sum s$) and a spin multiplicity of three ($2S+1$), that is, a triplet molecule for its lowest energy electronic configuration. Its low reactivity is due to a quantum rule that requires conservation of overall quantum spin state between reactants and products. As dioxygen has a spin state of 3, many reaction pathways for oxidation are unavailable because they would involve a forbidden change of spin state.

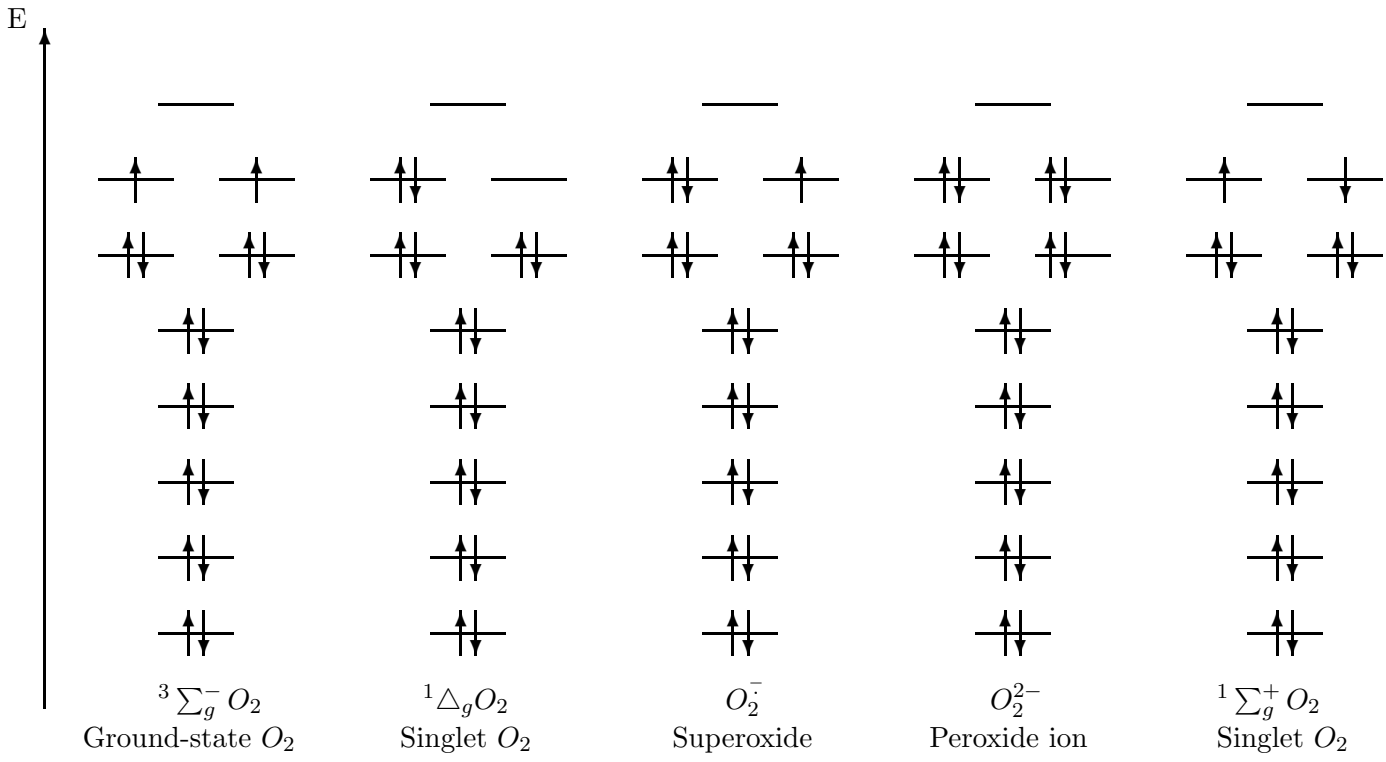

Figure 1.1: Bonding in the diatomic oxygen molecule. Redrawn from [73]. The $\sum$ denotes that the electrons have angular momenta in different directions (they belong to two orbitals) and $\triangle$ in the same direction (they are in the same orbital); and the superscripts of 1 and 3 mean respectively that they have opposed or parallel spins (singlet and triplet molecules) [11].

Inspection of figure 1.1 illustrates the properties of the dioxygen molecule. The first column shows the electronic structure of ground state dioxygen, with two unpaired electrons in two different molecular orbitals. The fact that they have the same spin (both arrows point in the same direction resulting in two unpaired electrons) explains the quantum number mentioned in the previous paragraph, the blocking of reactivity and the paramagnetism of dioxygen. To become reactive the molecule has to be "promoted" either energetically or electronically. The former involves the absorption of energy by dioxygen causing a change in its electronic structure (configuration) — by inverting the spin of one of the unpaired electrons resulting in the pairing of those electrons — so that it becomes singlet dioxygen, either ${}^1\triangle_g O_2$ or ${}^1\sum_g^+ O_2$ depending on the amounts of energy involved (second and last columns of figure 1.1). Electronic "promotion", on the other hand, involves either the mono-reduction of dioxygen which transforms it into the radical anion superoxide (O_2^- third column of 1.1), or bi-reduction, transforming dioxygen into the peroxide ion (H_2O_2 fourth column 1.1)[1].

Notwithstanding the relative inertness of ground state dioxygen, the two singlet oxygen forms, the radical anion superoxide and the peroxide ion (hydrogen peroxide), are all highly reactive, but to varying degrees.

1.2 Free radicals

Perusal of the literature will yield slightly differing definitions of what a radical is depending on which field of chemistry the text derives from. A radical can be defined most succinctly as a chemical species which contains one or more unpaired electrons [83]. These species have been designated free radicals in order to distinguish them from the term radical used in certain areas of organic chemistry, which has a totally different definition. They are so called because they are species capable of independent chemical existence containing one or more unpaired electrons, and it is in this sense that they are free. The existence of unpaired electrons within these free radicals causes paramagnetism (attraction to magnetic fields) and makes the species highly reactive.

Free radicals can be made most simply from any of the chemical elements capable of

[1] As a curiosity the formation of water from dioxygen involves tetra-reduction. There has been a wide ranging controversy on how this process occurs with some of the proposed mechanisms involving the possible production of both superoxide and peroxide ions.

independent existence belonging to odd group numbers in the Periodic table. This is the case because as the atomic orbitals are filled two by two there is always one electron left over, unpaired.

However free radicals can also be found in other circumstances — such as in complex molecules. In such cases when the number of electrons for each of the elements composing the molecule adds up to an odd number: such a molecule will be a free radical. There is also one other set of circumstances which can lead to free radicals: when, despite an even number of electrons within a molecule, the process of filling the orbitals results in there being two (or more, depending on the degeneracy of the orbitals) unpaired electrons in different orbitals. Such situations are caused by a rule relating to how orbitals are filled known as the Hund rule. According to this rule orbitals (atomic, ionic or molecular) should be filled from the lowest to the highest energy levels, only moving to the next level once the preceeding one is filled (one should also be reminded of the Pauli principle which states that an orbital can only contain a maximum of two electrons). A further consequence is that when there is more than one orbital in the same energy level these will have to have at least one electron in each orbital before any pairing can be concluded. This will lead to the occurrence of unpaired electrons characteristic of free radicals. Dioxygen is a prime example of this situation (refer to fig 1.1).

Reactions can also result in the chemical production of free radicals by the gain or loss of electrons by one of the participants in the reaction:

$$A + e^- \longrightarrow A^{\overset{-}{\cdot}}$$

or

$$A \longrightarrow A^{\overset{+}{\cdot}} + e^-$$

or by homolytic fission:

$$A - B \longrightarrow A^{\cdot} + B^{\cdot}$$

for example

$$H - O - H \longrightarrow H^{\cdot} + OH^{\cdot}$$

Based on the properties mentioned above, especially their reactivity, the number of reactions involving free radicals is very large. However, the possible reactions fall into four

major types:

- addition — this is simply when a free radical gets added onto the molecule it is reacting with, creating another free radical — e.g.:

  $R^{\cdot} + A \longrightarrow RA^{\cdot}$

- hydrogen abstraction — in this case the radical will, as the name indicates, "steal" a hydrogen atom from the substance it is reacting with. Such reactions tend normally to occur when the hydrogen is bound to an unsaturated atom — e.g.:

  $CH_2 = CH_2 + R^{\cdot} \longrightarrow RH + CH - C^{\cdot}$

- electron transfer — the unpaired electron of the radical is usually transferred from the radical to the other reactant. Sometimes, however, the transfer occurs the other way round — e.g.:

  $R^{\cdot} + A \longrightarrow R^{+} + A^{\dot{-}}$ or $R^{\cdot} + A \longrightarrow R^{-} + A^{\dot{+}}$

- recombination — these are the only type of reactions that do not produce a radical (this is explained further in the following text) because the reaction is between two radicals resulting in the elimination of both — e.g.:

  $R_1 + R_2 \longrightarrow R_1 - R_2$

As a result of the high reactivity of free radicals, reactions seldom happen individually, often occurring in quick succession. A widely known example of this is the fact that free radicals are often involved in chain reactions — an instance of which is lipid peroxidation. This process contains three steps, typical of chain reactions:

- initiation

  $$Lipid - H + R^{\cdot} \longrightarrow Lipid^{\cdot} + RH$$

  this consists of a simple hydrogen abstraction. This reaction leads to the appearance of new types of radicals which then allow the next step to occur.

- propagation

  $$Lipid^{\cdot} + Lipid - H \longrightarrow Lipid - H + Lipid^{\cdot}$$

another example of hydrogen abstraction. The availability of the lipid, and the possibility of repeating this simulation, will contribute to an increase in the production of radicals. This will continue until there is no more lipid available.

- termination

$$Lipid^{\cdot} + Lipid^{\cdot} \longrightarrow Lipid - Lipid$$

an example of a disproportionation removing radicals from the system, so terminating the whole process.

It can be seen from all the examples given above that the reaction of a free radical with a non-radical species produces a different free radical, which may be more or less reactive than the original radical.

1.3 Dioxygen free radicals

How does all this knowledge about the high reactivity of free radicals, and the type of reactions they are involved in, link up with the known unreactivity of dioxygen? The data presented earlier concerning the spin restriction state that the overall quantum spin state has to be conserved between reactants and products. Further analysis of the reactions detailed above illustrates this principle: the reaction classes presented always have one free radical on both sides of the reaction, meaning that the spin is conserved, except in the case of disproportionation where the spin is 0 on both sides (due to the cancelling effect of spin pairing). Because the vast majority of molecules have no unpaired electrons they have a spin multiplicity of 1. Dioxygen is unusual in that it has a spin multiplicity of 3. As a result, due to the spin conservation rule, the likelihood of dioxygen being involved in a reaction is very slight. To elucidate this point further: $O_2 + A$ will have a quantum spin number of 4, 3 from dioxygen and (in most cases) 1 from A; demanding a set of products with a quantum spin number which sums to 4 (for example two mono-radicals). There are only a very few situations in which this criteria can be met. Once dioxygen has been "promoted" (as detailed previously) this problem is solved, however, as then the resulting spin quantum number is only 2 or 1.

When dioxygen is "promoted" energetically to one of the two singlet forms the spin restriction is removed totally, because both forms, as the name indicates, have a quantum spin number of 1 and can react freely with most substances. Electronic reduction, on the

other hand, will transform dioxygen into radical anion superoxide (quantum spin number of 2) or peroxide (quantum spin number of 1), again far more reactive than dioxygen. Although all the promoted forms are much more reactive than ground state dioxygen, it is only radical anion superoxide which is, in fact, a dioxygen free radical. Despite the common misconception that the other species are free radicals, they are not — neither form of singlet dioxygen contains unpaired electrons, nor does peroxide ion.

The only substances that can react with ground state dioxygen or radical anion superoxide directly are transition metals. The reason for this is that a wide range of transition metals can alter their quantum spin numbers via changes in their oxidative state.

For example metal ions widely used in biological systems such as iron (Fe) and copper (Cu) can be found in oxidative states such as Fe^{2+} or Fe^{3+} (known as Fe(II) or Fe (III)) or Cu^{+} or Cu^{2+} (Cu(I) or Cu(II)). This gives alternative possibilities for the quantum spin number (5 and 6, and 3 and 4 respectively) which allows possible pairing with, for example, radical anion superoxide as in the following reaction:

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

with the quantum spin numbers:

$$2 + 6 \longrightarrow 3 + 5$$

It is also known that at high temperatures iron combusts spontaneously in the presence of dioxygen to form tri-iron tetroxide:

$$3Fe + 2O_2 \longrightarrow Fe_3O_4$$

obeying the spin rule. This is why certain types of metal ions are found in many enzymes active centres — iron and copper most usual in oxidising enzymes.

As a consequence of the wide range of roles and properties belonging to the dioxygen species it is now necessary to focus on each of them in turn. As each of the species has been covered thoroughly in the literature I will restrict myself to an overview which will include references to the significant publications.

1.3.1 Singlet dioxygen $^1\triangle_g O_2$ and $^1\sum_g^+ O_2$

Singlet dioxygen ($^1\triangle_g O_2$) is the first excited state of dioxygen at $92kJ/mol$ above ground state. Several reviews such as those of Kearns, Bland, Gorman and Duchstein have covered the essential aspects relating to this species [11, 33, 42, 56, 91, 101], ranging from the purely historical background to its physical properties and the chemical and biological characteristics.

There is considerable debate concerning the genesis of this species. There are two suggested types of mechanism: chemical, including electrochemical generation [136]; or physical, such as microwave discharge, atmospheric generation and the transfer of energy from photosensitizers (e.g. psoralens) to groundstate dioxygen, such as:

$$S + h\nu \longrightarrow S^*$$

$$S^* +^3 \sum O_2 \longrightarrow S +^1 \triangle O_2$$

Alternatively singlet dioxygen production has been proposed during the dismutation of superoxide, or of hydroperoxide ($2ROOH \rightarrow 2ROH +^1 O_2$) [19, 151]. Although there has been much debate upon this issue [11, 19, 56, 77, 101, 104, 105, 115] no consensus has yet been reached. Halliwell opposes the notion of biological production of singlet oxygen — he claims it just does not happen — dismissing apparent experimental proof as being the result of improper laboratory technique, including the inappropriate use of scavengers, and claiming the results obtained from these experiments have been misinterpreted [72]. However Khan and Koppenol have studied theoretically the possible production and reaction of singlet dioxygen [104, 114, 115] and several authors have determined the involvement of singlet oxygen with biological structures in carefully prepared media [14, 108–110, 118, 119, 154, 180]. More recently Kanofsky [99] has proposed the possible biological production of singlet dioxygen during the respiratory burst in phagocytes. In 1994 Halliwell himself mentions the production of singlet dioxygen — although without detailed discussion [74].

It is the fact that singlet dioxygen is very reactive that has led to the controversy concerning its production. Very small half lives have been proposed and these are highly solvent dependent, with values in the region of $20\mu s$ proposed by Kearns [101], rising to

$53\mu s$ in D_2O or falling to $4\mu s$ in H_2O [56]. Sies and Yu and [177, 200] have suggested that in aqueous media at 37^oC the half life is 1 to $10\mu s$. Such brief lifespans obviously make it extremely problematic to determine the presence of the species experimentally, and highlight consequent difficulties in proving its biological involvement.

In the event of there being biologically produced singlet dioxygen there is a considerable amount of data that implicates it in biological reactions. These include:

- extremely fast reactions with nucleotides, implicating singlet dioxygen in possible cancer generation [11, 200] The reaction of singlet dioxygen with membrane constituents leads to a peroxidation process (Fenton-type reaction) [121, 184]
- highly specific reactions with $\beta-$carotene, α-tocopherol and ascorbic acid — correlating with an *in vivo* role for the species [17, 36, 58, 121, 177]
- highly specific reactions of tryptophan and cholesterol with singlet dioxygen [180,143] but not with hydroxyl radical (different reaction products are obtained). This is significant because it allows for the design of experiments to demonstrate the specific biological involvement of singlet dioxygen [55, 71].

The controversy concerning the other variant of singlet dioxygen, ${}^1\sum_g^+ O_2$, is even greater — it has been suggested that it is even more reactive than ${}^1\triangle_g O_2$, with an even smaller lifespan (Kearns proposes a value of $10^{-11}s$ [101]) making it virtually impossible to simulate biologically. Values with the same order of magnitude have been proposed for the rate of decay from ${}^1\sum_g^+ O_2$ to ${}^1\triangle_g O_2$, which further supports the above argument. As a result the main interest in this form is in chemistry.

1.3.2 Radical anion superoxide (O_2^-)

As already mentioned this is the one-electron reduction state of dioxygen. Although the chemical existence of superoxide has been known for quite some time — as far back as the beginning of the century — the recognition of its importance in biochemical systems is relatively recent. It was the work of McCord and Fridovich (1969) that demonstrated the possible impact of this radical upon biological systems [138]. In a very elegant experimental approach they proposed a role for an, at that time, unknown enzyme that was, apparently, responsible for the dismutation of the superoxide radical. They also detected a copper ion within the enzyme's active centre. This fact, allied with the system within which the

enzyme was initially found, namely red blood cells, led to the name erythrocuprein. This enzyme, however, has subsequently been found to also have an extensive distribution in tissues other than red blood cells, being present not only in eukaryotes but also in prokaryotes.

A name change from erythrocuprein to superoxide dismutase was proposed to reflect both the wide distribution of the enzyme as well as the specific process it catalyzes. Superoxide dismutase has been widely described, characterized, and thoroughly investigated [46, 47, 62, 138, 150]. The discovery of superoxide dismutase has been paramount in facilitating understanding of the importance of superoxide radical in three significant areas:

- the simple fact of superoxide dismutase's existence in biological systems implicates the presence and biological importance of the necessary biological elimination of the superoxide radical
- the specificity has allowed detailed investigation of superoxide radicals' involvement in a wide range of biological processes
- the presence of superoxide dismutase has also led to a determination of the kinetic parameters for superoxide radical in reaction with any other metabolites *in vitro*.

Crucially the discovery of superoxide dismutase justified other work done both previously [37, 78] and later in the seventies [139]. More specifically — there had already been work which attempted to assess the damaging effects of high concentrations of dioxygen [78] which had been attributed to the possible *in vivo* production of superoxide radical, and the presence of superoxide dismutase verifies this hypothesis.

From 1969 onwards the majority of experiments investigating the possible existence of superoxide radical were designed in a similar fashion to that of McCord and Fridovich — that is having a known source of superoxide radical ([138] and further explanation to be included later) and by repeating experiments with and without superoxide dismutase (which, when studying biological systems, can be achieved by the presence or absence of specific inhibitors of the enzyme). Such experiments allowed the investigation of the participation (or not) of superoxide radical in a wide range of biological processes [46, 48, 61, 62, 138, 140, 144, 146, 162]. In certain cases, when the experimental approach to a

biological system was too complex, an *in vitro* model could be developed to the same end [97].

Properties and Reactions

As mentioned previously, superoxide radical can be obtained simply by the monoreduction of dioxygen:

$$O_2 + e^- \longrightarrow \dot{O_2^-}\ E^0(O_2/\dot{O_2^-}) = -0.33V$$

This production of the radical can be achieved via various well-documented types of processes including: photochemical; radiochemical and electrochemical [9, 48, 137, 138]. Chance *et al* have extensively reviewed the possible biological production of this radical [20] and have proposed a steady-state concentration for superoxide radical of $10^{-11}M$. The reasons behind suggesting such a small concentration are twofold. Firstly, superoxide dismutase catalyses the following reaction:

$$\dot{O_2^-} + H\dot{O_2} + H^+ \longrightarrow H_2O_2 + O_2\ k = 10^9 M^{-1}s^{-1}$$

Secondly, superoxide radical dismutates chemically (without superoxide dismutase), albeit with a lower rate constant ($k = 8 \times 10^7 M^{-1}s^{-1}$).

In addition to rapid dismutation, biological systems appear to avoid the production of superoxide radical (except in circumstances where it is advantageous, for example, phagocytosis [2, 8, 32, 124, 150, 175, 196]). They do this by favouring tetra-reduction reactions which result in the production of water, H_2O. In normal circumstances the production of superoxide radical can be considered "accidental", being the result of a "leakage" in the redox mechanisms (the spillage of intermediate metabolites before a process is completed). These facts, allied to the presence of superoxide dismutase, contribute to the low concentration of superoxide radical suggested above which, in normal circumstances, will not lead to any damaging effects.

Superoxide radical can be found in one of two forms, depending on the pH of its medium. Superoxide radical *per se* behaves as a weak base and, in solution, the following equilibrium will be established:

$$H\dot{O_2} \rightleftharpoons H^+ + \dot{O_2^-}$$

having a pK_a of 4.7. Assuming a pH of 7 for most biological media (despite this not always being the case) the predominant form of superoxide radical will be $O_2^{\cdot-}$, rather than its protonated form $HO_2^{\cdot}$ which only predominates in more acidic media.

Both the enzymatic and chemical dismutations of the superoxide radical are highly pH-dependent taking different forms and kinetic parameters according to the medium pH. At pH 7 the chemical and enzymatic reactions take the same form. At very low pH the chemical reaction becomes:

$$HO_2^{\cdot} + HO_2^{\cdot} \longrightarrow H_2O_2 + O_2 \; k = 8 \times 10^5 M^{-1} s^{-1}$$

whereas at high pH it is:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \; k < 0.3 M^{-1} s^{-1}$$

An important detail not included in the above reactions is the possible production of singlet dioxygen, as discussed in Section 1.3.1. Of all the dioxygen species presented earlier, despite being the only true dioxygen free radical, superoxide radical is not as highly reactive as singlet dioxygen. Although there has been much research into the possible biologically deleterious effects of superoxide radical, data published on the reactivity of superoxide radical in biological structures points towards surprisingly relatively low kinetic parameters [52]. In the light of this it has been difficult to defend the "superoxide theory of oxygen toxicity" [37, 38, 71].

The initial theory, as exemplified by McCord and Fridovich, concerning the harmful effects of superoxide radical led to work determining its kinetic parameters. Although the results pointed to the involvement of superoxide radical in biological systems the fact that these kinetic parameters were much lower than expected spurred discussion between Fee and Halliwell about which species *were* responsible for the observed destruction [38, 67].

During the determination of the kinetic parameters of the superoxide radical Bielski et al observed that the protonated form was far more reactive than superoxide radical itself. One of the reasons behind this is that the protonated form has no electrical charge and so can react efficiently with the lipids composing biological membranes.

Recently some publications have implicated superoxide radical in the inhibition of certain enzymes — possibly those playing key roles in metabolism [45, 48, 71, 186]. These

include catalase [112, 138].

The extent of the involvement of superoxide radical in the damaging of biological structures has not been fully established. Some researchers [37, 38, 186] believe the future will prove the destructive impact of superoxide radical upon biological systems.

1.3.3 Hydrogen peroxide

As mentioned earlier, hydrogen peroxide can be considered the divalent reduction of dioxygen. It can also result, however, from the univalent reduction of radical anion superoxide — any system producing the latter will also produce hydrogen peroxide. The previous section gave the steady-state concentration of radical anion superoxide in the cell as $10^{-12}M$, explaining that the radical undergoes a constant disproportionation, either chemical or SOD-enzyme-catalysed. By this process hydrogen peroxide is continually being created as an elimination product of radical anion superoxide. A consequence of this is that this species has been widely studied and there is extant a wealth of data concerning which reactions hydrogen peroxide is part of and its possible biological involvement [19, 22, 72, 90, 128, 130, 151].

Apart from the above generation of hydrogen peroxide there are other enzymes which also lead to its production, namely: a wide range of peroxidases and oxygenases such as 2-hydroxyacid and urate oxidases; myeloperoxidases; superoxide dismutase and monoamine oxidase [20]. A full list can be compiled using one of the several enzyme databases extant. This list would contain around 100 enzymes where their general nomenclature can be represented by $1.x.3.x$. All these contribute to a higher cellular concentration of hydrogen peroxide, about $10^{-9}M$ [20, 200].

It is important to re-stress that hydrogen peroxide, as mentioned previously, is *not* a radical — it does not contain unpaired electrons. Despite an apparent likelihood for hydrogen peroxide to be less reactive than radical anion superoxide, its diffusion rate through membranes is higher (there is even a channel on the erythrocyte membrane specific to hydrogen peroxide) giving it a wider arena in which to play a part. There are, therefore, grounds for arguing that hydrogen peroxide is, in fact, more reactive in practice than radical anion superoxide.

Problems arise in determining the mechanisms behind the action and toxicity of hydrogen peroxide. Although there has been direct evidence of hydrogen peroxide toxicity

towards bacteria [71, 75, 107] and animal cells [20, 23, 71, 128], other bacteria and photosynthetic algae generate and release large amounts of this metabolite, apparently without harm [71]. The differences between these two effects might be due to the balance existing between the mechanisms of hydrogen peroxide removing enzymes (e.g. catalase and peroxidases) and the rate of hydrogen peroxide conversion into more reactive species. There has been considerable debate concerning what such conversion would entail, and what radical species could be involved [71, 156, 176].

Careful analysis of results obtained from trying to elucidate the role of both radical anion superoxide and hydrogen peroxide in cell toxicity is necessary because:

- radical anion superoxide cannot easily diffuse across membranes, whereas hydrogen peroxide can;
- superoxide dismutase cannot eliminate radical anion superoxide from outside a cell, whereas catalase can displace the equilibrium hydrogen peroxide concentrations on both sides of a cell compartment.

When experiments are designed care has to be taken in using model microsomes as these can be formed with membranes from various cell sources, and the type of membrane can itself influence the reactions occuring.

Notwithstanding the difficulties associated with studying the toxicity of hydrogen peroxide, there is a wealth of available kinetic data for its reaction with other metabolites, ranging from inorganic substances to cellular components [14]. However, this still does not explain the extensive biological damage which sometimes occurs. There have been a variety of theories proposed, suggesting the apparent toxicity is not due to hydrogen peroxide itself but to other species which could be being produced from the accumulation of hydrogen peroxide. Suggested alternatives include singlet dioxygen, radical anion superoxide and, in the right conditions, hydroxyl radical. As hydroxyl radical is the most reactive of these three species, it has been nominated by the majority of researchers as the radical principally responsible for the damage observed, although proving its involvement has been problematic due to characteristics of the hydroxyl radical which will be discussed further in the following section.

1.3.4 Hydroxyl radical $OH^{\cdot}$

The reactivity of hydrogen peroxide and radical anion superoxide in biological systems does not explain the extensive damage being reported and their apparent role in processes which cause illness. Some authors, however, still maintain that these two species are the metabolites mainly responsible for the initiation of oxidative stress [38,186]. As discussed in chapter five, this is only true in certain conditions.

Evidence indicates that the intracellular anti-oxidant defences are very important in maintaining cellular integrity. Broadly speaking these fall into two categories:

- enzymatic mechanisms, such as SOD, catalase, peroxidases in general and GSH peroxidase in particular, are all specifically involved in the removal of radical anion superoxide, peroxides and, in particular, hydrogen peroxide from the various cellular compartments;

- chemical defences, for example $\alpha-$tocopherol, $\beta-$carotene, ascorbate, flavenoids and some proteins (caeruloplasmin), which are generally present in most cellular media, play an important role in mopping up any extant radicals within the system (for reviews on how these systems work see Halliwell [74], Krinsky [121], Sies [177] or Duchstein [33]).

These mechanisms do not only eliminate both hydrogen peroxide and radical anion superoxide but indicate that these radical species could have a serious impact on the system if they were to be present in any greater concentrations, which is why it is important to monitor their concentration sizes closely.

Allied to the biological framework there is chemical evidence that hydroxyl radical might be formed in certain conditions. Hydroxyl radical has been found to be extremely reactive and a wealth of evidence has been reported illustrating not only the possible interactions this radical has with a wide range of substances, but also the extremely high kinetic parameters determined for such reactions. All this data has prompted the creation of hypothesis concerning the role of the hydroxyl radical in biological systems, and experimental models have been produced to study whether the hydroxyl radical can be produced *in vivo* and the conditions in which this could occur.

In 1894 H.J.H. Fenton proposed that the oxidation of malic acid is promoted by ferrous iron and mediated by hydrogen peroxide [39, 193]. Forty years later Haber and Weiss

suggested that hydroxyl radical is produced in the so-called Haber Weiss reaction [63]. Some authors [44] present this reaction as being:

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

Others [159] consider the production of hydroxyl radical to be part of a cycle rather than a single reaction. This cycle is also the mechanism proposed by Haber and Weiss for the disproportionation of hydrogen peroxide, and, in absence of a metal catalyst, it can be written as [63, 114]:

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

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

or, when a metal catalyst such as Iron(III) is present [159], as:

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

$$Fe^{3+} + H_2O_2 \longrightarrow H^+ + HO_2^{\cdot} + Fe^{2+}$$

Rotilio *et al* proposed a value of $10^{-4} M^{-1} s^{-1}$ for the kinetic constant for the reaction producing hydroxyl radical in 1977, after a study using OH radical scavengers [164]. His paper also reflects that at the time there were other authors who had obtained different values, either directly or indirectly, for this kinetic constant. Dainton and Rowbottom proposed a value of $3.4 M^{-1} s^{-1}$ [27] for the reaction, and Bray [13], McClune and Fee [137] and Halliwell [66] agreed it was smaller than $10 M^{-1} s^{-1}$. McClune and Fee made an attempt to determine the rate constant for the *Haber Weiss* reaction, but without success. Their paper was, however, focussed on the study of the superoxide disproportionation [137]. Knowing that hydrogen peroxide is one of the products of the dismutation of radical anion superoxide ($O_2^{\cdot -}$), they studied the effect of this product on the dismutation reaction, with the results indicating that no inhibition (decrease) occurs and so no reaction between $O_2^{\cdot -}$ and H_2O_2 is observed [137]. The major information that can be obtained from this paper is the values for the kinetic constants for the dismutation of the superoxide anion in aqueous solution. It is important to note that these values are dependent on the pH conditions and, in this case, on the value of the extinction coefficient used for the calculation of the kinetic parameters.

Halliwell proposes a mechanism for detecting the possible production of $OH^{\cdot}$ based on the possible inhibitory effect H_2O_2 could have on the degradation of Nitro-blue tetrazolium (NBT) to formazan with a k of 5×10^4 in the presence of $O_2^{\cdot -}$. If the rate of degradation of NBT were to be affected when H_2O_2 was present, a reaction between superoxide and hydrogen peroxide could be proposed possibly leading to the production of hydroxyl radical (although he did not propose any methods for the direct detection of this species apart from the use of mannitol which could act as a radical scavenger). He was aware that some dismutation can also occur and that this was a factor to take into consideration when interpreting the results. When he detected no inhibition he assumed this indicated that no production of hydroxyl radical could be achieved in such conditions, although he still claimed that there was plenty of evidence for the possible production of this radical, though via an unknown mechanism.

Other values for the kinetic constant when catalysts were present were proposed by Halliwell in his book [71] as being $76 M^{-1}s^{-1}$ for Fe^{2+} and 4.7×10^3 for Cu^{+}. The first value derives from Walling who reviewed the stoichiometry and mechanism of the Fenton reaction and the possibilities for producing hydroxyl radical [193]. This, however, is not the original publication. McCord and Day proposed a value of $1000 M^{-1}s^{-1}$ for the reaction catalyzed by Fe^{2+} when this ion is in the form chelated by EDTA. They also give numbers for a possible efficiency of hydroxyl radical production from superoxide, but these are affected by possible mechanistic complications [140]. A different value of $62 M^{-1}s^{-1}$ for the Fenton reaction was also proposed by Wilson [197]. This was determined by Keene in 1964 [102]. The value for the reaction when copper is present was originally determined by Chiou [21].

The opposite effects that the iron-EDTA complexes might have on hydrogen peroxide have to be taken into consideration. Whereas Fe(II)-EDTA might catalyse the production of hydroxyl radical (as has been proposed above), Fe(III)-EDTA has been reported, by Halliwell, to catalyse the disproportionation of hydrogen peroxide [65] with a kinetic constant of $6 \times 10^4 M^{-1}s^{-1}$. Pryor [158] stresses that this could possibly be a reaction which competes with the production of hydroxyl radical, although Fong *et. al.* have criticised Halliwell's approach stating that the experimental conditions used [41] might have contributed to an erroneous interpretation of these results. These appear to have been later accepted by Halliwell as in an overview on the role of catalytic metals on the production of

free radicals written in 1990 [72], he goes on to propose that those Fe(III) complexes (without specifying which) can also react further with hydrogen peroxide, possibly via several stages, but ultimately producing some more hydroxyl radical. The proposed mechanism could be:

$$Fe^{3+} + H_2O_2 \longrightarrow ferryl \longrightarrow perferryl \longrightarrow OH^{\cdot}$$

with every step being dependent on the availability of hydrogen peroxide. In this event, the production of hydroxyl radical would not only be possible through the Fenton reation, when the iron has an oxidation number of two, but, also through the decomposition of hydrogen peroxide, via a not yet known or proven mechanism, with the iron ion with an oxidation number of three. This has been proposed when the iron ion is attached to nitrilotriacetic acid [72]. Bacon *et al* postulate a role for this complex (Iron(II)Nitrilotriacetic acid — FeNTA) in the damage produced to DNA which ultimately induces the formation of carcinoma cells [5].

Bearing in mind that it is already complex to prove the production of the hydroxyl radical via the Fenton reaction, then the difficulties of proving that these two reactions can occur is even more problematic. Possible complications might arise from the fact that other reactions are also possible with most of them, not just competing between them, but, ultimately leading to the consumption of $OH^{\cdot}$, such as:

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

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

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

These reactions, with the others proposed above, do not constitute the full range of reactions that could occur in systems producing free radicals (bearing in mind all the interconversions they undergo). Inspection of the literature would present the reader with a huge number of possible reactions from which a choice would have to be made in order to understand a mechanism or develop a system for further study.

Various methods have been developed to facilitate the study of dioxygen free radicals, and they are summarised in Rice-Evans and Diplock's recent book [163]. Wilson [197,198] claims that pulse radiolysis experiments are the approach *par excellence* for both the

production and the determination of rate parameters using competition kinetics analysis. However, for each study there is a specific method suitable — which one will vary according to the charecteristics of the system concerned. Although Rice-Evans and Diplock detail the problems concerning the experimental study of these species, and in particular those with very low concentrations, the development of models to demonstrate the involvement of hydroxyl radical remains controversial.

The possible site-specificity of hydroxyl radical metabolism has been suggested as being an obstacle to the experimental study of this species [3, 60, 69, 70, 197, 199]. This means that the radical is produced in an enclosed environment, and subsequently readily consumed, which could account for the difficulties in using indicators (radical scavengers or antioxidant enzymes) [107] to demonstrate the role of this species, either because they are unable to access that environment or because they are too rapidly consumed to be detected [189]. This raises two important issues related to this hypothesis:

- the concentrations of the hydroxyl radical present in biological systems are too small to be measured;
- due to its characteristics (its high reactivity and diffusion) the timescales involving hydroxyl radical reactions are also very small.

Wilson [197] states that when hydroxyl radical is produced in a biological medium, the species will have a very short lifetime (a value of less than $1\mu s$) and will react in the immediate vicinity of where it was formed. Walling is also in agreement with these findings. His work addressed the identification of the radical species involved in the mediated "oxidation" of organic substrates under metal complexes, and he claims that there is enough evidence to prove the presence of dioxygen free radicals given that they adequately account for the results obtained [193].

1.4 Layout of this Thesis

It can be seen that there are significant experimental difficulties in establishing the involvement of free radical species in particular reactions and also in determining kinetic constants with any degree of accuracy. When a number of such imperfectly known reactions are combined together, it becomes difficult to make qualitative predictions of what

will happen. It therefore seemed appropriate to test some of the proposed reaction schemes by computer simulation. For these reasons the concepts and basis of modelling and simulation will be introduced first in chapter two, then a review of the relevent applications will be presented in chapter three, at the end of which there is a statement redefining the aims of the thesis.

Chapter 2

Modelling in biochemistry

Research in biochemistry during the second half of the century has resulted in the increasing number of reactions depicted on the metabolic maps displayed in almost all labs like trophies. Three types of information can be obtained from the topology of metabolism, the identification of:

- the metabolites;
- the enzymes involved;
- the interconnections between the several metabolic pathways.

Despite the fact that this intricate reaction network was compiled from the most diverse sources, it cannot show the experimental conditions under which the results were obtained nor the sources of the data. A metabolic map conveys all the magnificence and the importance of a human being's metabolism, and also reveals the final aim of research work: the knowledge of the functioning of the human "machine". This is fed as much by simple curiosity as by the desire to understand how metabolism behaves in response to all external environmental changes: in other words, how life itself goes on.

Metabolism is the collection of mechanisms which maintain the interaction between what is normally termed the living thing and its environment. It maintains a continuous circulation of matter, energy and information in order to operate and regulate the biological processes responsible for certain functions of the system [79]. It is therefore important to look at the metabolic map in a different light, bearing in mind that what one sees is not a result of a simple linear combination of pathways working to the same final aim. There

is an unknown number of interdependencies, not necessarily structural, which render the whole network too complex (non-linear) for its behaviour to be predictable. It is similar to trying to understand the contribution of a small section of a road map to the global communication system. However, one can gain information about how metabolism works by combining all the wealth of kinetic data produced by the reductionism that has affected research for many years. A study of the dynamic behaviour of metabolic pathways in cellular environments leads to an elucidation of the relationship between the structure and the function of pathways [79].

Until now too much work (research) has been done to describe the parts (reductionism), but attempts to study larger parts of metabolism will have to be made so a greater understanding of how things work together is possible. One of the possible ways of integrating the knowledge available is the construction of models. Notwithstanding all the possible limitations of such an approach (differences from real systems, unknown parameters) the author thinks that this is one of major tools in modern research.

2.1 What is a model?

A model can be of several different natures (classes, types) such as, conceptual, experimental or mathematical. A definition of each of these classes is necessary before any further development of this thesis in order to make the different situations clear where the word "model" is used.

There is an intrinsic complexity related with the use of this word, which is apparent from what has been just said. Its imprecise use at different levels of science can result in a loss of rigour of the language and the reasons for this are deeply embedded in the history of the natural (chemical, biological), physical and pure (mathematical) sciences.

So while a conceptual model has its origins at a high level of abstraction only touching the pure sciences superficially (it can be considered in the domain of philosophy), a mathematical model is at the level of the physical and pure sciences, and the experimental at the level of the natural sciences (it is difficult to draw a line here as historically the study of the physical phenomena was at the base of the development of all sciences).

The easiest way to explain all these ideas is actually to follow the reasoning behind the process of model building. Initially an idea or an image of how a certain phenomena occurs develops in the human mind, this is normally followed by an attempt to instantiate

```
model building <--- A
               <--- B <-- A
               <--- C <-- B <---A
```

Figure 2.1: The source of model building. 'A' represents a conceptual model 'B' an experimental model and 'C' a mathematical model.

this abstraction by defining a set of relations (sometimes an amalgamate of intuitive rules) which will relate and/or describe the physical phenomena. The end product is a set of concepts (conceptual model) that can be later used in a different level of modelling. The normal means to test or to prove the validity of such ideas or concepts (theories) is to devise and propose protocols (experimental model) that can represent (model) the phenomena initially thought (this can be regarded as an instantiation of what the human mind had thought).

The mathematical modelling can occur either after the experimental, or before when an intermediate step is needed for a better planning of the experiment, or sometimes as the final step (without subsequent experiments) when a theoretical study is being made. It consists, as its name suggests, of sets of equations that quantitatively attempt to describe some kind of observed events (our physical phenomena). An example of a mathematical model is the simple equation that was proposed to describe the position (s coordinates) of a moving body through time (t) when submited to a variable speed (v) and acceleration (a):

$$s = s_0 + vt + 1/2at^2$$

where s_0 is the initial position.

2.2 Model creation

The creation of models is a highly varied and flexible process and only dependent on the initial ideas and the final "object" to model. Figure 2.1 illustrates this by the different starting points and the possible paths before the creation of the model.

When the "objects" being modelled are biological in nature and we are interested in

Table 2.1: Alternative system classification (adapted from [167])

Types of system	Mathematical representation
Static or dynamic	The first is described by algebraic equations or by finite difference equations; the second by differential equations
Continous or discrete time	If the time is continuous the system is described with differential equations, whereas if discrete finite difference equations will be used.
Linear or non-linear	They are both defined in terms of ordinary differential equations (ODEs); however if the system is linear its solutions will be linearly related to their inputs.
Lumped or distributed parameters	ODEs when the system is dynamic with lumped parameters and continous. On the other hand when the system has distributed parameters, is continous and dynamic the mathematical representation is through partial differential equations.
Varying or invariant time	Dynamic systems decribed by difference (or differential) equations with coefficients as functions of time. Time invariant (constant parameters) dynamic systems are also described by difference equations but with constant coefficients.
Deterministic or stochastic	Deterministic systems have fixed (non-random) parameters or inputs, whereas stochastic systems have random parameters or inputs.

establishing a mathematical framework to achieve a result that portrays the process or event in a realistic way, there are a number of factors that have to be taken into consideration. First and foremost a general understanding of how the components (structural and functional) of the process behave. Depending on these results a mathematical framework can be chosen so as to describe not only the general behaviour of the event, but also to depict accurately the evolution of all the parts. An example of the difficulty of this task is illustrated by all the options available on Table 2.1. It can be seen there are only a few limited types of mathematical representation, which can be summarized as: finite difference equations; differential equations; ordinary differential equations, and partial differential equations. Although the choice might seem easy because of the limited range of equation types, it is quite difficult if one wants to achieve an accurate representation of the system components.

After the appropriate framework has been established the model development can take two distinct, but related forms:

- a mathematical formulation of a system without all the precise information about its structure, either because it is unknown or because the modeller wants to make an approximated approach. It is then adjusted to the real system by means of sim-

ulation (or experimentation) and recursive modification, in other words by iterative comparison of the model results with real data;

- a formulation of the system involving a full mathematical description in terms of structure (kinetic data) and function (flow of matter-energy), and subsequent analysis of the relations between the structural and dynamic data of the real system.

Modelling is, then, the creation of a system through the analysis of real data. As a result the system model will comprise a set of mathematical equations that, via simulation, will generate output from an initial set of conditions. Two distinct steps must be taken to accomplish this task for models describing systems of reactions:

- the formalization of the model in mathematical terms, using one of two possible kinds of approach, given that the parameters are not distributed and there will be no need for a partial differential equation formulation (see Table 2.1):
  - a stochastic approach [129, 147], or
  - a deterministic approach [50];
- choosing a time scale for the model and deciding which components are variable and which constant over this period (autonomous).

The conceptualization of reactions in mathematical terms is dependent on the model's purpose and on the parameters under study. Casting the model closer to a real system would require the system to be defined in detail, as a microscopic reactor. Within the microscopic reactor the reactions are the result of collisions in a three dimensional space as long as the events have enough energy (the collisions are events determined by chance). This type of approach is termed stochastic [129, 147]. The mathematical description of these events includes a large number of equations, the knowledge of specific thermodynamic parameters and of the relationships between particles (laws of diffusion, potential energies, kinetic energies, reaction parameters etc) [1,85,178,181]. The output information from the simulation of such system models is large and allows an extensive analysis, but so much input information is needed that, since some of it is not yet known, assumptions have to be made. However, simplified methods, based only on the rate equations of the reactions and the calculation of the probability, can be applied, but this still implies considerable processing time, making the study of large systems almost impossible.

The deterministic approach to conceptualising a reaction can be termed macrocosmic as its mathematical basis relies on the chemical kinetic rate laws [50, 149]. Thus the mathematical description of the model corresponds to a set of differential equations. Each of these represents the dynamics of a metabolite of the system and is a combination of all the factors that contribute to the generation, or consumption, of that metabolite.

The second step to bear in mind when modelling is how the properties of the components of the system are defined, that is whether they are autonomous. This is especially important when dealing with enzyme systems, as their regulation needs to meet two types of requirements [79]: those of homeostasis and of homeoresis. The former relates to the constant production of metabolites to maintain a pattern of concentrations inside an organism. In such cases the system can be termed autonomous, since the structure and kinetic parameters are independent of the time scale, and the regulatory mechanisms in the system are closed. The latter relates to the possible changes in the enzyme parameters (V_{max} and K_m) which might occur during early forms of development, differentiation and morphogenesis. Because the processes are time dependent such a system is nonautonomous and must include an explicit time variable [79].

2.3 Simulation:

> "Feign ... pretend to ... mimic ... imitate the conditions of ... for training"
>
> Oxford Current English Dictionary, 1982 ed.

The above is the normally understood definition of *simulate*. In scientific terms, however, it is the production of results for a specific experiment by means of a model.

There are several ways of performing simulations, but the ones we are interested in necessitate the use of digital computers. Here the models, or problems, to be studied have to be coded into a suitable language for these machines before any production of results. This operation can involve the development of a computer program, or the translation of the problem into a set of instructions that can be used by an extant program.

Any simulation program normally consists of:

1. initialization;
2. the simulation itself;

3. output.

The first and third points are where interaction between the machine and the user occurs. The initialization is when the parameters of the model for a specific problem under study are set, either by reading an information file or by direct typing, and checked for any possible inconsistencies. The output consists of the information being given by the program, presented in graphical form or as a data file.

Until recently the availability of programs for the simulation of reactions was very limited and in most cases the range of applicability dubious, as the majority of these programs were developed to treat the problems their writers were interested in. Thankfully this trend has changed in the last few years and the availability of programs for deterministic models has grown (see Chapter 4).

2.4 Differential equations

As previously mentioned, the mathematical representation of a deterministic system is based on the construction of a system of ordinary differential equations (ODEs), with as many equations as the number of chemical components of the system (though this can, depending on the circumstance be simplified).

For example the chemical reaction:

$$A + B \longrightarrow C + D$$

and the equations describing the time (t) change for the concentrations of the reactants are:

$$\frac{d[A]}{dt} = \frac{[B]}{dt} = -k[A][B]$$

and the products:

$$\frac{d[C]}{dt} = \frac{d[D]}{dt} = k[A][B]$$

So a system describing the concentration changes of all the chemical componentes would have, for the above reaction, four equations that would have to be solved simultaneously.

If instead of a single reaction, let us have a look at an example containing three

reactions, such as:

$$A + B \longrightarrow C$$

$$C \longrightarrow A + B$$

$$C \longrightarrow A + D$$

For this system of reactions the system of ordinary differential equations would also contain four equations but this time a little more complex:

$$\frac{d[A]}{dt} = -k_1[A][B] + k_2[C]$$

$$\frac{[B]}{dt} = -k_1[A][B] + k_2[C] + k_3[C]$$

$$\frac{[C]}{dt} = +k_1[A][B] - k_2[C] - k_3[C]$$

$$\frac{d[D]}{dt} = k_3[C]$$

and again, for determining the concentration time profiles of the system components, these equations have to be solved simultaneously.

So, the general mathematical form of a deterministic metabolic model is:

$$\forall S_i \in System \; \frac{d[S_i]}{dt} = \sum_n k_n \prod_m [S_m^{a_{mn}}]$$

where S_i is a metabolite, k_n a rate constant and a_{mn} the reaction order. However this equation can take a even more general form:

$$\frac{dS}{dt} = N.v$$

where N is the system's stoichiometry matrix, v the rate vector and S the concentration vector.

The process of integration of differential equations normally includes the reduction of the equation to a familiar form (possibly involving variable substitution). However, due to the complexity and, often non-linearity, of the equations in biological models this is not possible, and so it is practically impossible to achieve an analytical solution (though where analytical solutions exist they are usually to complicated to use). The only possibility is

to use approximate methods, which can be graphical or numerical.

Sometimes, instead of time evolution, one is interested in determining whether there is a steady state and what the concentrations of the metabolites are at that point. A steady-state is, in Wyman's words, a metabolic wheel in a continuous turn, with steady net flow through the system. It follows, from first principles, that such sustained motion requires support from the outside. A steady-state can happen therefore only if the system is open and several or all reactions are "pushed" from outside. If the system is isolated, without such input, then it can approach only an equilibrium state, determined by the initial conditions [161]. When such studies are required the system of ordinary differential equations is transformed, there is no longer a change of concentrations through time so:

$$\forall S_i \in System \; \frac{d[S_i]}{dt} = \sum_n k_n \prod_m [S_m^{a_{mn}}] = 0$$

or,

$$\frac{dS}{dt} = N.v \; \leftrightarrow \; N.v = 0$$

and the system is transformed into a simple system of simultaneous equations that can be solved by various methods; however the mostly widely used is the Newton method [24].

2.4.1 Numerical methods

It is not unusally possible to find close-form solutions for non-linear or time-varying dynamic systems. It is nevertheless possible to find responses of fixed linear systems analytically for systems of any order, although it is impraticable with systems over 3^{rd} or 4^{th} orders. However, generally solving systems of ODEs for discrete points in time involves the use of approximation methods based on numerical solutions. Hamming [76] and Conte [24] amongst others have discussed in detail all the problems and applications of numerical solutions for systems involving ODEs.

All methods for obtaining numerical solutions involve predicting the state of the system a small time later in the future ($\triangle t$) from the current (an perhaps past) states of the system. A critical problem is the choice of the interval $\triangle t$; if this is too short, the simulation is inefficient and time consuming. In addition the large amount of calculation results in numerical errors. If $\triangle t$ is too large the predictions become less accurate. Hence the real problem is to choose an appropriate combination of extrapolation method and $\triangle t$.

The choice of $\triangle t$ becomes particularly difficult when some variables are changing rapidly, and others slowly. Then it is difficult to choose a single value of $\triangle t$ appropriate for both sets of variables. Such systems are termed "stiff" and special methods have to be used to integrate them, usually involving continuously adjustable $\triangle t$ values [24, 80, 88, 87, 127].

2.5 Stochastic Methods

From the non-mathematical point of view a stochastic process is any probabilistic process, that is, any process developing in time and controlled by probabilistic laws so that predictions of the outcome are not unique, but fluctuate randomly on successive determinations [30]. The stochastic method used to simulate systems of simultaneous reactions is the *Monte Carlo* method. This method arose during the second world war mainly for application to the study of atomic collisions [165].

The *Monte Carlo* method of simulation has been widely used in the fields of chemistry, biochemistry and biophysics. Examples of these applications include simulation of protein dynamics (changes of conformation, vibrations and relative movements of different chains in the same protein); integration or minimizing functions (e.g. the molecular energy function), and simulation of chemical reactions, which is the problem we are interested in.

The application of the *Monte Carlo* method to the problem of chemical kinetics consists of the representation of a given reaction set (it may be only one reaction, or as many as the user is interested in studying, bearing in mind the limits of the machine being used for the simulation) as a spatial distribution of the species involved in a predefined volume. To perform this action one must put aside the normal notion of concentration and consider the whole system and the species depicted within as discrete numbers of molecules or atoms. The whole problem can be understood as a representation of different species as different populations, and the changes due to a reaction are represented by the population levels [160]. The way it really works is based on the statistics of random events and the probability of their (reactions) occuring in concert, simultaneously, consecutively or repeatedly [160]. It must be clear enough that this method does not supply a way to solve a set of differential equations nor does it provide closed form rate equations [160].

Sometimes it is possible to apply a stochastic element to a deterministic system, to introduce an element of randomness into the problem to be solved. In these circumstances the term *Monte Carlo integration* can be applied.

Any stochastic simulation involves observing a random phenomenon and so, for the consistency of the application of this aproach, one must be particularly careful about the source of randomness. This is normally a sequence of numbers that have an independent uniform distribution inside a predefined range. The concept of random numbers is probably due to Kolmogorov's fundamental theorem of stochastic processes [190].

The first random number generators were based on physical processes, such as the 18th century Buffon's needle experiment, used to estimate the value of π; from census reports, as in the table of 40000 digits produced by Tippet (1927) by taking numbers at random from those reports; from electronic noise, such as RAND (1955), which was a table of a million digits produced from that source and which was used as the random input to the British "Premium Bond" [165]. However, it soon became evident that these processes had their own problems; they were prone to biases and dependencies, and ultimately, mechanical problems were detected in the process to produce RAND [165]. It was then necessary to turn to other methods to achieve a better way of producing a set of random numbers. The idea came to turn to mathematics and to use non-linear recursive schemes. A method based on the "middle square" of a given number was proposed by von Neumann [165]. This introduced a new dimension in the discussion about random numbers, as the method was based on deterministic rules so causing concern about the randomnesss of the set. Given the fact that known mathematical rules were being used for the production of random numbers, it was more appropriate to call them pseudo-random or quasi-random. A sequence of pseudo-random numbers (U_i) is a deterministic sequence of numbers assigned to a given interval (Exp: [0..1]) having the same statistical properties as a sequence of "pure" Random Numbers [165] i.e.:

- statistical tests applied would not reject the null hypothesis
- test of non predictability

A review of the several different types of mathematic random number generators can be found in the paper by Marsaglia [132] and the books by Ripley, Sedgewick and Press *et. al.* [157, 165, 174].

One more method has being developed with the science of cryptography and is based on pseudo-random acoustical noise.

Most digital computers have access to standard random number generators, but one

must be aware that different kind of generators might suit different problems, either because of their sequence length or the range of values produced, making the decision of random number generator an important one.

Chapter 3

Introduction: modelling of free radical reactions

3.1 My previous models

My experience in the application of simulation to the field of dioxygen free radicals goes back to my degree project. First Lehninger [125], then Hunter and coworkers [92–94], showed the effect of mixtures of oxidized and reduced glutathione on isolated mitochondria. Flohé later extended these studies, incorporating the action of known anti-oxidant enzymes (glutathione peroxidase, catalase and superoxide dismutase). They all observed that there was a pronounced swelling of the mitochondrion in the presence of high concentrations of glutathione or ascorbate. Flohé showed that this effect was inhibited by the anti-oxidant enzymes [40]. Both glutathione and ascorbate are known to behave as anti-oxidants but in these circumstances a pro-oxidant effect was observed. Even though it was possible with the experimental conditions to establish the framework of action of the enzymes, the mechanism that induced the mitochondrion to swell was never clear. Although the elucidation of the mechanism of action of the several anti-oxidant enzymes constituted an important development for the future of the field of dioxygen free radicals, that information proved difficult to use for establishing the possible mechanisms that lead to the swelling. Catalase and glutathione peroxidase did considerably reduce the time needed for starting lipid peroxide formation (by reducing the concentration of hydrogen peroxide) and in the process did inhibit the increase of the swelling. Some forms of glutathione peroxidase also scavange lipid hydroperoxides produced (in the presence of glutathione) which can be

considered a reparative event albeit without reversing the swelling. This was the reason why I aimed to develop a simpler model, believing an adequate computer simulation could help elucidate the mechanisms that lead to lipid peroxidation. This approach necessitated choosing a representative set of reactions and a suitable simulation method.

The choice of a suitable set of reactions is problematic for one basic reason: the extreme reactivity shown by the free radicals. For this reason the number of reactions reported to date is very large and still increasing. For the models to be simple and easy to understand the number of reactions included had to be limited, and so representatives of the four main sets of processes relevant to the problem were selected:

1. generation and free radical interconversions;
2. free radical attacks on a particular substance such as an unsaturated lipid and the consequent chain reaction (initiation, propagation and termination)
3. protection mechanisms not involving enzymes, and
4. biological enzymic protection mechanisms (e.g. with peroxidase, catalase and superoxide dismutase).

The simulation of the system was a far bigger problem - no adequate computer programs were available at the time so I wrote my own. This program was written by a non-professional programmer who was only interested in obtaining results and not in distributing software to the scientific community. The result was a computer program that was model-oriented, making its successful usage dependent on an in-depth knowledge of the program.

For each new set of reactions chosen for a particular simulation the complete set of differential equations defining the system had to be written and then translated into the computer language Pascal via a procedure which was an integral part of the program, with all the consequent fine tuning for necessary new variables and/or parameters (for example concentrations, kinetic parameters etc.) created during the translation process. The Jacobian matrix also had to be explicitly stated and coded into Pascal due to the numerical method implemented. Only once these tasks had been accomplished could the compilation of the whole program proceed. Eventually a run of the program was possible, although tests were necessary to verify whether the model implementation was correct.

Although there were programs (some spreadsheets had some in-built mathematical functions that could be used for solving very simple problems) apparently available at the time for solving problems involving systems of differential equations, such as:

- Eureka the Solver for the PC and Mac
- Teka Solver (Mac version only)
- Mathmatica (a very early version for the PC)

attempts to use any of these programs revealed limitations first in the user interface and then in the capability of the program to deal with such problems. I had difficulties obtaining any results at all with a small set of reactions, and the programs were unable to handle large sets. These problems were due in part to the lack of computer memory (in those days personal computers did not have a large working margin) and in part either to the performance of the numerical methods used to solve such models or to the choice of the methods implemented.

Last but not least the choice of parameters (i.e. concentrations, kinetic constants and simulation times) was a major source of uncertainties, in particular the kinetic parameters for some of the reactions. I had no knowledge of some of the major reviews of Dorfman (1973), Brummer (1981), Bielsky (1985), Buxton (1988) and the two of Alberta Ross (1988 and 90) [10, 14, 18, 31, 152, 153] which cover most of the known reactions in which radicals are involved and present their kinetic values. The choice of initial concentrations was made according to Flohé [40] and two different types of simulation time were chosen:

- 100 seconds in an attempt to emulate the experimental studies
- 1 second or less to mimic the initial mechanisms.

After a period of testing both the program and the model that included the fitting of unknown parameters, simulations with the full scale model could be done. This contained up to 29 metabolites, including the several radical species, lipid, chemical anti-oxidants and biological anti-oxidants (enzymes and their complexes), and 38 reactions, some of which were modelling the action mechanisms of certain anti-oxidant enzymes. The number of metabolites and reactions was dependent on how many of the blocks of processes (presented previously) were being simulated.

As mentioned above two different time scales were chosen, one with times generally longer or equal to 100 seconds which gave results that were comparable with those published by Hunter and Flohé [40,92]. The levels of lipid peroxides obtained in the simulations were of the same order of magnitude and the role the different anti-oxidants had in the system showed similar behaviour. The validity of such an approach could be contested on the grounds that it had made no further contribution to the understanding of the problem in hand. Some people would say: "Why do this if you get the same results?" The answer is that it shows that the chosen reactions are sufficient to lead to the observed processes (the model was an adequate description of the process). Also the flexibility of the approach relative to that of a physical system is a great advantage, allowing for control over the majority of variables.

The results of the simulations for much shorter times showed considerable promise. Damped oscillating behaviour was observed for some metabolites as well as a very sharp peak (corresponding to increased concentration) for the hydroxyl radical. The first was later found to be spurious, resulting from the method's inadequate numerical accuracy, the behaviour disappeared as soon as the accuracy for the simulations was improved by reducing step size. The observed peaks in the dynamical behaviour (concentration time profile) of certain metabolites, however, remained, no matter what the conditions for the simulations were, even with small time increments (defined in the previous chapter as δt). This initial peak correlated to a fast increase and then decrease of the concentration of the hydroxyl radical at the very beginning of the simulation in the presence of high concentrations of glutathione, even with the full enzymic protection present. The concentration of hydroxyl radical attained was sufficient to initiate lipid peroxidation as some lipid peroxides were detected as well (their variation showed the same sharp peak as the hydroxyl radical in the presence of glutathione peroxidase).

These remain the most striking results of this approach and suggest that the explanation for the swelling of the mitochondrium is hidden somewhere in the early processes that occur as a consequence of increasing concentrations of glutathione, before the enzymatic mechanisms have any influence. This also indicates the usefulness of this approach in finding a direction to follow when the experimental research has not yet yielded any definite answers. There is only the need for a computer (nowadays desk-top models can cope with sufficiently complex models and there is still the possibility of access to more

powerful mainframes), the right software (which was a problem at the time, but now there are many options available) and a basic knowledge of chemical kinetics and mathematics.

My initial approach had shortcomings. The first was the numerical method used. Although this was chosen specifically to cope with the problem (the stiffness resulting from the wide range of values included in the parameter set, and a subject developed in the previous chapter), accuracy had to be controlled and tested in every simulation to investigate the consistency of the results. The accuracy of this method is directly dependent on the time increments so the program user had to play with both to optimize results. This is why some observed oscillating behaviour disappeared when the time step was decreased, increasing the accuracy. A second problem arose from an unfamiliarity with some of the literature concerning kinetic parameters. However, all the unknown parameters were fitted and thoroughly tested, and, as the results indicate, they could not have been far from the real values, as was later confirmed.

Although the previous weaknesses could all be addressed, one major issue threatens to undermine the approach used. The occurrence of concentrations below $10^{-10}M$ of some metabolites of the model, such as hydroxyl radical, singlet dioxygen and some radicals in the lipid peroxidation chain, call into question the validity of the deterministic approach used and suggest that a different approach should be used for studying the initial processes of such problems.

3.2 Other models of oxygen free radical reactions

A number of previous attempts have been made at using a theoretical approach to explain the importance of free radicals, either to establish the possibility of production of certain species (*e.g* hydroxyl radical), or to set up a framework that enables the study or understanding of a process (*e.g.* lipid peroxidation).

These attempts fall into two main groups, albeit with a common theoretical basis in the mathematical representation of chemical kinetics using:

1. a rate law (a differential equation) for a single reaction,

2. a set of differential equations representing a set of simultaneous reactions.

While the first, using a rate law for a single process (**one single reaction**) to estimate the rate of production or consumption of a species involved in the process, seems to be

straightforward, it has serious limitations, to be discussed below. In this way Halliwell [71, 72] has estimated the production of hydroxyl radicals for justifying his qualitative hypotheses about its biological effects, while others have used it [111] as a foundation for a qualitative study of lipid peroxidation and its influence on the cell cycle.

The second, presented in the previous chapter as a deterministic approach (as the single reaction fundamentally is), consists of writing the whole set of equations describing the time behaviour of the system components and then integrating it over time. Several authors attempted this type of modelling with various results, and although all started with the same theoretical basis in the end they used differing approximations and assumptions which made their mathematical formulation different also [4, 111, 162, 186, 191].

3.2.1 The Single Reaction Approach

This approach is based on simple chemical kinetics and would be entirely valid for a single process occurring in a closed system. Assuming the general chemical reaction:

$$A + B \longrightarrow C + D$$

the following rate equation can be written:

$$v = \frac{d[C]}{dt} = \frac{d[D]}{dt} = -\frac{d[A]}{dt} = -\frac{d[B]}{dt} = k[A][B]$$

where k is the kinetic constant for the reaction. Therefore in the case of the Fenton reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\cdot} \tag{3.1}$$

the rate of production for the hydroxyl radical will be of the form:

$$\frac{d[OH^{\cdot}]}{dt} = k[H_2O_2][Fe^{2+}]$$

In such instances if the kinetic parameter 'k' and the concentrations of hydrogen peroxide and ferrous iron are known, the instantaneous rate of production of the hydroxyl radical can be predicted.

Halliwell and Gutteridge [71–73] used the above equation to estimate the rate of pro-

duction for hydroxyl radicals and obtained a value of $7.6 \times 10^{-11} Ms^{-1}$, given the assumption of micromolar concentrations of both ferrous iron and hydrogen peroxide[1], and $76 M^{-1}s^{-1}$ [193][2] as the kinetic parameter (which is the proposed value when Fe^{2+} is not chelated). How is this value to be interpreted? It is a rate which indicates that 7.6×10^{-11} moles (76 picomoles) of hydroxyl radical will be produced per litre per second. This seems at first to be a very small amount. However, it has to be seen in the right perspective. If the Avogadro's number (6.023×10^{23}) and the average volume for a cell (from 10^{-12} to $10^{-11} l$ for a liver cell) are taken into consideration (as Halliwell states they should be [71]) a value between 46 and 460 is obtained. This value is the number of hydroxyl radicals produced per second per cell, and has opened the doors to other researchers [3, 60, 70, 69, 107, 199] for the elaboration of hypotheses on the mechanisms of dioxygen toxicity; justification of unexpected experimental results; and, to those acknowledging how small this number is in terms of cell contents, attempts to devise other methodologies for studying the mechanisms of dioxygen free radical reactions that can be highly localised.

While this approach appears to be very simple and direct it has several limitations with potential for errors. The equation for the change in hydroxyl radical concentration is defined as an instantaneous rate. What does this mean? In a single reaction products and reactants are always changing their concentrations (unless the reaction is at equilibrium), so the rate is dependent on the instant of time at which it is calculated, and therefore cannot be considered a constant.

It is, moreover, difficult to accept that the Fenton reaction occurs in isolation. Neither the original authors, nor subsequent workers, have taken into account the other substances which would be competing for the hydroxyl produced, for example the rapid reaction of hydrogen peroxide with hydroxyl radical which would probably use most of the radical that would otherwise be available to initiate other processes. The way Halliwell calculated the value would only be acceptable if the system were at steady state and closed, which is not possible because a system at steady state is open.

The rate of change for any substance in a system is a result of all the simultaneous

[1] Volkov and Lebedev (unpublished results) use the same approach as Halliwell as a process of including a source of hydroxyl radical in their model. However they assume a different concentration for both metabolites (in the millimolar range) and include in the model the same value obtained by Halliwell for the rate of hydroxyl production, which is wrong.

[2] This is the published kinetical value for the Fenton reaction with the iron not chelated. Babbs and Steiner use a different number which corresponds to the reaction with the iron chelated ($3.2 \times 10^5 \pm 5 \times 10^5 M^{-1}s^{-1}$).

or coupled reactions, consequently the full rate equation for a certain substance will be the sum of all the rate laws that contribute to its production and consumption. The concentration of any chemical substance X in the system can be defined as:

$$[X] = f(t, v_i, .., v_n, ..., X, Y, Z)$$

where the concentration of X is a function of time (t), the rate laws of the reactions involving X ($v_i, .., v_n$) and all the other components of the system (Y, Z). From this point of view, the single reaction approach is trying to isolate something (X) that cannot be isolated.

A natural consequence of this definition is that all the other components of the system (metabolites) are defined the same way, the only exception being for those metabolites that are to be considered external, that is, those metabolites that will behave as sources or sinks to the system and so will emulate the effect of constant pools without suffering changes of concentration. The effect of this is to open the system to its neighbourhood allowing for flow of mass; however a far more important result of this imposition is that other types of studies can be undertaken, such as steady state analysis.

As opposed to the use of the single reaction approach by some researchers, there have been some other groups who used a suitable method for handling a model containing a set of simultaneous reactions, which will be discussed in the following section.

3.2.2 An integrated approach (deterministic)

Mathematical formulation

The differences between an integrated approach and the single reaction approach are based on the fact that the former tackles the criticisms directed at the single rection approach, as described previously. Instead of isolating a reaction and a named metabolite from its system all of the elements composing the system (metabolites and reaction) are used for constructing a set of equations which will be of the form:

$$\forall S_i \in System \; \frac{d[S_i]}{dt} = \sum_n k_n \prod_m [S_m^{a_{mn}}]$$

where S_i represents a metabolite and $[S_i]$ its concentration; k_n the kinetic constant for reaction n; a_{mn} is the kinetic order of metabolite S_m in reaction n. The sum represents

the balance between the production and consumption of metabolite S_i such as

$$\frac{d[S_i]}{dt} = \sum_n production - \sum consumption$$

These equations can still be written in matrix form which conveys the idea of integration much more easily as the whole system topology can be represented by its stoichiometry matrix N and the kinetic information by the rate-of-reaction vector v thus:

$$\frac{d[S]}{dt} = N.v$$

This equation represents the basic mathematical framework describing the changes in concentration for all the system metabolites. This basic set can be developed further by:

1. Including the description of special system properties. Alterations would embrace the editing of the actual set of equations or the addition of other formulae which will emulate specific processes. The first accounts for the introduction of external metabolites, also known as constant metabolite pools, either because they were irrelevant to the overall behaviour of the system or because of the need to define a directional flux from a source to a sink. However, the major effect of such alterations is to simplify or reduce the set of equations.

2. The second type of alterations, normally undertaken with the use of "forcing functions" that are time dependent, can be used for emulating two different effects, which depending on the parameter affected, can be either a modulation or a perturbation. The former is achieved by altering the concentration of an external metabolite either for just an instant of time or for longer to establish a desired behavioural pattern (sinusoidal for instance). When the parameter affected is any of the internal metabolites then the observed effect is a perturbation of the system, and can be used for testing its stability (or for investigating, by emulation, the system response to certain known physiological changes). It is important to note that alterations to the rate laws, or definition of new ones, cannot be considered in this group, as they are part of the system and dealt with in the rate-of-reaction vector. It is true that the modeller will in certain situations create new rate laws, because those provided by the programs in use are not satisfactory or sufficient, but they do not represent an

extension to the basic framework defined above.

3. There is an important type of adjustment sometimes made to the rate laws so the model can account for a non-uniformly distributed system. Such an adjustment is made to avoid changing the mathematical basis of modelling as defined above. A partial derivatives formulation rather than an ODE should be used when modelling heterogeneous systems, that is, when the diffusion effect is an important factor; as a result the numerical method for solving the set of equations (an important consideration regarding the availability of computer programs) also has to be changed.

4. A totally different type of alteration from that defined above can still be conceived; however, that will leave the system in a different form. As mentioned in the previous chapter, when the system is open to a directional flux a specific state can be achieved, that is even when the reactions are still "working" but the net fluxes at the several nodes of the system are zero. The system in this situation is said to be at steady-state, and, as mentioned in the previous chapter, to investigate this situation it is necessary to reduce the equations composing the system to $d\mathbf{S}/dt = 0$ which transforms the initial ODE problem into a system of non-linear equations. The data that can be collected from such studies is different from that of the ODE formalisms because the dynamical behaviour is lost. The data corresponding to the steady-state can be seen as the final values of the dynamics of the system, so these can be obtained using the differential equations when the simulations have a sufficient time variable, but the opposite is not achievable.

Review of previous implementations

Considering the amount of information that can be gained by attempting computer simulation of models under experimental study it is rather surprising to find that there has not been much computer modelling done in the field of dioxygen free radical reactions. Although the methods and techniques for studying free radicals have greatly advanced from the revolutionary work of McCord and Fridovich in 1969 [138] to the book published in 1991 by Rice-Evans *et al* compiling the currently used "Techniques in free radical research" [163]; a considerable amount of controversy still seems to exist not only concerning how experiments are set up but also about how they are interpreted [38, 67, 72] (with disagreement over the attribution of the toxicity roles to the several species possibly

involved), leading to accusations that some results were artefacts [72, 99, 100] (problems related with the extrapolation of *in vitro* results to *in vivo*). These problems are caused by a combination of technical difficulties and the properties of free radicals which *per se* would constitute a perfectly fertile ground for a theoretical approach. Rice-Evans *et al* have recently [163] acknowledged not only the existing technical obstacles but also the care and the proper planning required because of the high-cost instrumentation needed for some specialized techniques when they state that, "free radical species are generally short-lived ... and thus direct measurement and identification are often impossible" [163]. This is just the kind of situation where the aid of computer modelling and simulation providing the exploratory work for a project would mean that the planning of experiments could be more cost effective resulting in financial savings (a very important factor when the current economic situation is taken into consideration).

The use of computer simulation to aid the understanding of biological problems already has a long history spreading over many areas of research, as pointed out in the previous chapter, so it is surprising that it has only recently been extended to the field of free radicals. To be fair, some work was published during the 1970*s* which was concerned both with the mechanisms of combustion (involving both the production and consumption of dioxygen free radicals which have extremely fast kinetics [29, 15, 160]) and the theoretical (or conceptual) problems of how to simulate them (related to the concentration levels and their implications [54]). These are the first examples of how to use a stochastic approach when dealing with reactions with fast kinetics. Even though the work was not directly related to the biological involvement of free radical reactions, it provided a potential framework for dealing with such problems and, with the right kind of application, could have been used for modelling and simulating specific biological systems.

Bartholomay in 1962, Jachimowski *et al* in 1964 and Kibby in 1969 did make an attempt to illustrate the possibility of applying such methods to biochemical models [7, 96, 106], although without success. This stochastic modelling approach was intended to overcome the problems encountered during the simulation of free radical models and, as it constitutes per se a whole new section of this thesis, it will be discussed in more detail later in connection with my application of it to biological free radicals (Chapter 6). The use of this type of approach was the original intention at the start of this project (because of the problems detected with my models prior to the beginning of this project, specifically

those related with the concentration of some radicals in the system). Only the technical difficulties encountered forced a further development of the deterministic approach.

It is important to stress at this point that the final form of a model is not just the end product of the several stages or approaches to modelling but that it is highly dependent on the background of those doing the modelling and, to a certain extent, of those who will execute the simulations. There will always be a strong 'subjective element' to any model, contrary to any impression that this is not the case suggested by the presentation of the mathematical frameworks underlying the models attempting the study of system dynamics. This point becomes more apparent if the different ways in which a certain pathway or mechanism can be specified is considered, as well as, the mathematical complexity of the rate laws governing those processes.

For these reasons classifying the various previous models was found to be extremely difficult. All the the research dealing in some way or another with the biological role of dioxygen free radicals is normally found to be concerned with one of the many different possible aspects of their biological impact.

Although I will introduce here all the relevant work found, I will refrain from presenting it in depth as most of the information gathered will be covered in a critical comparison in Chapter 5, along with my own findings.

The reason why all the possible factors involved in model development were emphasized above will be better understood when the existing models are reviewed and compared with that presented at the beginning of this chapter (under the heading of "My previous models"). The task of evaluating all these models constituted a definite problem as it was very difficult to compare their general properties, their composition and the mathematical methods used for simulating such varied models involved with modelling problems dealing with free radicals. After careful examination of all the models it was possible to identify a set of rules that could be used as a criteria for comparing the reviewed work and my own model. These rules can be divided into three major groups under the headings of model composition and the modelling and simulation techniques. The first, model composition, comprises eight items considered necessary to characterize the models in respect of the reaction composition, including specially the source and the interconversion reactions of free radicals used; which type of dioxygen free radical damage is being studied; which chemical and enzymic anti-oxidants are present; which species are being monitored; the

final number of metabolites and reactions in the model; and the biological counterpart (the metabolic process that the modeller is attempting to emulate or study by choosing such a set of reactions). The second and third criteria, the modelling and simulation techniques, were not split into further items. This fact is justified specifically by the lack of information provided by the modellers regarding the modelling and simulation techniques used. The references reviewed showed little or no interest whatsoever in covering these two important steps, probably due to lack of available space. Closer analysis of the set of rules chosen for comparing the models, and of the tables, will reveal a couple of points that are not included, namely the data the models were based upon (kinetic parameters and metabolite concentration), nor its respective sources; and the type of simulation used (performed). Only the species monitored, which could be considered one of the several parameters normally included in the necessary specification for the simulations, is reported in the tables. Also not included is information on which of the two types of simulation, steady state analysis or dynamical behaviour, was performed, and, in the case of the latter being chosen, what the simulated time was.

One other point to take into consideration is that, at this point, I am only interested in presenting the models that have been used for studying oxygen free radical reactions. I do not intend to discuss in great depth the work that has gone into their development, i.e. the simulations performed as part of choosing parameters (kinetic constants, metabolite concentrations, simulation settings) for the model, nor the results obtained. These will be, as mentioned elsewhere, discussed at a later stage along with my own findings.

Because some specific comments need to be made about each model the data will be divided into six tables, one for each model reviewed (five in total) and one for my previous model (Table 3.1) which has already been presented and analysed at the beginning of the current chapter.

Those with an interest in the field of simulation all have in common a belief that modelling can be used as a means to achieve an integration of all the experimental information available in the literature. Tappel's group were the first to show awareness of this fact by conceiving a simple model representing the major events involved in lipid peroxidation using an integrated approach [191]. The reasons for choosing both this process and approach were firstly that lipid peroxidation is a well established process with a reasonable wealth of information on its possible mechanisms and kinetical parameters; and secondly that a

		Model Reviewed
		Moniz-Barreto
Model composition	Generation system	None: a constant supply of peroxide and the superoxide anion was assumed
	Radical conversion	Yes
	Radical damage	Lipid peroxidation
	Chemical antioxidants	Vit-E, GSH
	Enzymic antioxidants	SOD, Catalase, Glutathione Peroxidase
	Species monitored	Hydroxyl radical and Hydroperoxides including $L^{\cdot}$ and $LOO^{\cdot}$
	Number of metabolites and reactions	29 38
	Biological Counterpart	The study of the mitochondrial swelling in presence of high glutathione concentrations and the role played by the enzimic anti-oxidant defenses
Modelling technique		Description of the system in the ordinary differential equation format. No steady-state aproximation used.
Simulation technique		Built own Pascal program implementing a semi-implicit third order Runge-Kutta as the integrating numerical method

Table 3.1: General characteristics and properties of my model.

		Model Reviewed
		Tappel *et al*
Model composition	Generation system	Cyt P450 system Halogenated hydrocarbon inducers
	Radical conversion	No, they are implicit to the model
	Radical damage	Lipid peroxidation
	Chemical antioxidants	Vit-E, GSH
	Enzymic antioxidants	Glutathione Peroxidase
	Species monitored	Hydroperoxides (LOOH) with emulation of the TBARS method
	Number of metabolites and reactions	10 (8) 7
	Biological Counterpart	The study of the lipid peroxidation process
Modelling technique		A simplified set of equations describing the change of concentration of all species in spreadsheet format
Simulation technique		Use of a spreadsheet program (Excel)

Table 3.2: General description of the modelling and simulation characteristics of the model from Tappel *et al* [191]

simulation program was thought to be a way of synthesizing data from a wide range of experiments to transform what normally are variables in experimental systems into simulation parameters that can be fitted [191]. This process can be used for the prediction of results or planning of experiments, an important issue that not only this group but the majority of the groups reviewed failed to promote.

The development of Tappel's model can be said to have had three distinct stages. In the first they identified the major components of lipid peroxidation, including both the substrates and processes (reactions) involved. In the second they combined all these components, achieving a pictorial representation of the whole process so it could be translated into a spreadsheet model containing the description of the information network. In the third all the parameters (kinetic constants, metabolite concentrations etc) taken from the literature, when available, were included (initialized) and simulations performed to find and fit those missing.

In the case of Tappel's model the kinetic information does take a form that is different from all the other models reviewed in this section. To fully understand what they did it must first be noted that they advocated, when working with simpler (not necessarily smaller) models, establishing quantitative cause and effect relationships between parts of

the model [191] to gain insights into how all the parts of the model work and contribute to the overall result, rather than trying a more exhaustive approach to modelling. For this reason they aggregated suitable parts of the mechanisms under study obtaining a set of overall reactions that corresponded to a general description of the process rather than a mechanistic one. All the other modellers reviewed here chose to declare explicitly the set of chemical (or enzymatic) reactions describing their processes under study. Tappel *et al* define their model in such a way that implies that it does not use real reactions. As a result of this they cannot simply apply the experimental values found in the literature – forcing them to fit the model, through trial and error , until they obtain values which are closely related to experimental data but which also work within the system.

Tappel focusses his system around LPX with no specific chemical reactions (and as a result no block type separation, unlike my work); his system has lipids, two types of activators/inducers of peroxidation (enzymic(CP450)/chemical), then the chemical anti-oxidation in the form of Vit-E and GSH (it is unclear whether he uses GSH directly or with the enzyme), and the enzymic anti-oxidation in the form of GSHPx (no inclusion of SOD or Catalase or other Peroxidases), and finally the LOOH leftovers tranformed into thiobarbituric acid-reactive substances (TBARS).

Another interesting issue that arises from their work is their exercise of judgement in establishing a rapport between the model, the simulation and some relevant publications on lipid peroxidation. In other words, despite the model design being based on a wide network of information, the simulations were directly compared with three chosen experimental references [43,194,195]. In order to aid this comparison Tappel *et al* introduced an additional step to the model emulating the experimental determination of hydroperoxides by the thiobarbituric acid-reacting substances method (TBARS) [191]. As a result, unknown parameters could be fitted directly to the published experiments. Although useful information was gained from their approach and their implementation has heuristic value (as an illustration of simulation using widely available tools) there are certain aspects that were overlooked but that due to their relevance to the development of my work, I will examine at a later stage.

Babbs and Steiner [4] were the next to realize the potential of computer simulation not merely as an aid to the experimental approach, like Tappel *et al*, but also a tool for research in this rapidly growing field. As I have argued myself they pointed to the intrinsic

		Model Reviewed
		Babbs and Steiner
Model composition	Generation system	Xanthine oxidase system ($O_2^{\cdot-}$) Hypoxanthine oxidase ($O_2^{\cdot-}$)
	Radical conversion	Yes, all radical species are present Fenton reaction used for hydroxyl production
	Radical damage	Lipid peroxidation (LH $\rightarrow$ LOOH) Protein damage (RH $\rightarrow$ ROOH)
	Chemical antioxidants	Vit-E, GSH
	Enzymic antioxidants	Catalase and SOD
	Species monitored	Hydroperoxides (LOOH). These can derive from arachidonate, linoleate, oleate, methyl or unspecified.
	Number of metabolites and reactions	32, although a table indicates only 27 32 but the full model included up to 109
	Biological counterpart	The study of the first few minutes of reoxygenation after ischaemia
Modelling technique		Description of the system in the ordinary differential equations format with parameter separation (steady-state aproximation for fast reactions)
Simulation technique		Built own C program with two different types of numerical methods (Gauss-Seidel for steady-state and Euler for ODE's)

Table 3.3: General description of the modelling and simulation characteristics of Babbs and Steiner's model [4]

technical challenges of studying oxygen free radicals either *in vivo* or *in vitro*. In particular, they rightly noted the extremely low concentrations of some of those radical species and their extremely short life spans [4], that result in experiments becoming increasingly more challenging technically, and they advocated addressing such problems with suitable tools [4] such as modelling and computer simulation. One of the stated advantages was that the model framework would allow the study of the time profile of any chemical species belonging to the system, establishing its involvement in the whole process. This included species like the hydroxyl radical that would otherwise be extremely difficult to determine experimentally.

Compared to Tappel *et al*, Babbs and Steiner make more connections to modelling and simulation work that has been done before, even though not specifically concerning the biological involvement of free radicals. For example, Tappel *et al* only refered to some simulation papers in other biochemical applications, whereas Babbs and Steiner mentioned other attempts to model and simulate the action of free radicals (combustion flame mechanisms and photochemical models) using appropriate methodologies. However I would argue that both sets of authors chose to devise their own modelling methods that were in some respects (as it will be shown in Chapter 5) inferior to those used in the papers they cited.

Babbs and Steiner argued that although the implications of oxygen free radicals had been widely covered in the literature, the specific importance of certain radical species had not been properly established. They stated that the results of work from multi-varied experimental approaches indicated the possible roles of free radicals in such processes as lipid peroxidation, reperfusion injury, and so on, but that no consensus on specific mechanisms appeared to exist. They proposed that computer simulation could, in this case, provide a means of answering these questions [4].

Whereas Tappel *et al* built a model around a specific process to gain insight into its parts (lipid peroxidation), Babbs and Steiner created a model which *a priori* is not specific for any particular biological process, and, although they were particularly interested in studying reperfusion injury, they designed their model around oxygenation damage to membranes. Whereas Tappel *et al* had based their model on a generalized description of the overall steps in lipid peroxidation Babbs and Steiner started from actual chemical reactions which may or may not be linked to the specific biological process under study.

They selected those reactions thought to describe the mechanisms of dioxygen free radicals involvement in such processes as reperfusion injury (an oxidative stress situation). Although they could have included a large number of reactions to emulate the damaging effects to several biological structures, they focussed only on lipid peroxidation. Such a task is not trivial in the light of an ever increasing number of publications reporting new theories of dioxygen free radical involvement in other processes and proposing different mechanisms and reactions. Overviews of all the available reactions, illustrating of how difficult the selection process is, can be found in the reviews of Dorfman, Brummer, Bielsky, Buxton and Ross [10, 14, 18, 31, 152, 153]. To a certain extent this task can be simplified if the choice is made by selecting first those reactions that already have published values for their kinetic constants. If the model is still unsatisfactory it can be completed with those missing reactions and unknown parameters which can, in the same fashion as Tappel's, be fitted later during the simulations.

In contrast to the spreadsheet method used by Tappel *et al*, Babbs and Steiner wrote their own computer program embedding the model in the same fashion that I had. They translated the full set of reactions into a suitable programming language (in the form of C routines) and added the other C routines necessary for numerically solving the system. Their program should be classed as model dependent like mine, though in their program it is possible to select subsets of reactions for a simulation. This apparent flexibility by coding a large reaction set (109) into the program, but leaving the selection of the reactions for the simulation to the user in the form of an input file. This file contains a zero or a one (a flag) for each reaction and so designates the current system chosen for simulation. Adding extra reactions would, however, require alterations to the program.

Although Babbs and Steiner chose what they considered to be the most suitable integration from those available and coded it into the program, their choice does constitute a major source of criticism and will be discussed again at a later stage.

On the other hand Babbs and Steiner were aware of the problems that this type of model raises. They were dealing with sets of reactions that in normal circumstances occur in different cellular compartments: the lipid peroxidation chain reactions take place in the membrane but the location of the other reactions will depend on where the free radicals are produced or even on the localization of some enzymatic mechanisms known to be sources of free radicals (*i.e.* radical anion superoxide). If the system is assumed to be

homogeneous, an important simplification is being made, and this should be acknowledged and studied so that its impact in the model can be investigated. Babbs and Steiner were the only group to face this question, and they found a shortcut to overcome this so-called problem[3] without a drastic change in the mathematical formulation of the model. They introduced a factor in the kinetical equations which describes the partition coefficient of each of the species present in the system between the phases.

Like all the other groups (except Suzuki and Ford who I will discuss later), they used lipid peroxidation as the biological process consuming the free radicals (though some other groups also included some chemical or enzymic protection mechanisms). The concentration of peroxides throughout the simulations was monitored and its levels compared with those obtained experimentally.

The 109 chemical reactions of the Babbs and Steiner model are divided in similar blocks to mine, that is:

- generation and free radical interconversions;
- free radical attacks on a particular substance such as an unsaturated lipid and the consequent chain reaction (initiation, propagation and termination);
- protection mechanisms not involving enzymes, and
- biological enzymic protection mechanisms (e.g. with peroxidase, catalase and superoxide dismutase).

The exception is (I) the source of superoxide in the form of xanthine/hypoxanthine. They have (II) radical interconversion (not totally identical to mine as they put initiation and propagation of chain reactions together; including the production of OH radical and its attack on lipids), then (III) the chemical anti-oxidants in the form of Vit-E (they name it AH, as Remacle *et al* did) and GSH, and the enzymic protection (IV) in the form of only SOD and catalase. They have elaborated the chain reactions thoroughly with a wide range of reactions for chain termination as well as initiation and propagation. Due to their creation of the two compartment model they also had to decide on the values of the partition coefficients for all of the component species of the system. They confined

[3]It is important to remember what are the consequences of modelling non-homogeneous systems: the need to consider second order derivative type of mathematical framework and different numerical methods for its solving.

		Model Reviewed
		Remacle *et al*
Model composition	Generation system	Univalent reduction of dioxygen ($O_2^{\cdot -}$) Xanthine oxidase system ($O_2^{\cdot -}$)
	Radical conversion	Yes, all radical species are present Fenton reaction used for hydroxyl production
	Radical damage	Lipid peroxidation H_2O_2 inhibition of SOD LOOH inhibition of Glutathione peroxidase
	Chemical antioxidants	Vit-E
	Enzymic antioxidants	Glutathione Peroxidase, SOD, Catalase
	Species monitored	No time course graphs. Stability studies made by monitoring Hydroperoxides (LOOH) its derived radicals and the enzyme activities
	Number of metabolites and reactions	6 (with additional 12 as parameters) 16
	Biological Counterpart	The study of the relantionships between the pro-oxidant substances and the anti-oxidant enzymes
Modelling technique		Transcription of the model into a ODE system. Steady-state aproximation ($\frac{dS}{dt} = 0$) determination of the eigenvalues using the Routh-Hurwitz criteria.
Simulation technique		Not known

Table 3.4: General description of the modelling and simulation characteristics of the model from Remacle *et al* [162]

the lipid species (LH and its derivatives) and the antioxidant AH (Vit-E and its radical derivatives) to the lipid compartment; and all the other components, except $OH^{\cdot}$ H_2O_2 and O_2 which can be equally distributed between the two compartments if present, were confined to the aqueous compartment. A consequence of these decisions was that much greater care has to be given to the definition of the kinetics in order to avoid the occurence of impossible reactions (between immiscible species) and to include the correction of the concentrations.

Remacle *et al* followed in 1992 with another computer simulation application developing a model that can be considered a hybrid of the previous two [162]. It was much more simplified than Babbs and Steiner, but not to the extent of Tappel's. It did not contain as many reactions as the former (it only had 16 compared with the 109 included in the general model of Babbs and Steiner) but its mathematical formulation followed the

same methodology, although the compartmental question is not addressed. While Tappel *et al* designed his model around lipid peroxidation (where lipid peroxidation is again one major component for the investigation of the biological action (impact) of dioxygen free radicals), and Babbs and Steiner around four major groups of free radical reactions (described above), Remacle *et al* focused the design of their model on the balance that should exist between the production and destruction of dioxygen free radicals. The central free radical species for this process were considered to be the radical anion superoxide, hydrogen peroxide, hydroxyl radical, ROOH, ROO radical and R radical and, consequently the mathematical system was written with these species in mind. The reasons behind such a small selection were not only to keep the system simple but were also based on the relative differences in the relaxation times of free radicals compared to other species present in the system. In other cases (iron ions for example) they were assumed to be stable enough to be considered constant. The full set of chemical species is, in this case, split between two groups, one containing those mentioned above with their concentrations described by the differential equation framework (variables); and the other containing all the remaining chemical species with concentrations that were kept as constants (parameters) – falling into the same category as the kinetic constants and other parameters necessary for a full description of the model.

Although the models of Remacle *at al* and Babbs and Steiner might appear to be assuming the same behaviour (steady-state approximation) for the existing free radicals, Babbs and Steiner varied between fast and slow changing (radicals and non-radicals) with a mathematical parallel in the steady-state assumption for the radical species and normal ODEs for the rest. Remacle *et al* considered all the non-radicals as remaining constant (which means that they are of no interest to the system) and focussed on the radical species where, in the end, a steady-state assumption was also made (it is like having a magnifying glass on Babbs system). While both Tappel *et al* and Babbs and Steiner gave lipid peroxides a central role for the output (or even further in Tappel's with TBARS), in the case of the model from Remacle's group they are taken as just another variable of the system and are used for stability studies. The full set of reactions could still be divided into three sets: the first (I) can be seen as the production and interconversion of dioxygen free radicals; the second (II) as the lipid peroxidation (viewed very simplisticly, neither very detailed nor thoroughly described); and the third (III) as including both the enzymic

and chemical anti-oxidant protections. The enzymic protection included for the first time all the three main enzymes namely catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPX). Remacle *et al* went even further, where Tappel *et al* and Babbs and Steiner only modelled the damaging effects of dioxygen free radicals on membranes they also emulated some effects that the radicals can have on the enzymes present in the system. These effects are inhibitory and are executed by incorporating a factor into the kinetical equations of SOD (hydrogen peroxide inhibition) and GPX (hydroperoxide inhibition).

One final comparison with the previous two applications is the apparent lack of interest Remacle's group has concerning the concentration time profiles for all the variables defined (see above). Remacle *et al*, as stated above, developed the model based around the free radical species present without commenting on the results obtained for their concentrations or on the role played by some of the most discussed radicals (*e.g.* hydroxyl radical). Their main focus was the role of the anti-oxidant enzymes and the possibility of establishing a parallel between their activity levels and physiological effects. Building a qualitative framework with all the available data made it possible to compare with known physiological conditions, and to establish a pattern of the efficiency of the three anti-oxidant enzymes and the stability of the system according to the concentration levels found. In this situation it is also possible to predict if the system is close to a stress point.

Remacle's group model is the most complete for the dioxygen free radical production. Although the xanthine/hypoxanthine oxidase system is not included, despite being considered by Babbs and Steiner and later by Suzuki and Ford as the biological source of superoxide par excellence, they have emulated it with two different steps, one for the one electron reduction and the other for the the two electron reduction (which emulates the action of other oxidases).

The last application to appear in the literature was that of Suzuki and Ford [186] where once more both a different type of modelling and of simulation were used. The central point for this study was also different from that of the previously reviewed authors. Suzuki and Ford were innovative in the way that they defended and justified the use of a theoretical application to illustrate the possible misconceptions in the field of dioxygen free radicals. Whilst all the previous models were designed with a very strong basis both in the data and the knowledge provided by years of research in this field, and also having specific physiological processes in mind, Suzuki and Ford distanced themselves from all this, at

		Model Reviewed
		Suzuki and Ford
Model composition	Generation system	Xanthine oxidase system (O_2^-)
	Radical conversion	Yes, all radical species are present Fenton reaction used for hydroxyl production
	Radical damage	$OH^{\cdot}$ affects all the steps in an hypothtical biological pathway, whereas O_2^- inhibits only one (rate limiting step).
	Chemical antioxidants	None
	Enzymic antioxidants	None
	Species monitored	None. The flux through the pathway is the data provided in the presence and absence of the radicals.
	Number of metabolites and reactions	20 28 (although this is unclear)
	Biological Counterpart	None. Purely theoretical model (except for the radical source) designed to support the superoxide theory of oxygen toxicity.
Modelling technique		The complete model was assembled using the theory of Network Thermodynamics (in the form of a circuit diagram)
Simulation technique		Use of Spice 2 program (Simulation Program with Integrated Circuit Emphasis, vers. 2) running on a VAX 8650

Table 3.5: General description of the modelling and simulation characteristics of Suzuki and Ford's model [186]

least partially, and concentrated on designing a hypothetical biochemical process which could be affected by free radicals, with the intention of studying the efficiency of both hydroxyl radical and radical anion superoxide [186]. The choice of a theoretical pathway was justified on the grounds of generalization and, because it is also much easier to model such a simplified pathway. The contact with reality was made in the form of a small block of reactions that emulated the possible production of radical anion superoxide and its interconversion to the other species.

Suzuki and Ford defended the idea that the toxicity of a certain species is not just a reflection of its reactivity but is also dependent on several other factors, such as: the concentration levels attained by the species (it is not sufficient to be highly reactive if there is none available); and, their most important point, the specificity of the toxicity (there is no point in having an indiscriminate toxicity, due to high reactivity, if this is not affecting central points of the metabolism – which they called controlling steps). It was for these reasons that they chose to start their modelling from a theoretical/mathematical approach – by proposing a hypothetical pathway in which all the characteristics known to be necessary to describe the behaviour of the two species could be incorporated. In this way they cannot be criticised concerning missing links. Having a totally theoretical model this problem is avoided as all the links can be said to be included in a certain step.

Whilst the previous models aimed to address particular physiological problems, Suzuki and Ford's model was not an attempt not only to understand the problem of the reactivity of dioxygen free radicals, but also to deal with their toxicity in general. They presented an evolution of the ideas on radical anion superoxide and how the initial assessment of the importance of this species changed in favour of the hydroxyl radical (this is also developed in this thesis as another argument in favor of modelling and simulation). Like myself, they presented evidence for the reactivities of both the superoxide and hydroxyl radical and discussed one other question not yet raised by any of the other authors: the balance that must exist between between reactivity and diffusibility. This question had been raised previously by Pryor [158, 159] who said that any hydroxyl radical produced during an oxidative stress situation would readily react randomly in the close vicinity of its production. This fact has been acknowledged by most authors in the field who nevertheless still argue in favor of an important role for the hydroxyl radical in dioxygen free radical toxicity, based on the experimental evidence. It is the lack of "hard" evidence proving not

only the involvement but also the production of that radical which has created so much discussion over the years, and has led to the development of theories (not yet proven) for continuing to believe in the involvement of hydroxyl radical in dioxygen toxicity. This had created the ideal conditions for a theoretical approach and it is at this point that Suzuki and Ford stepped in. Their final aim was to show that there was no reason for supporting the hydroxyl radical as the major toxic species and they proposed as the central species the radical anion superoxide (meaning that they advocated a return to the initial theory explaining dioxygen toxicity).

The simplest pathway they suggested was a linear sequence of reactions with first order kinetics, the number of those steps being arbitrarily set to 10. This represented any metabolic process transforming the initial species "A" ultimately to species "K" (although they did not specifically designate this one) via a linearly ordered chain of events. One source of ambiguity is the fact they started by nominating the letters as hypothetical biological events but end by using them as hypothetical metabolite names.

To include in the model the differing characteristics of both the radical anion superoxide and the hydroxyl radical they first nominated one of the steps in the aforementioned pathway as a "rate limiting step" (their term). This was achieved by, again arbitrarily, attributing equal values to all the first order kinetic parameters except to that of the step chosen as controlling, which had a lower value than the others. The controlling step was also the one chosen to be the only one affected (inhibited) by the radical anion superoxide, representing both the specificity of the radical's toxicity and its inhibition of a central part of the metabolism. To represent the general toxicity of the hydroxyl radical they allowed this species to affect (inhibit) all of the steps of the pathway without exception. The inhibition was assumed to happen when one of the radicals reacted with any of the pathway components, with a pseudo-first order kinetics, producing one single inactive species that accumulated in a pool without further ramifications (in effect this was considered an external metabolite). The overall effect the radicals had on the pathway was to draw their components away.

The different reactivities of these two radicals could be represented by differing values of the kinetic constants, so Suzuki and Ford assumed that a difference of two orders of magnitude between the interactions of the two radicals would suffice, with $10^7 M^{-1} s^{-1}$ for the reaction involving radical anion superoxide and $10^9 M^{-1} s^{-1}$ for all the reactions wtih

hydroxyl radical. The rest of the model, as mentioned before, was based on reactions taken from the literature with their corresponding kinetic values. To avoid introducing more arbitrary values for the hydroxyl radical or the radical anion superoxide (O_2^-) concentrations Suzuki and Ford included in the model the necessary reactions for radical production and interconversion. They started with a known biological source of radical anion superoxide, also used by Babbs and Steiner, in the form of the xanthine/xanthine oxidase system (in fact they use the aldehyde oxidase EC 1.2.3.1) which was linked to reactions producing hydrogen peroxide and ultimately hydroxyl radical in the presence of iron ions.

A recent paper by Volkov and Lebedev dealing with the possible involvement of oxygen free radicals with the cell cycle timer [111] also used a theoretical approach. The details of the model and the approach used can be found on the Table 3.6.

To summarize these studies:

- All but one refered to previous applications of modelling and simulation, but all in different fields, with Tappel *et al* criticising non-specifically the lack of uniformity of modelling standards and how results are presented. Babbs and Steiner cited the previous use of modelling and simulation approaches to problems involving free radical reactions but in fields such as flame chemistry, photochemistry and environemtal chemistry. Suzuki and Ford refered to the work of Babbs and Steiner as a proof of the usefulness of mathematical modelling and computer simulation in free radical research; however they used a new technical approach to the modelling and simulation (Network Thermodynamics). However, Volkov and Lebedev do not refer to the existence of any previous applications.

- All refered to the use of a simulation approach as the means of investigating a particular problem that is difficult to understand experimentally due to the chemical properties of free radicals such as: lipid peroxidation (Tappel *et al*); reoxygenation or reperfusion injury (Babbs and Steiner); superoxide theory of oxygen toxicity (Suzuki and Ford) and lipid peroxidation as a cell cycle timer (Volkov and Lebedev).

- The work of Suzuki and Ford and Volkov and Lebedev can be said to have an essentially theoretical approach - being a theoretical attempt to prove the different reactivities of $OH^{\cdot}$ and O_2^- without citing a specific experimental parallel.

The models reviewed above and summarised in tables 3.1-3.6 form the bulk of the simu-

		Model Reviewed
		Volkov and Lebedev
Model composition	Generation system	They assume 2% continuous production of (O_2^-)
	Radical conversion	No, they are implicit, being ultimately concentrated in one rate of radical initiation
	Radical damage	Lipid peroxidation via the action of hydroxyl radical and a Fenton-like reaction occuring between the lipid hydroperoxides (LOOH) and Fe^{2+}
	Chemical antioxidants	Tocopherols in general
	Enzymic antioxidants	Glutathione peroxidase implicitly (without inhibition)
	Species monitored	None in particular. Parameter analysis is the main objective. They do present a selection of time courses.
	Number of metabolites and reactions	7 (with another two being considered as constants) 17
	Biological Counterpart	Lipid peroxidation as a possible cell cycle timer
Modelling technique		The model was first written in terms of an ODE system and then normalized to estimate the characteristic time and finally simplified to obtain a set of equations with dimensionless parameters on their right hand sides. The system obtained provides a qualitative mathematical description of the kinetics of lipid peroxidation
Simulation technique		Not disclosed

Table 3.6: General description of the modelling and simulation characteristics of Volkov and Lebedev's model [111]

lation work published in the field of dioxygen free radicals and their impact on biological systems.

Some more work reporting either the possible involvement of free radical reactions or advocating the use of modelling and/or simulations for the study of free radicals can also be found in areas like food technology [86, 126, 168], the study of combustion kinetics [15, 54] or the effect of radical attack on DNA [145, 192]. The reports in the food technology area have as a major concern the problems related to food preservation or irradiation and although the approach used can help in demonstrating how to build models in that area, these are still far from being of any pratical use for this project. The articles on combustion kinetics proved to be vital in finding the right approach for tackling the problems of simulating systems of dioxygen free radical reactions through a specific type of Monte Carlo simulation. In these respects the reports on radiation and DNA proved to be far too complex to be used within the framework of this project.

3.3 Plan of this work

It can be concluded from the previous sections that some researchers have realized the growing importance of modelling and simulation as tools for the study of the possible mechanisms involving free radicals in biological systems, studies otherwise difficult to undertake experimentally. However, although both modelling and simulation were shown to be possible with varied methodologies, some problems remained unanswered and need to be tackled. These are related to the prediction of the actual role of specific free radical species on oxidative stress, including the assumptions made when modelling and the tools used for simulation.

From the state of research at the the beginning of this project, three major areas of research seem to be possible.

3.3.1 The search for simulation tools

To be able to proceed with the modelling that I had envisaged at the beginning of this project a new simulation tool had to be found, that is, a computer program that could deal appropriately with the mathematical form of the chosen models without being model dependent. In order to do this, selection criteria were chosen against which all the programs available were tested. The resulting set of rules, the underlying reasons for their

choice, and the presentation of all the programs tested constitute the first major step of this project which is developed in the next chapter.

3.3.2 The deterministic simulation

The second stage of this project is the development of my previous model in parallel with those reviewed in the previous section. All these models used a deterministic approach. This process was undertaken to investigate the following points:

1. to address the known weaknesses of my models and so test the effect on my results of using currently accepted parameter values;

2. to test the validity and the applicability of the other models

3. to investigate how much information on the mechanisms for free radical formation and the initiation of lipid peroxidation can be gained by using a deterministic model. It will be shown that, after some initial problems simulating the models, the results obtained were in agreement with experimental results. They also allowed some useful insights into the dynamical behaviour of metabolites (concentration profiles of some radicals) and into the fluxes through the reactions composing the model (namely the flux through the radical generating reactions)

4. to investigate the possibility of applying metabolic control analysis to my models

5. to aid understanding of the mechanisms leading to the production of hydroxyl radical, it was decided to limit the size of the models, and so, based on the knowledge of the existing reactions, a new model was built around the Haber Weiss reaction. This new phase of the project continued using deterministic simulation, and the different steps of the modelling included:

    - the study of the Haber-Weiss cycle (including the effects of pH)
    - the addition of reactions to allow the interconversion of the different free radicals forms (including singlet dioxygen) both
        - with non-catalysed Haber-Weiss (no metal ions present in the system)
        - with catalysed Haber-Weiss (simulating the presence of either iron or copper in the system)

- the addition of a set of reactions that can emulate the process of lipid peroxidation, with initiation propagation and termination (again in the presence and absence of metal ions)

3.3.3 The stochastic simulation

The third and final part of the project was centered on a totally different modelling approach, Monte Carlo simulation, which actually was the initial aim of this project. This is defined in the previous chapter as a form of stochastic modelling and was chosen for its ability to deal with systems with a very small number of particles, or in other words extremely dilute metabolite concentrations. Some work on this approach was started at the same time and along the same lines as the deterministic modelling described above.

Chapter 4

Simulation tools

Some comments on the availability of computer programs for the simulation of reactions were made in the previous chapter in relation to my previous simulations. It was noted that the majority of those were developed with specific models in mind, which made their adaptation to different models difficult. The problems ranged from the difficulty in understanding how to use the program (user interface) to situations where, after some effort, the program would prove inadequate for the problem in hand, either because lack of control over the output or because of the inadequacy of the numerical methods used by the program.

Thankfully this trend had started to change by the beginning of this work and it was therefore important to reassess the situation. In part this was due to developments in the microcomputer world (which allowed the production of new versions of known programs), and in part to the growing interest of some biochemists in modelling, which resulted in programs being written, by biochemists, with biochemistry in mind.

In this chapter I present first the requirements for choosing an adequate simulation package and the strategy chosen to test them, and then a critical reappraisal of the several programs tried.

4.1 Requirements and strategy

In the quest for the most suitable program to deal with models of free radical reactions several programs were tested. This task was to be broken into two different steps. The first was to separate the available programs according to their characteristics and properties

and the second was the actual choice of parameters to be used in testing. These can be summarised as:

- the technical requirements, and
- the chosen sets of reactions (models) that the programs would have to deal with.

In Chapter 2 I described how a chemical problem can be written as a set of differential equations, which for a system of free radical reactions is stiff. Although there is a wide choice of computer tools for mathematics, they do not all deal with ODEs so those that allow for modelling and simulation had to be selected. Obtaining programs is nowadays a much easier task and the available choices are:

- commercial packages;
- programs described in the scientific literature as used for similar problems, usually created by the authors and available on request, and
- shareware or freeware programs that are made available over the internet on distribution (ftp) sites indexed under mathematical or biological tools.

For those programs that might at first sight have been suitable, the next step was to inspect a more detailed technical description about the package. Factors examined were:

- the form of input required, as illustrated by any examples distributed with the program, that is, the way the models had to be defined, and how easy it was to acomplish this task.

This factor contributed to the rejection of some programs as my preferred form of input was to enter the set of reactions (the model), which makes the simulation package front end a lot closer to a "real" test tube, rather then a set of mathematical equations. However writing the stoichiometry matrix was also acceptable as an option. Some may see this an unreasonably restrictive factor of selection but it is justifiable not only because of the gain in presentability of the model to others (chemists and biochemists in particular), and of flexibility of working with it (possibility of changing the model by editing adding or deleting reactions and or metabolites without having to rewrite the equations describing the model which can be troublesome with big models due to the large number of variables

and parameters present), but also because this requirement has no adverse effects on the accuracy of the results. To meet this requirement the program had to be able to parse the chemical model into the appropriate mathematical form without any intervention from the user. To complete the description of the model a set of parameters including the kinetic constants, the initial concentrations for the metabolites and the time required for the simulation have also to be defined and the prefered form was one which offered the possibility of defining parameter names (specially attributing names to metabolites) that could be used by the program when producing the simulation output. It was also useful if the user were allowed to define more than one type of simulation, that is, whether it was possible to construct a parameter space (to study the overall impact of certain variables in the model, for instance the effect of pH), define a batch of simulations, or be able to combine simulation and steady state determination.

- The type of results (output) obtainable, in what form and how flexible they were, that is whether the user had any control over the results. Most programs normally offered the choice of graphical or numeric output for the solution of the problem and so this requirement did not constitute a discriminator.

- Whether there was information about the numerical method implemented or whether a choice of methods was available whether it was offered to the user or chosen automatically by the program

- Whether the available numerical method was one that could deal with stiff ODEs (which was sometimes wrongly claimed). Numerical methods have already been discussed in Chapter 2, so I simply reiterate that I was looking for the Gear method or any variation of it.

- The amount of computer memory left available by the program for working with the models, which in cruder terms means the maximum number of metabolites and reactions available to the user.

If the programs appeared to meet all these requirements on paper, they were tested in use to see whether they could accomplish all the requirements and for this task some models were chosen. One, partially because of its simplicity, consisted of reactions 4.1, 4.2 and 4.3 (these represent the simplest Henry-Michaelis-Menten type of reaction between an enzyme and its substrate):

$$Enzyme + substrate \longrightarrow Enzyme - substrate - complex \tag{4.1}$$

$$Enzyme - substrate - complex \longrightarrow Enzyme + substrate \tag{4.2}$$

$$Enzyme - substrate - complex \longrightarrow Enzyme + product \tag{4.3}$$

The main reason for this choice was that an exact solution for the integrated time course can be calculated and compared with the results from the simulation. The input of this model also revealed how easy it was to use the program and how praticable to change the parameters to repeat simulations with other data.

If and when the program proved statisfactory in terms of results, usability and practicability on this model, another model was tried. This had to be a model of free radical reactions so that it would be closely related to the real problems in this wide field. The choice of a set of reactions from the hundreds available (commented and developed somewhere else in this thesis) was based on my existing knowledge of the dynamical behaviour of certain sets of reactions. The chosen set correspond to the first block of reactions (interconversion of oxygen free radical species) from the model described in Chapter 3. This model, compared with the one just presented for the Henry-Michaelis-Menten, includes a large number of reactions and metabolites, and when transformed into a set of differential equations exhibits the problems of stifness.

After all the discussion on the advantages and disadvantages of the deterministic and stochastic approaches in Chapter 2, it has to be noted that the above discussion applies (see below) only to packages dealing with the deterministic approach. Some effort was made at finding packages that could deal with Monte Carlo Simulation, but those programs that were found were invariably directed to the simulation of chemical structure rather than chemical kinetics. This will be further developed in Chapter 6.

4.2 Review of the available programs

Sometime before the begining of this work some time and effort was spent on a project with Thierry Letellier at building a computer simulation program (SiDyBios: A **Si**mulator of

the **Dy**namics of **Bio**logical **s**ystems (unpublished results)) directed at solving biochemical problems (time course of reactions and steady state analysis) but dealing with all the requirements described above. This project was abandonned before completion largely because other programs had become available and more specifically because there were two other programs that overlapped with our plan.

The two programs responsible for stopping the development work on SiDyBios were SCAMP [170] and GEPASI [141] which were at a much more advanced stage of their development and gave the opportunity to start modelling and simulation immediately with programs already subjected to a considerable amount of testing. This was not the case with SiDyBios which still needed some development and testing to achieve the same input and output facilities that the aforementioned programs offered.

At the time of assessment the available programs could be divided into two main groups according to the scientific community for which they were designed, that is, mathematicians (engineers) or (bio)chemical scientists. This separation can be more easily understood as the last group just wants simple simulators (chemical or biochemical kinetics) which will be a specific task of the more general mathematical tools available in the programs for the first group. In this first group we have Mercury [173], MathCad [134], MLab [183] and MatLab [135]; whereas in the second we have the two mentioned above, SCAMP and GEPASI, and then FitSim [202] and SCoP [113], though this last one was initially developed for and by engineers and was later extended to "meet the needs of biomedical simulators" [113].

All the programs in the first group were rejected because even though they could meet some of the necessary requirements, they failed on the grounds of the relations with the user. They could deal with a wide range of mathematical problems since they had a great deal of flexibility over the definition of the equations composing the models, and they also had the possibility (with varying degrees of difficulty) of specifying the amount and form of output desired. Although the programs proved to be useful for a variety of tasks the main reason for their rejection was the incovenient way the model had to be specified, that is, as a series of equations instead of reactions.

The next three sections will discuss in somewhat more detail the available tools mentioned above. The first, under the heading of "Mathematical modelling tools" will present, assess and criticise Mercury [173], MathCad [134], MLab [183] and MatLab [135], tools

that were mentioned above as designed for mathematiciens, over their suitability for the simulation of chemical kinetic models. Even though the remaining programs were all classified under the same interest group (biologists), as they all are metabolic modelling tools, two different sections have been created so the chosen programs can be presented in an independent section. The first will be under the heading of "Metabolic modelling tools" and the last under "GEPASI and SCAMP". The former will in the same style as the previous section, disscuss the non suitability of FitSim [202] and SCoP [113] for the current project; and the latter will present GEPASI and SCAMP.

4.2.1 Mathematical modelling tools

Mercury [173] is an outgrowth of version 1 of Eureka the Solver (Borland International), available for both the PC (IBM compatibles) and Apple Macintosh, which was made available to another software developer who carried out further development. The final result is a package that can deal with a reasonably wide range of mathematical problems such as:

- evaluating mathematical expressions
- solving for the roots of an equation
- solving a system of equations
- maximizing or minimize a function, with or without constraints
- evaluating derivatives and definite integrals

However, having been designed for educational use, the program does not accomodate large problems or very complex models . It also proved to be the most limited of the programs tested in terms of memory problems.

The only striking difference between the other mathematical programs investigated, is that MathCad [134] is available for the PC running under Microsoft Windows graphic operating system whereas the other two, Mlab and Matlab [183, 135], are for the PC running under the Microsoft DOS operating system. The program descriptions do not differ much in essentials from that of Mercury, although they are more powerful and so can be used for even more complex and larger problems (these ranged from elementary maths to transcendental equations including probability and statistics, linear algebra,

optimization, cluster analysis and combinatorics). They are also designed for far more demanding users and include:

- a better description of what they can do and how (by means of far more extensive and detailed help);
- the possibility of solving problems either numerically or symbolically;
- curve fitting;
- a precise description of the numerical method available (MathCad: 5^{th} order Ridder) and in some cases even a choice (MLab: Gear, Adams or a mixture of the two) of methods.

4.2.2 Metabolic modelling tools

This section will present in more detail the programs previously listed as metabolic modelling tools that were eventually eliminated from consideration for not complying with the discussed requirements. It is not my intention to present an exhaustive review of all the available programs (for a wider review of the field refer to Mendes [141] and Sauro [170]) but only of those known and tested at the begining of this project.

FitSim (possibly from **Fit**ing **Sim**ulation) [202] is a computer program that fits simulated data, the result of a computer simulated model, to experimentally measured progress-curves by an iterative weighted least squares procedure. That is, user-defined theoretical parameters are fitted to the real progress curves producing an optimized kinetic model of the system under study. This goes further than required for this project as there was no suitable experimental data to work with. However, included in the distribution, and necessary for producing the simulated data, was another program (KinSim, [6]) from which all the kinetic simulation routines in FitSim are derived and which might have fulfilled the criteria. Basically FitSim is designed to run as a supervisor program to Kinsim, each sending messages and requiring answers from one another, but with the control of the whole simulation centered around FitSim. It reads the input (model) sending it to Kinsim for transcription into a mathematical model that can be simulated. Once this is done the results are passed back to FitSim for the first iteractive fitting. This cycle can be user-controlled depending on the accuracy or tolerance required. The positive attributes of this program are the possibility of defining the input model as a set of reactions and

the integration routines available in Kinsim. The Gear method for stiff systems of ODEs was available. As the program was originally designed for fitting of enzyme kinetics, the accepted input models must be based on the mechanisms of enzymes (single enzymes), and though there is the possibility of defining a large number of chemical steps (40), it turned out to be difficult to use the program for other kinds of models.

SCoP (**S**imulation **Co**ntrol **P**rogram) [113] is a program developed for micro- and minicomputers. Its functioning is similar to SCAMP (refer to next section) in that it takes a user-defined model and builds a simulation program. That is, the models are translated into C subroutines then compiled and linked to SCoP to produce a menu-driven interactive program. They differ in that whereas SCAMP accepts reactions as input, SCoP does not, it being necessary for the user to define the dynamic equations in a form of computer language. The program developers have attempted to aid this step by allowing for user-defined name parameters, so that some reality can be gained from using, for instance V_{max} as one of the components of an hyperbolic function, but it still does not fulfill the requirement for the existence of a parser which would translate the input in the form of a reaction network into the appropriate set of equations which describe the dynamics of the model. Fitting of experimental data can also be made to the simulated model via SCoPFit, thus allowing the user to work on the optimization of the model parameter values.

Some of the other programs or tools that came to my notice during this project included:

- BIOSSIM, developed initially by Garfinkel [51] and latter by Roman and Garfinkel [166], consists basically of a metabolic simulation language resembling SCoP. The program was written in Fortran and relied on non-standard compiler features, making it difficult to implement and use the program on PCs (Fell, personal communication).

- SIMFIT, developed by Holzhütter and Colosimo [89], is a program designed for the same purpose as FitSim [202], primarily for the fitting of models to experimentally obtained kinetic data. However it can both simulate and solve for the steady-states of theoretical models [141]. It was written in Turbo (i.e. non-standard) Pascal (Borland International) for PCs under MS-DOS. The user has to write the ODEs into a Pascal subroutine, though there is an interactive user-interface for assigning

parameter values, run-type and length [89]. Early versions did not have integration routines for stiff systems of equations.

- Essyns was developed by Irvine [95] to use the power law formalism of the "Biochemical Systems Theory" introduced by Savageau [171,172], but it is mainly designed for the calculation of steady-states or dynamic responses to perturbations in the regions around them [141]. Apart from the fact that it is difficult to work out the exact translation between the normal chemical kinetics and their power law representation, it was the absence of methods for calculating progress curves without reference to a steady state which made it unsuitable for this project.

- MetaModel, developed by Cornish-Bowden and Hofmeyr [25], is also unsuitable because it is designed only for the calculation of steady-states using relatively simple numerical approximation techniques.

- MetaSim can not be considered as program because it can not run on its own. It consists of a set of computer language (C++) definitions (libraries) that can be used by programmers to build programs for simulating metabolic pathways [185]

- Mist [35], which could have been considered as a suitable tool, was not available in its final form in time for this project. A previous form of the program, which was model dependent, was tested and looked promising at the user interface level (it was quite easy to use), however, the simulation engine seemed to be very slow (Fell, personal communication).

4.2.3 SCAMP and GEPASI

There were two additional reasons for the final choices made, apart from the fact that both programs eventually met the necessary requirements, the first being that both programmers were known to me and easily contacted when difficulties or errors were encountered and could be requested to make alterations or additions; the second was that although they are so different in their user interface design, both eventually use independent implementations of the same numerical methods (see below for further development) which allows for comparison and validation of results.

GEPASI [141] (an acronym for **GE**neral **PA**thway **SI**mulator) is designed to be a metabolic modelling package, i.e., a software system for modelling chemical and or bio-

chemical reactions [141]. Version 2 of the program current when testing was carried out (the development of the program continued and this version has been replaced by the new GEPASI version 3 [142]). The program allows the study of both the dynamics (progress curves) of the system and the steady-states, either separately or in conjunction. If a steady-state is reached, then the theoretical formalism of Metabolic Control Analysis [16,81,82,98] can be applied. The program also has reasonably high limits for the number of reactions and metabolites allowed (45 of each) and it offers the user a choice of either 35 predefined rate equations (from first order chemical to allosteric interaction) or defining their own. Last but not least, it does have very flexible means of parameter space exploration which can be sequential, when the density or dimensions of the grid are user defined, or randomized [141]. It runs under the Microsoft Windows graphic operating system even though it could not claim to use all the Microsoft Windows capabilities. This program was until recently the only available based on a graphical user interface philosophy which is an advance on the old menu driven programs and a long way from the majority of programs which were language based.

The user interface was developed in GEPASI to make it easy for an even inexperienced user as the program directs the user through the tasks that need be done before a simulation is possible by enabling and disabling menus on the main window. The package contains three different programs:

- one that is used for defining the system (GWTOP);
- another that will read that information and allows for definition of all the kinetic parameters, simulation parameters, simulation type (GWSIM);
- and when everything is correctely defined a simulation can be run by GEPASI (the third program), which is the simulation engine only and gives the name to the package.

For a particular model to be simulated it is necessary, by opening the appropriate windows, to:

1. use the first program to:
    - type the reactions sequentially;
    - define which metabolites are internal and external;

- attribute the correct kinetics to the reactions (or to define new ones);
- save this information into the 'model topology file'.

2. Then, using the second program, which links in the previous file that provides the model information, lists of all the available parameters are constructed and presented to the user who initializes the data (concentrations and kinetic parameters), saving all this information into a **sim**ulation file.

3. Lastly, depending what type of simulation one wants (dynamics or steady-state), defines the amount and type of output. The program then writes a file containing all the input information, passes it to the simulation engine and, when the simulation is finished, allows the user the choice of seeing the results (again this is dependent on what type of simulation was performed).

The input files written by GEPASI, even though editable, are cryptic as seen in Figure 4.1, and hence difficult for the user to adapt for subsequent simulations if they should want to step straight into the simulation engine. The set of reactions initially entered are written into the files as the stoichiometry matrix describing the system, and other relevant information for the simulation. On the other hand when new reactions are to be added to an existing system (using GWTOP) the topology changes and so another simulation file has to be created (GWSIM can not attribute more than one **top**ology file to each **sim**ulation file) and it is tedious to have to repeat the initialization (specially for big models or for incremental development of models) through the user interface. It is important to note that the benfits of using GEPASI, however, far outweigh the disadvantages, and versions developed afterwards have eliminated most of these difficulties.

SCAMP (from **S**imulation and **C**ontrol **A**nalysis **M**odelling **P**ackage [170]) is another computer package. Like GEPASI it contains several programs that cover the whole range of steps from the user-defined model to graphing the user-required output, and of these SCAMP is the first program to be used. It is designed essentially as a metabolic modelling program, so that although the author demonstrates the possibility of representing and solving certain generalised numerical problems, its main capabilities and strengths are in modelling and simulating biological problems. SCAMP, unlike GEPASI with its GUI (graphical user interface), has a language-based user interface (like BIOSSIM), that is, the input the user has to provide is in a structured file separated into sections by keywords. In

```
version 2.05
testp (comparison with mcarlo)
3 4
-1  1  1
-1  0  1
 1 -1 -1
 0  1  0
 5  0 R1
 7  0 R3
 7  0 R2
-1 -2  3  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0
-3  4  1  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0
-3  1  2  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0
 0  0  0  0
 0  0  0  0
 0  0  0  0
1 s1
1 s2
1 s3
1 s4
 2.e+008
 2000.
 2000.
2.5e-006
4.e-005
2.5e-006
0.
mM s
0 1 100 1.000000e-004 1.000000e-006 1.000000e-012 1.000000e-009 12 5 0
0 0 0 0 0 0 0
0 0 16 0 0 1 0 1 0
C:\APPLIC\GEPASI\EXAMPLES\RESULTS.DAT
0 1 0 2 0
0 0.000000e+000 0.000000e+000 1 0
8 0.000000e+000 0.000000e+000 1 0
```

Figure 4.1: GEPASI input file (simulation file as created by GWSIM) for a Henri-Michaelis Menten model as defined by reactions 4.1,4.2 and 4.3

effect the model is described in terms of a metabolic command language. This resembles any high level computer program language (Pascal in particular) and defines the title of the model; the type of simulation (time course or steady state determination); the variable metabolites (those the program will write ODEs for); the set of reactions (with user defined names) and the corresponding kinetics (simple chemical and basic Michaelian enzyme kinetics are predefined, so the user has only to declare the necessary parameters); the initial numeric values of all the variables and parameters known to the model; the required number of points for a progress curve; and finally the information to be output (the program calculates and keeps records of all the variables of the model, but it is up to the user to specify the amount of information required or else no output will be produced). There are specific keywords to introduce each of these sections.

After this input file has been written with any text editor, the user must compile the model. SCAMP translates the metabolic command language into an intermediate code. If any errors are detected, whether syntactical or in self-consistency of the model definition, they are reported by SCAMP and can be inspected in a listing file. Once the compilation phase is successfully completed the user had two options of how to run the simulation, either by using another program distributed in the package called "CODEGENR" (run code generator) to write a coded representation of the model that is subsequently interpreted by "RUNEXEC" (run executable), or by using "CODEGENC" (C code generator) which generates a C program file that can be compiled and run on any machine[1] (though the latest versions of SCAMP do not support C-code generation).

Initial difficulties of using SCAMP are analogous to those of using any new computer language, which will not discourage computer users who are programmers, but which will constitute a difficult phase for computer illiterates and this is, in my view, the most striking difference between GEPASI and SCAMP.

A similar criticism to that made about GEPASI could be made about SCAMP with respect to editing existing models if it was not for the general increase in speed to current computers. That is, when one wants to edit (add, modify or delete) the system of reactions composing the model it is at the level of the command file (metabolic command language)

[1]The initial version of Scamp was made for the Prime minicomputers and was written in Pascal and produced Pascal code. It was the interest it generated and the demand for a version that could be used on different platforms (mainly IBM and compatible PC's) which forced a rewrite of the program and consequently the change of the programing language to C. This fact allowed the creation of the present flexibility.

that these modifications will have to be made and so all the steps above described have to be taken before simulation is possible. However, the unchanging elements of the command file can be re-used, so only the alterations need to be typed in. If one wants only to change the initial parameters (concentrations or kinetic constants) for the simulation, changes can be made at a different level (by editing a user-readable data file) which does not require the repetition of the whole process.

In addition to the points presented above, Both SCAMP and GEPASI do include integration methods that are capable of dealing with stiff systems of equations. GEPASI initially provided an integration routine based on a Runge-Kutta method (a 3^{rd} order Calahan [122]) specially developed for stiff systems. However this was a fixed step method and eventually a more appropriate routine was employed, based on a method initially developed by Hindmarsh (LSODE [87]) and latter modified by Petzold [127] to include an automatic detection of the type of ordinary differential equations and the appropriate integration routine to use for their solving. With SCAMP the user has the possibility of choosing which integration routine will be used for solving the system of ODEs. Although LSODE was not initally available in SCAMP because other methods thought to be good enough to deal with stiff systems were already implemented (fixed Runge-Kutta, adaptative Runge-Kutta, Kaps 4T, Kaps 4A, fixed semiIRK and adaptative semiIRK [170]), the models I was interested in simulating created problems for those methods and eventually LSODE was also made available.

4.2.4 Discussion

In general most of the progams could deal in some way or another with relatively simple models but for the reasons presented above it was decided to use GEPASI and SCAMP for most of our modelling and simulation. So far proof or evidence of why these tools do meet for all the necessary requirements has not been shown. It was deemed unnecessary and tedious to present in the present section all the information gathered on the use and the results obtained for the programs discussed in the previous sections when implementing the two types of models chosen for the testing. It will only be shown how to implement the Henri-Michaelis-Menten model (composed of reactions 4.1 to 4.3) in GEPASI and SCAMP, and the problems encountered when trying to simulate the models incorporating free radicals will be highlighted.

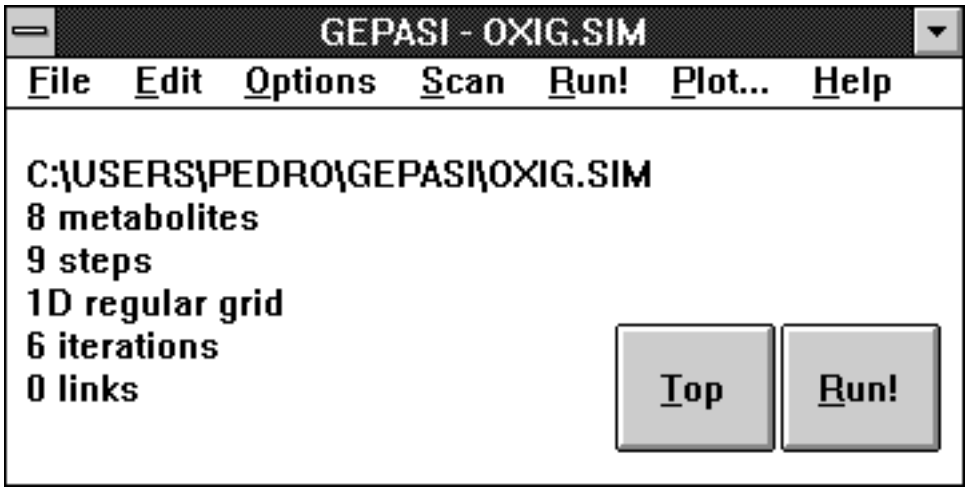

Figure 4.2: Gepasi user interface: controlling program GWSIM

The required criteria for chosing a suitable simulation package, discussed at the begining of this chapter, can be summarized in the folowing 3 rules:

1. existence of a parser which translates the reaction network into the appropriate set of equations which describe the dynamics of the model;

2. availability of a numerical method suitable for the problem to be solved

3. existence of control commands for the simulation, to alter parameters and to direct the presentation of output

On these grounds, GEPASI and SCAMP were the only programs that dealt with all the above requirements when modelling and simulating the three reactions that constitute the Henri-Michaelis-Menten model (reactions 4.1 to 4.3). The necessary simulations could be done with some of the other simulators, but the costs of achieving that because of awkward user interfaces were high. The only other program that could not be totally rejected at this level of testing was KinSim [6].

Figures 4.2 to 4.4 highlight the ease with which the simulations can be done with GEPASI. Figure 4.2 and 4.3 show a screenshot of the two programs mentioned in the previous section as GWTOP and GWSIM, and it is by selecting the menus on these two programs that all the necessary information for a particular simulation is defined. In order to define the set of reactions for the present model one can select the reaction editor and simply type the reactions as shown in Figure 4.4. After all the information is entered in the respective dialog boxes and before the simulation is actually executed, GWSIM will

Figure 4.3: Gepasi user interface: controlling program GWTOP

Figure 4.4: Gepasi user interface: the reaction editor dialog box

ask the user to save the file creating what was described in the previous section as an input file, but is a GEPASI simulation file.

All the differences between GEPASI and SCAMP became clearer by inspection of figure 4.5 where one can see a SCAMP command file with all the separate sections clearly defined and ended by keywords and preceded by comment lines (identified with an # and which can be introduced anywhere in the input file for more clarity).

Refering to figures 4.3 and 4.5 one can see that even though the two packages have totally different user-interface designs, the way the information for the model is defined agreed with the criteria defined previously. The reaction scheme is typed as if they were being writen in any reasercher's notepad, and then the values for all the parameters (seen only in case of SCAMP) for the simulation and output required.

With respect to KinSim, attemps at the input of the Michaelian model looked initially promising as can be seen in Figure 4.6. However at first it was not very clear what were the output coefficients (here defined as F1 to F3); these were latter discovered to be scaling factors to allow the visualization of the time courses of the required metabolites on the same screen, but their initial setting was not straightforward without a prior knowledge of the range of concentrations expected for the components of the system. It might be noted from Figure 4.6 that only two reactions were introduced against the three that were used for both GEPASI and SCAMP. They could have been entered in this form in GEPASI and SCAMP to resemble that used in KinSim, but not vice versa and this is a point that let KinSim down as this is the only type of kinetic representation available. This is is not very flexible, especially where enzyme rate equations are needed.

In all these models, s1 is the enzyme, s2 the substrate, s3 the enzyme-substrate complex and s4 the product. After advocating the benefit of using the standard names for metabolites and enzymes it might seem a bit odd that I chose to use apparently meaningless names; however, they were chosen to be consistent with some of the work done for a future chapter (Monte Carlo Simulation).

In order to validate the programs, the parameters of the model had to be chosen carefully so that the simulator could be tested adequately. The normal form for the rate of reaction for an ezyme catalysed reaction is:

$$v = \frac{V_{max} S}{K_m + S}$$

```
Title testp (comparison with mcarlo) ;

# Remember, statements starting with a '#' symbol are
comment lines, just like this one;

# specify simulation;
Simulate;

# declare the floating metabolites;
Dec
   s1 , s2 , s3 , s4 ;

# define the reaction network;
Reactions;
            s1  + s2 - s3 /k1/;
            s3 - s2 + s1 /k2/;
            s3 - s4 + s1 /k3/;
eor;

# Initialise all the existing parameters and variables;
Initialise;
     k1  = 2.0e8;
     k2  = 2.0e3;
     k3  = 2.0e3;
     s1  = 2.5e-8;
     s2  = 4.0e-5;
     s3  = 0;
     s4  = 0;
     CSUM1 = 5.0e-6;
     CSUM2 = 3.75e-5;
ei;

# Simulate up to the time point of....;
timeend = 4.0e-2;

# print out the time and concentrations of s1 to s4;
# Note, 'screen' indicates the screen;

print_sim TIME,s1,s2,s3,s4  (testp/2, screen);

# And don't forget the 'END' statement;
END;
```

Figure 4.5: SCAMP input file (metablic language created with any text editor) for a Henri-Michaelis Menten model as defined by reactions 4.1, 4.2 and 4.3

```
! testp comparison with mcarlo
$testp
s1 + s2 == s3
s3 == s1 + s4
*OUTPUT
s1*F1
s3*F2
s4*F3
```

Figure 4.6: KinSim input file for a Henri-Michaelis Menten model as defined by reactions 4.1, 4.2 and 4.3

where v is the instantaneous rate, V_{max} the limiting rate (or as it is normally known the maximal rate), K_m the Michaelis-Menten constant, and S the enzyme substrate. For the present model, the steady-state assumption was considered and so the rate v can be written as:

$$v = \frac{k_3[E]_0 S}{\frac{k_1+k2}{k_3} + S}$$

where the k_1 to k_3 are the kinetic rate constants for reactions 4.1 to 4.3. Under high substrate conditions (that is when $S >> K_m$) the above equation can be simplified to:

$$v \approx V_{max} = k_3[E]_0$$

and so v can be calculated precisely based on the chosen parameters and compared with the simulation results. Any significance differences between this value and that obtained by simualting would mean either that the simulator was not correct or that it was badly implemented in the program.

After the kinetic parameters had been chosen, the substrate concentration had to be set so it was at least a couple of orders of magnitude greater than K_m, and the initial enzyme concentration had to be set low enough that it could be considered to be saturated by the available substrate and so "working" at maximal velocity.

With these considerations in mind the values chosen for the kinetic constants were at $2 \times 10^8 M^{-1}s^{-1}$ for reaction 4.1, $2 \times 10^3 s^{-1}$ for reactions 4.2 and 4.3; and the initial concentrations of $2.5 \times 10^{-8} M$ and $4 \times 10^{-3} M$ for enzyme and substrate respectively. The

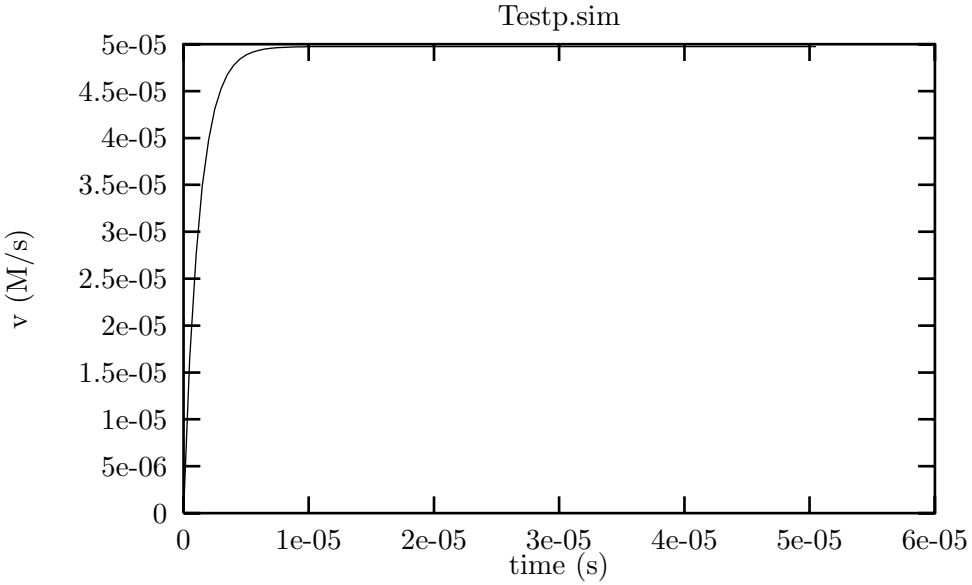

Figure 4.7: Change of flux (rate) through reaction 4.3 for conditions where $[S] >> K_m$.

simulation end time had also to be tested so an appropriate time range could be attained for monitoring the change of rate of production of product (in this model reaction 4.3 is considered to be rate limiting for p production, so the enzymatic rate will equal the flux through this reaction). The necessary value for the time variable was found to be $5 \times 10^{-5} s$. A graphical output was easily obtainable for both GEPASI and SCAMP and a plot of the flux through reaction 4.3 versus time can be seen in Figure 4.7. This graph shows two distinct phases: first a rapid increase of the flux in a very short time that corresponds to a pre-steady-state phase before the enzyme gets saturated, after which the rate remains fairly constant for the rest of the simulation corresponding to the $E - S$ complex being at steady state due to the presence of a high substrate concentration. The value of that rate is $4.98 \times 10^{-5} M s^{-1}$ which is close to the calculated V_{max} ($5 \times 10^{-5} M s^{-1}$).

These results demonstrate the applicability of both SCAMP and GEPASI for the modelling and simulating of simple biochemical problems, but their capacity to deal with models of free radical reactions was still to be proven.

Two different sets of free radical reactions for implementing on both SCAMP and GEPASI were chosen. The model developed before the start of this thesis had enough experimental evidence to prove the general validity of its results and so it was a first natural choice for testing the capacity of the programs. However, this model, described in the previous chapter, is very large and very stiff (contains a wide range of parameters)

and so another smaller model based on the work of Babbs and Steiner [4] was also used.

The implementation of the two models was straight forward in both programs. For my model it was a simple task of typing all the reactions with all the necessary parameters in a SCAMP command file language (GEPASI was not used at this time as the current version was still under development), but it took two distinct stages for the model based on Babbs and Steiner work. A more detailed comment on this author's work will be found in the next chapter, so it will suffice to say that from a large set of reactions available for the simulations, Babbs and Steiner were able to select subsets of these for specific simulations and their paper is concerned with one of these particular cases. It is in these circumstances necessary to identify the reaction subset used, which is not entirely clear before attempting to write the SCAMP command file once more a simple task. Even though both these two models demonstrated the flexibility of modelling with SCAMP, they also revealed the problems that this program had with stiff systems. These were discernible by inspection of the results obtained or the lack of them, that is, the output for some of the monitored chemical species was given as NAN[2]. These results showed that the numerical methods implemented in SCAMP at the time were not the appropriate for dealing with such problems. Simulations were still possible but they were tedious and time consuming. The process for simulating was to decrease the chosen time several orders of magnitude, down to microseconds real time (simulations that still took up to three hours) to start with and then concatenate consecutive simulations until the required time was achieved.

It was difficult in the end to validate the simulation tool (SCAMP) for both models: mine because the concentration profiles produced were for a time interval far smaller than the ones I had obtained with my own program (to which I returned to try to reproduce the results for smaller time intervals so a comparison was possible), and Babbs and Steiner because, even though some results were obtained they were not directly comparable with those obtained in their paper. However, some indications of agreement was obtained because the same concentration levels for lipid hydroperoxides were attained. The reason why the two sets of results were not fully comparable is because they were achieved through two totally different methodological approaches. As mentioned elsewhere, SCAMP offers

[2]An abbreviation that stands for "not a number". It is a standard (IEEE) generated result by the processor when the result of an operation can not be represented, such as when a number exceeds the maximum (or minimum) permited, that is the number is outside the range.

a choice of numerical routines (the choice was limited at the time of testing, but has since increased) based on established mathematical methods whereas Babbs and Steiner, to overcame the problems of system stiffness, mixed two different methods [4] assuming that the time scale of concentrations change could be split into two distinct classes, with inherent costs. This is a subject that will be further developed in the next chapter.

4.3 Conclusion

Why select at this stage a program that could not simulate for the required amount of time (SCAMP) and another that hardly existed (GEPASI)?

This was in fact the reason for continuing to search for other packages and did not constitute reason enough for finally eliminating them as potential tools especially as:

1. available programs were scarce, and

2. as was latter revealed by testing, the other programs showed similar if not worse behaviour and did not comply with some of the other requirements such as the form of model definition.

However by the time other programs had been researched and tested, SCAMP had developed into a much better tool and due to an extension of the available numerical methods, could cope with the simulations required. GEPASI, though not complete at the time of the search was developed in time to become an important tool for the rapid modelling of small systems. Currently a version 3 of Gepasi is available. This version has addressed the criticisms concerning the flexibility of using existing models to develop new ones [142].

A general feeling left by all the work done on this subject and also observed by others (including inevitably the authors of some of the programs) is the dissatisfaction of the inexistence of a standard when it comes to simulation techniques. McGill [57] and Lacy [123] have made attempts at defining some sort of standards in what concerns modelling and simulation work. McGill tried to address the problems of graphical representation but without a clear cut conclusion in what should constitute a standard. Lacy on the other hand discusses at length all the different concepts related to systems theory, and proposes different frameworks for approaching the study of such systems but ultimately

does not propose a methodology for the description of a model nor the representation of a problem. All the mathematical background as seen in this and previous chapters is well established and a wide range of options are available, however a definition of what type and form of model specification is not extant making the design of a computer program the more complex and dependent on the programmers background.

Chapter 5

Deterministic simulation

Following the plan of work that is presented at the end of Chapter 3, having completed the first part of that process (Chapter 4 "Simulation Tools"), the next stage was to make use of those results to proceed with the development of my previous models, devising and experimenting with new sets of reactions. This development consisted of two destinct stages — firstly a comparison of my model with those put forward by Tappel *et al* [191], Babbs and Steiner [4], Suzuki and Ford [186] and Volkov and Lebedev [111], followed by a re-evaluation of the Haber-Weiss cycle, and of the interconversion reactions.

For comparison between the models to be possible a descriptive list of characteristics against which they will be measured somehow has to be devised. Choosing the best way to present all this data (the models simulated, parameters used and the corresponding results) is a complicated task, especially when the project in hand is theoretical, and there are no apparent restraints for the testing of variables and parameters alike. Tappel *et al* conveys the same idea by stating that in computer simulation one factor in a system can be emphasized without much concern for the effect of other factors [191]. This quote highlights both the negative and positive aspects of simulation, as contact with reality can easily be lost (this is especially important if the model was an attempt to depict an *in vivo* or *in vitro* situation or process). When this factor is taken into account, however, some interesting insights into biological processes can be gained [191].

All the results obtained were invariably produced by computer simulation and so consist of numerical lists depicting the concentration changes of selected system components over time. They can be grouped into distinctive classes, according not only to all the developmental stages that modelling undergoes, but also to the simulation of structurally

different models.

The model development involved the choice and grouping of adequate sets of reactions for studying the designated biological processes. The starting point for this can either be extant models, or, if these prove inadequate for highlighting the required mechanisms, initiating the study of a new set of reactions, which could be a subset of the previous models. The latter brings with it all the problems of having to prove the correctness of the models. For particular situations, as will be shown later in the chapter, the deterministic simulation technique, though applicable to the models in mathematical terms, proved to be inadequate for my aims, causing a change of simulation technique which will be dealt with in the following chapter.

On this basis the results in this chapter have been organized to reflect all the research put into the development of the appropriate models, which were designed to allow the study of the mechanisms leading to the production of the hydroxyl radical, and the comparative efficiency (and importance) of all the radical species in initiating deleterious processes (e.g. lipid peroxidation). More specifically the following points were those initially under investigation:

- Haber-Weiss cycle;
- Fenton-type reactions;
- the effects of the metal cations Fe and Cu;
- OH radical concentration levels, half life and the possibility of the initiation of lipid peroxidation;
- efficiency of lipid peroxidation initiation;
- comparison of that efficiency with that of other potential initiators such as radical anion superoxide, hydrogen peroxide, singlet dioxygen, and also autoxidation.

5.1 Deterministic simulation of known models

Notwithstanding the criticisms of my own model, it was decided to transcribe it into the SCAMP command language (GEPASI was also used, but at a later stage), alongside those developed by Tappel *et al* [191], Babbs and Steiner [4], Suzuki and Ford [186] and Volkov

and Lebedev [111]. These have all been presented in Chapter 3, where the problems they were trying to simulate (i.e. the biological processes under study) were identified along with the model topology (set of reactions), parameters (concentrations and kinetic constants) and the modelling/simulation techniques used. These are all summarised in Tables 3.1 to 3.6.

The decision to proceed with the work in this direction was justified by the wide range of information that these new models provide and so it was important to establish to what extent they could be used to achieve my goals. It was also relevant to compare them with the model I had devised so the weaknessess and applicability of each model could be known.

The possible conclusions to be drawn at the time were diverse and dependent on the foundations used by the different authors to devise their models. These consisted of attempts at either the investigation or validation of the known mechanisms that lead to the production of lipid hydroperoxides [4, 111, 191], or the role played by the different radical species in the initiation mechanisms for oxidative stress [186]. For example, one of the models was developed with the aim of proving the usefulness of computer simulation by comparison with the well established results obtained experimentally. It was then only natural to try to formalize some basis of comparison between the aforementioned models and the one I had previously developed. Specifically, from those four models, the ones proposed by Babbs and Steiner [4] and by Suzuki and Ford [186] were considered to be of interest for further investigation in the light of the way in which they were devised and the results they achieved. The other two were considered either difficult to implement in the form they were conceived [191], or downright ill defined [111]; however some work was attempted and will be described in the next sections.

Apart from the biochemical problems under consideration, mentioned in the previous Section (5.1), some other points of a more technical nature were under scrutiny at this phase of the project:

- addressing the known weaknesses of my models by using different numerical methods to integrate the system equations, replacing some of the parameters that at the time of the model development had to be fitted, and observing if the results were the same with the real parameters;

- testing the validity and the applicability of other models;

- comparing the dynamical data of my model with that of Babbs and Steiner [4] to investigate how much information could be gained about the mechanisms of free radical formation and the initiation of lipid peroxidation;
- investigating the possibility of applying metabolic control analysis [16, 81, 82, 98] to my models by studying whether the models attain a steady-state.

It will be shown that, for gaining a more specific and detailed insight into the mechanisms contributing to the production and consumption of the hydroxyl radical, the models used were inappropriate (too large for example) and so the following section will discuss the available reactions, and subsequently the development of smaller models will be described. Using the chosen simulation tools the time profiles of the relevant species was determined and the effects of what are considered the crucial parameters in those models were studied.

5.1.1 Further investigation of my models

Transcription of my previous models into a SCAMP command file

The first task was the transcription of my model into the SCAMP command language. This process was undertaken in several stages, firstly to ensure any initial problems related with the learning of the language were overcome, and secondly to avoid the inclusion of any discrepancies in the definition of the model. The stages were the same as those I followed initially when developing my own model (chapter 3):

1. generation and free radical interconversions;
2. free radical attacks on a particular substance, such as an unsaturated lipid, and the consequent chain reaction (initiation, propagation and termination);
3. protection mechanisms not involving enzymes, and
4. biological enzymic protection mechanisms (e.g. with peroxidase, catalase and superoxide dismutase).

The first SCAMP command file was written with the reactions belonging to the first stage and the corresponding kinetic parameters. This file was then compiled and executed

```
Title oxig5 ( report ) ;

# Remember, statements starting with a '#' symbol are comment lines,
just like this one;

# specify simulation;
state;

# declare the floating metabolites;
Dec
   O2S , O2_MIN , HO2 , H2O2 , OH , LIPID , LIPID_OO , LIPID_OOH ,
   LIPID_O , GSH , GS , GSSG ,  LIPID_OH , GSOO , GSOH ;

# define the reaction network;

Reactions;
                O2_MIN + HO2 + $H_PLUS - H2O2 + O2S  /k1/;

                O2S + O2S - $O2 /k2/;

                O2S + O2_MIN - O2_MIN + $O2 /k3/;

                H2O2 + H2O2 - O2S + $H2O /k4/;

                O2_MIN + H2O2  + $H_PLUS - O2S + OH + $H2O /k5/;

                OH + H2O2 - $H2O + O2_MIN + $H_PLUS /k6/;

                O2_MIN + OH + $H_PLUS - O2S + $H2O /k7/;

                OH + OH - H2O2 /k8/;

                HO2 = O2_MIN + $H_PLUS /k9,kr9/;

                $LIPID_H + OH - LIPID + $H2O /k10/;

                LIPID + $O2 - LIPID_OO /k11/;

[LIPIDOOH]      LIPID_OO + $LIPID_H - LIPID_OOH + LIPID /k12/;

                LIPID_OOH + O2_MIN - LIPID_O + $OH_MIN + $O2 /k13/;

                LIPID_OOH + HO2 - LIPID_O + $H2O + $O2 /k14/;

                LIPID + GSH - $LIPID_H + GS /k15/;

[GSOH_H2O2]     GSH + H2O2 - GSOH + $H2O /k16/;

                2 LIPID_OO - LIPID_O + LIPID_OH + O2S /k17/;

                2 LIPID_OOH - 2 LIPID_OH + O2S /k18/;

                GS + GS - GSSG /k19/;
```

(continues on the next page)

```
                GS + $O2 - GSOO /k20/;

                2 GSOO - GSSG + 2 O2S /k21/;

                $H2O - $H_PLUS + $OH_MIN /k22/;

[GSOH_LIPIDOOH] GSH + LIPID_OOH - GSOH + LIPID_OH /k23/;

                GSOH + GSH - GSSG + $H2O /k24/;

[OH_HW]         GSOH + O2_MIN - GS + OH + $OH_MIN /k25/;

                LIPID_O + GSH - LIPID_OH + GS /k26/;

                $LIPID_H + GS - LIPID + GSH /k27/;

[OH_GSOH]       GSOH - GS + OH /k28/;

                GSSG - 2GSH /k29/;

                LIPID_OH - $X /k30/;
eor;

Initialise;
 k1  = 8.5e7;     k2  = 1.10e12;     k3  = 3.6e7;     k4  = 1.0e-10;
 k5  = 1.0e-4;    k6  = 2.3e7;       k7  = 1.0e10;    k8  = 5.5e9;
 k9  = 2.0e-5;    kr9 = 1;           k10 = 5.0e8;     k11 = 1.0e8;
 k12 = 1.0e5;     k13 = 1.0e2;       k14 = 3.0e4;     k15 = 3.0e8;
 k16 = 1.0e5;     k17 = 1.0e7;       k18 = 1.0e-5     k19 = 2.0e8;
 k20 = 1.0e2;     k21 = 1.0e5;       k22 = 1.0e-14;   k23 = 2.0e4;
 k24 = 5.0e8;     k25 = 1.0e10;      k26 = 1.0e5;     k27 = 5.0e3;
 k28 = 1.1e10;    k29 = 1.0e-5;      k30 = 1.0e-2;
# the concentration is expressed in M
 X = 1;                  O2     =1.0e-4;         O2S    =1.383867e-17;
 O2_MIN =1.0e-11;        HO2    =4.925510e-16;   H2O2   =3.0e-9;
 OH     =4.788491e-13;   H_PLUS =1e-7;           H2O    =66;
 LIPID_H=1.0e-3;         LIPID  =2.228641e-12;   LIPID_OO=2.228643e-12;
 LIPID_OOH=1.53939e-09; LIPID_O=6.86145e-15;     OH_MIN   =1.0e-7;
 GSH      =7.238272e-4; GS     =4.890675e-08;    GSSG     =2.767733e-04;
 LIPID_OH =1.05678e-05; GSOO   =7.054719e-08;    GSOH     =2.394246e-17;
ei;

print_sta O2S,O2_MIN,HO2,H2O2,OH,LIPID,LIPID_OO,LIPID_OOH (res1.res);
print_sta LIPID_O,GSH,GS,GSSG,LIPID_OH,GSOO,GSOH (res2.res);
print_sta [LIPIDOOH],[GSOH_H2O2],[GSOH_LIPIDOOH],[OH_HW],[OH_GSOH] (res3.res);

# And don't forget the 'END' statement;

END;
```

Figure 5.1: SCAMP command file including all the blocks of reactions that were defined for my previous model.

using the programs distributed with SCAMP. Only when this proved successful was a simulation possible. Another block of reactions from the list was then added to the command file, with consequent adjustments in terms of parameters, and the process of compilation and execution was repeated. This was intended to continue until all the reactions and metabolites had been included. After the addition of the third block of reactions it became impossible to simulate the whole model at the time, the reasons for which will be discussed further below. Thus the enzymic protection that had been included in my own model was never transcribed into the SCAMP command file (not even after this problem was overcome). A copy of the SCAMP command file representing the largest model that could be simulated can be found in figure 5.1 with the full set of reactions, kinetic parameters and initial concentrations. All the parameters were the same as those I had used previously when developing my model [148].

The time course simulations

The simulations undertaken with these models can be divided into two groups: the first including those where the dynamic behaviour or the time course for the system was required, and the second where the steady-state was investigated. The first type of simulation occured throughout all stages of the model implementation whereas the second was only attempted when the previous had been successful. The initial intention was to repeat the same type of work I had performed before so the results could be compared and the program tested. That work consisted of simulations either with a choice of end time of less than a second or with a time interval between one hundred and a thousand seconds. Unfortunately it soon became clear that the numerical routines available in SCAMP at the time would not allow for such a wide choice of simulation times. That is, there was no problem in simulating the models for very short time intervals but this was all that was possible because the numerical routines, though assumed to be developed to deal with stiff systems, did not contend successfully with the problems that were being posed. Even the choice of half a second as the time for the simulation was too large, forcing the breaking of this into further intervals sometimes of the order of microseconds or less.

Although annoying, this did not constitute an inpediment to the continuation of this work as there was a quite significant aspect that could still be investigated. One of the major results that I had obtained with my model which was important to investigate

Metabolite	Concentration ($moll^{-1}$)	
	initial	final
O_2^-	1.0×10^{-11}	9.9×10^{-12}
H_2O_2	3.0×10^{-9}	1.3×10^{-21}
$^1O_2$	1.0×10^{-20}	4.6×10^{-14}
$OH^{\cdot}$	1.0×10^{-20}	1.0×10^{-15}
$HO_2^{\cdot}$	1.0×10^{-20}	1.0×10^{-17}
$Lipid^{\cdot}$	1.0×10^{-20}	7.2×10^{-14}
$LipidOO^{\cdot}$	1.0×10^{-20}	7.2×10^{-12}
$LipidOOH$	1.0×10^{-20}	3.5×10^{-11}

Table 5.1: Concentration of the selected metabolites from the Scamp command file at a time point of 10s

further was a very sharp peak in concentration observed for the hydroxyl radical (almost like a peak in a HPLC graph). This peak could only be detected when the total time interval chosen for the simulations was of half a second or less; it was invariably located close to the time origin, and ocurred even when in the presence of all the anti-oxidant mechanisms (chemical and enzymic). Some initial attempts at isolating the time interval where this process occurred had already been undertaken with my old program, although without much success. This led to situations where I was working either close to, or beyond, the possible accuracy of both the method and machine.

Although it has been stated that using SCAMP for simulating my models for short time intervals (less than $60s$) was the only available choice at this time, this task still proved to be difficult for two basic reasons:

- the available version of SCAMP at the time required non-zero initial concentrations, ruling out the possibility of simulating systems that were void of free radicals, and
- the numerical method did not allow simulation for more than a nanosecond when dealing with the full model.

The implications of the first are non-trivial because of the underlying reasons for undertaking this type of modelling and simulation, namely the study of the mechanisms leading to the production and propagation of free radicals. The imposition of non-zero initial concentrations for those species, which under normal physiological conditions are zero, was unacceptable. A possible method of overcoming this obstacle would be to set those concentrations with sufficiently low values that they could realistically be considered

to be zero. This was attempted but was revealed to be problemmatic in practice, apart from the fact that it invalidated any possibility of comparison between the results gained from this process and those resulting from my previous program on the grounds of different initial conditions. Inspection of the numbers presented in Table 5.1, representing a selection of the concentrations of certain system metabolites for both the beginning of the simulation and for a given time point, illustrates this point. The concentrations at the end of the simulation are in the same region as those considered for the initial concentrations. In these circumstances it was not possible to analyse the system behaviour and draw any conclusions about the possible production of hydroxyl radical as its concentration did not vary during the simulation. The interest in continuing to use such a simulation tool can be justified by the fact that improved numerical methods were later incorporated into SCAMP, so it was possible to circumvent this problem.

The other problem mentioned above, relating to the available numerical methods, was based on the fact that it was not possible to obtain the time profile for any of the species after only one attempt, even for a time interval as short as a micro-second. The solution to this initial obstacle was to concatenate consecutive simulations chosen with even shorter time intervals (depending on the model being used, this could be as low as a nanosecond), which became a highly time consuming and cumbersome process for obtaining any results. Because of all these problems, and while waiting for further versions of SCAMP with other optional numerical methods which eventually dealt successfully with such models, it was decided to investigate some other characteristics of the model.

The steady-state determination

The other possible use that could be made of SCAMP at the time was to explore its capacity for determining the steady-state of a system (the concentrations of its components, the fluxes through the individual reactions and analysis of the control through the pathway). This was the only remaining way to confirm with an independent simulation tool the results obtained with my previous model.

Initial observations of the time courses for some of the metabolites (lipidperoxide and its precursors in particular) showed signs of an asymptotic approach to a fixed concentration triggering interest in determining whether a steady-state could be attained and how long it would take. This was investigated by successive simulations where the reaction time

interval was increased from $10s$ up to $1000s$ and the results were analysed. At an early stage of the development of the model I had undertaken some simulations with a longer time period than the initial choice of a second or less, up to $10000s$. This produced a set of results giving an idea of the concentrations for some of the metabolites when the system is nearing steady-state. Some caution had to be exercised, as the actual steady-state has never been calculated. Using this process it was possible to gain an idea of the steady-state concentration of the species involved in lipid peroxidation. However, SCAMP offers the possibility of determining the steady-state as an integral part of the program, without necessitating any major changes to the command file describing the model. Consequently this was an obvious task to perform in the light of the other difficulties encountered.

Nevertheless there were problems in this undertaking. Although there are methods that tackle stiffness when solving ODE systems, solving systems of non-linear equations is not the same: the available methods are not as robust. Both SCAMP and GEPASI later came up with optional methods for dealing with such problems when the standard methods failed. All the attempts to determine the steady-state were unsuccessful, no matter which conditions were chosen. When the program has problems converging on a solution it is possible to attempt to overcome this by feeding values closer to the steady-state into the system. There are two ways of doing this, either by initalizing the variables with non-zero values, or by running a dynamical simulation of the system for a big time interval coupled with the determination of steady state at the end (this is the actual the method employed by SCAMP and GEPASI to circumvent the problem). This still proved unsuccessful, however.

The system's Eigenvalues

The difficulties in determining steady-state stem from two distinct sources: either there is a steady state, but the rate of convergence is so low that the values obtained by simulation are still too far from the steady state for the numerical procedure to converge; or the system is actually unstable to the extent that it is unable to ever reach steady-state.

There is a method which makes it possible to determine when such situations occur, and it is based upon the calculation of the system's eigenvalues. The basis behind this approach, and an explanation of how it can be utilized when examining biological models, is put forward by Heinrich *et al* [80] and Reich and Selkov [161]. Remacle *et al* illustrate

Eigenvalue s
$-1.216 \times 10^{3} + 0i$
$-4.195 + 6.657i$
$-2.006 \times 10^{1} + 0i$
$3.054 - 0i$
$9.003 \times 10^{5} + 0i$
$8.505 - 0i$
$-1.012 \times 10^{2} - 0i$
$2.444 \times 10^{4} - 0i$
$-4.195 - 6.657i$
$4.882 - 0i$
$1.083 + 0i$
$7.166 \times 10^{-1} - 0i$
$-6.796 \times 10^{-2} - 2.042 \times 10^{-1}i$
$-6.796 \times 10^{-2} + 2.042 \times 10^{-1}i$
$-6.947 \times 10^{-1} + 0i$
$-3.794 \times 10^{-5} + 0i$
$-3.961 \times 10^{-4} + 0i$

Table 5.2: Eigenvalues for the floating metabolites present in the model described by oxig5.cmd.

this technique by applying it to free radical reaction simulation [162] and recently, in a more complex approach, Volkov and Lebedev [111] have used it in studying the cell lifecycle.

The impossibility of determining a steady-state obviously raises questions about the system's stability. Instability comes from system structure and kinetics, and is a measure of how the system evolves, and to what extent it is influenced by its components. Although all the reactions had been selected carefully, and assembled from known sources, some concern remains about the wide range of values selected for the parameters and the implications of this on the system behaviour. These relations are described by the eigenvalues.

The systems under study are systems of non-linear differential equations and these possess a multitude of solutions, the character of which is highly dependent upon the choice of parameter values. The parameter values affect the system's stability, and produce a specific set of eigenvalues which can be obtained by calculating the Jacobean matrix of the system. This matrix contains both the real and imaginary parts of each eigenvalue. These are a local estimate of the timescale on which a perturbation to the system will decay (negative values) or grow (positive values). A system near or at a stable steady state will have only negative values, though if these correspond to long time scales, any deviations

from the steady state will take a long time to disappear. Any positive value means the system is locally unstable — perturbations will grow and the system move away from its present state. If there are any complex pairs of eigenvalues, these predict an oscillatory behaviour.

Eigenvalues need not be real numbers: they are complex numbers of the form $a + bi$ where a is usually nominated the real (R) and b is imaginary (I). These numbers can be positive, negative or zero, depending on the character of the system. The imaginary component being other than zero indicates that the system is oscillating. If it has a negative value the oscillations will be dying away — on the other hand if it is positive the oscillatory behaviour is continuous. If the number is zero there is no oscillation.

Analyzing the significance of the real part of the eigenvalues is more problematic — not only can they be looked at individually, but they also have to be considered as whole sets. Taken individually they are always positive or negative. A positive value means that the species it represents will not attain steady-state, whereas a negative number indicates that the time-course will die away as the species approaches steady-state. However, the eigenvalues also have to be examined as a whole rather than in isolation alone. Steady-state for the system is only indicated when the real eigenvalue components are negative. In any other case (when one or more positives occur) a steady-state is not possible.

The eigenvalues can also convey further information. The inverse of all the real components of the eigenvalues within a system provides an idea of how long the different species within the system will take to reach steady-state.

Although SCAMP did not offer the option of working out eigenvalues directly, it was possible to include a procedure for their calculation before the compilation step. These results can be found in table 5.2 which shows that the eigenvalues all contain both real and imaginary parts — as is consistent with the problems found for calculating the steady-state: the system is unstable and will not converge to such a state. One other conclusion that can be drawn from these results is due to the very wide range of values obtained. As the inverse of the real part of the eigenvalue can be taken as a measure of the necessary time for some of the components to attain steady-state, discrepancies between all the different species can be seen. In fact the system can be split into two major parts: one containing those species that would quickly reach steady-state, the other those which would take indefinitely longer. This is one of the reasons, or the source, of the system instability

and the impossibility of calculating the steady-state, as well as being a measure of the 'stiffness' of the system. The range 10^5 — 10^{-5} observed represents the stiffness; the 10^{-5} indicates the system will take more than $10^5 s$ to relax.

These results confirm some of the criticisms made of the model by Heinrich (Heinrich, personal communication) namely, that the range of timescales of the variables was too great and the best way to simulate such models was to apply a technique which consists of variable separation [80]. This criticism did offer a potential route for overcoming the numerical difficulties encountered with the model transforming it in a suitable way that would certainly make simulation and the calculation of the steady-state easier. However, the use of such a technique would mask the study of the behaviour of extremely fast reactions (*e.g.* production of hydroxyl radical). This observation also applies to the method used by Babbs and Steiner [4] and that by Volkov and Lebedev [111] and is the reason why this direction was not pursued.

5.1.2 The study of the model proposed by Tappel *et al*

Table 3.2 shown in Chapter 3 summarizes the general composition of the model, the approach used for the mathematical modelling and the simulation type. (Further general details about the model are given earlier, in Section 3.2.2.)

The model developed by Tappel *et al* is, in spite of its contribution to the field of simulation, the least flexible for further use due to the way it was devised and it also does not provide information about the technical background. Nevertheless it can be a valid approach, and, in the circumstances the author points out, it can be extremely informative.

Two main areas of criticism remain: the first concerns the simulation technique and the numerical methods used. Although Tappel *et al* frequently mention the need for the requirements for simulations in the field to be defined (but without specifying them) they themselves do not satisfy all the technical requirements, in terms of the specification of models, algorithms and computer programs. While it is satisfactory to use a computer program to simulate a problem it is necessary to show an awareness of the difficulties raised by such models. In addition they criticise the lack of "industry standards" while not acknowledging McGill's paper [57] which presents a set of rules to be taken into consideration when a model is to be simulated. Lacy [123] also proposed some guidelines for approaching modelling and simulation research, which he also ignores.

Then there are problems associated with the model itself.

- There is a lack of kinetic information. It is only possible to reproduce their results by replicating their model. Because there are no references to elementary reactions it is impossible to create a SCAMP file. Although Tappel *et al* include a pictorial representation of the model in the paper, a table with some "valuable parameters" and, in the appendix, the spreadsheet describing the program [191], all this information is still insufficient to define the model. For this to be complete it would have to allow for the possibility of exporting it to another platform (simulation computer program) for the simulations to be repeated, which is not the case.

- Some elements of the model itself were unclear, namely the difference between metabolites and processes. A specific example concerns the modelling of the activity of the enzyme GSHPx (Glutathione peroxidase) — it is not clear if it is the enzyme that is modelled or the process it catalyzes.

Although the above model is proposed as an approach providing some quantitative insights into the mechanisms of lipid peroxidation and undoubtably integrates a wealth of knowledge in this field, the model created, is based on rather qualitative premises. It is true that some quantitative data is provided with the spreadsheet model, where values that are defined; as concentrations of some of the species present in the model can be identified, however the way the reactions and kinetic parameters are defined lacks precision. Table 5.3, an excerpt from Tappel *et al* model, illustrates this problem. As shown in the table, the concentrations, some processes and parameters are identified by names like: 'total hydroperoxide formed'; 'autoxidation' and 'activator degredation factor'. Bearing in mind that when modelling a set of reactions a system of ODEs is normally created taking the general form: $\frac{dx}{dt} = f(i)$, it is not clear in this case what is considered to be a rate or a concentration. This criticism disabled attempts to translate this model into a SCAMP command file for further inspection.

To summarize, the model of Tappel *et al* primarily provides an example of how to use personal computers (specifically a generalized spreadsheet program) in the modelling and simulation of biological problems. It also illustrates the insights that can be gained from such an approach, such as the possibility of fitting a model to experimental data with consequent determination of specific parameters. Finally it makes possible research at a much lower cost, allowing the parameters to be changed in order to study their impact on

Table 5.3: Excerpt of the model of Tappel *et al* [191]

Equation number	Equation
1	Remaining polyunsaturated fatty acids = polyunsaturated fatty acids (PUFA) - (accumulated hydroperoxides formed by action of activated inducer on PUFA + accumulated hydroperoxides formed by autooxidation)
2	Effective activator = ((concentration of activator 1)(activity of activator 1)) + ((concentration of activator 2)(activity of activator 2))
3	Activator loss = effective activator × activator degradation factor × total hydroperoxides formed
10	Hydroperoxides formed by action of activated inducer on PUFA = (remaining polyunsaturated fatty acids × peroxidizability of polyunsaturated acids × activated inducer × peroxidation rate)/ remaining antioxidant
12	Autoxidation = (remaining PUFA × autoxidation factor × accumulated total hydroperoxides formed)/ remaining antioxidant
19	Amount of TBARS from accumulated remainning hydroperoxides = accumulated remaining hydroperoxides × yield of TBARS from hydroperoxides

the system. Ultimately, however, their approach cannot contribute to the further study and understanding of the elementary mechanisms of biological processes.

5.1.3 The study of the model proposed by Babbs and Steiner

This model is introduced in Chapter 3 where its general characteristics and application are detailed. The effort that has obviously been invested in the development of the model and its associated computer program — which was kindly made available by the authors — is undoubtedly impressive. Babbs and Steiner show an awareness both of the wealth of extant data concerning free radical reactions and of the necessary steps for a computer implementation of their model.

Initial analysis of Babbs and Steiner's work led to attempts to use their program to investigate its applicability to my research. The design of their program allows for any choice of reactions from a full set that has been hard-coded. Babbs and Steiner's model, during its development, had contained up to 107 reactions, including: free radical production; interconversion; attack on biological structures and protection. After subjecting the model to a sensitivity analysis of its parameters the authors found that not all these reactions were necessary or important, and this allowed them to reduce the number of final reactions. However, they did not eliminate the redundant reactions from the pro-

gram, and this fact permits the user to select the reactions they want for their models and consequently for their simulations.

Because it was possible to choose any of the 107 available reactions it was logical to select those most similar to the reactions I had used in my previous models. The problem arose when I tried to set up the simulation parameters for the resulting model as it was not clear how to do this — there was no documentation available and the program only had a very rudimentary user interface.

Subsequently it became evident that an exact investigation into the production of the hydroxyl radical would not be possible with the approach used by Babbs and Steiner. They had elected to separate the full set of reactions into two major types, dependent upon the order of magnitude of their kinetic parameters. Most of the reactions leading to the production of radicals and their interconversion were deemed to be extremely fast ($k > 10^7$) and so Babbs and Steiner assumed that these would reach equilibrium. All the other reactions were assumed to be slow enough for them to be simulated through time. This technique allowed them to proceed with the simulation of most reactions without major numerical problems, and at specified (user-defined or default) time intervals the concentrations of the species dependent on fast kinetics were recalculated and fed back into the main body of the simulation.

While Babbs and Steiner's method allows for both speed and flexibility it was unfortunately inappropriate for research into my models. It was important, however, to attempt to investigate whether any information could be drawn from translating their model into SCAMP and simulating it. As was the case with my own models this did not lead to further progress on the objects of this research and it was abandoned.

5.1.4 The study of the model proposed by Remacle *et al*

A general description of this model can be found in Chapter 3 and Table 3.4 gives a resume of its properties and characteristics. The two most important features of the approach used by Remacle et al. can be summarized as follows:

- a dynamic analysis of the whole system rather than a temporal investigation of free radicals and their reactions;
- the modelling of specific data representing an interraction between the components of the system, i.e. enzyme activities are affected by the concentration of certain

metabolites (enzyme inhibition).

Their approach is unique in that they have not built a model for investigating the effects of free radicals but have focussed on studying the framework of the antioxidant enzymes SOD, catalase and GSHPx. Having derived a set of kinetic equations, including the main cellular free radical reactions, Remacle *et al* tested the evolution and stability of the system with a fluctuation of a radical species, while taking into consideration that two of the enzymes (SOD and GSHPx) can be inhibited by hydrogen peroxide. This was achieved by including extra factors in the rate equations of those two enzymes.

The inclusion of enzyme inhibition in the model shows that Remacle et al were aware of experimental data concerning the problems in studying these enzymes. They do not discuss in any detail how these interactions will affect the system as a whole. In Chapter 2 the possible problems of simulating heterogenous systems are explained, and Remacle *et al* do not seem to have come to grips with these.

Analysis of the applicability of the method used by Remacle et al to this research indicated that it was not immediately suitable for the type of simulation my work entails. Before it could be used in this arena the model would need to be converted into a SCAMP command file. This process revealed fundamental inconsistencies in their model formulation which meant that I could not use it for research into my own models. The system of ODEs they presented if based on the reactions they present in the paper is correct except for one equation, that defining the change of concentration of hydrogen peroxide. In their equation the concentration of hydrogen peroxide is independent from the concentrations of both radical anion superoxide and dioxygen which is inconsistent with the reaction scheme given.

Remacle et al present a possible methodology for establishing a framework for the study of model stability and this demonstrates the importance of the role played by the system components on the general behaviour of the system. However, ultimately this type of approach does not allow any information to be obtained about the temporal behaviour of the system's metabolites.

Other possible criticisms that can be levelled at this approach include:

- an incomplete choice of reactions for the production and interconversion of free radicals;
- no kinetic information or metabolite concentrations are provided;

- the concentration of iron ions is considered to be constant (but isn't specified) making it impossible for anyone other than the authors to investigate oxidative stress;

- the concentrations of radical anion superoxide and hydrogen peroxide are assumed to be much smaller than the Michaelis constant for their associated enzymes (allowing the assumption of first-order kinetics for these enzymes). No details are given concerning the concentrations of glutathione (GSH) or of the Michaelis constant of GSHPx.

In conclusion, it was not found to be possible to gain any information about hydroxyl radical metabolism using this model.

5.1.5 The study of the model proposed by Suzuki and Ford

This model was developed to establish a comparative study of the toxicities of both radical anion superoxide and hydroxyl radical, and its general characteristics are given in Chapter 3 and Table 3.5.

Suzuki and Ford consider the experimental evidence available illustrating the effects of radical anion superoxide on enzymes such as epinephrine catalase, lactic dehydrogenase, creatine phosphokinase and others [186], to be of sufficient importance to justify a prominent role in radical anion superoxide toxicity. Although this radical is less reactive than hydroxyl radical they claim that their respective toxicities are reversed. Based on the experimental evidence mentioned above they defend the superoxide theory of oxygen toxicity, claiming that it can be illustrated by mathematical modelling.

The methodology Suzuki and Ford adopted in formulating their model and the simulation approach they used has already been described in Chapter 3. The most important feature of their work is that they implemented what seems to be a complete set of reactions, not only for the production of both radical anion superoxide and hydrogen peroxide, but also for the interconversion of these into hydroxyl radical. Another interesting aspect is the simulation approch used — network thermodynamics. They claim that this is a powerful modelling technique based on a topological representation of the system rather than a series of equations. This allows the coupling of 'flows' and 'driving forces' to be analyzed in terms of circuits and the models are written in the form of a SPICE circuit diagram.

Figure 5.2: Response coefficients for the radical anion superoxide and the hydroxyl radical with changing the kinetic parameter for the interaction of the superoxide radical with the theoretical pathway

Despite Suzuki and Ford not having given numerical details there is a possibility that their approach might overcome the stiffness of the models formulated with differential equations — but this has yet to be verified. Attempts were made to use the SPICE formulation but it proved to be counter-intuitive and overly time-consuming.

Notwithstanding the importance of the model developed by Suzuki and Ford, there are some significant aspects of the modelling and simulation that have been overlooked by the authors. Primarily the definition of the model and its parameters has a fundamental flaw: one of the steps is designated as a 'controlling' step, and is also considered to be the only one inhibited by radical anion superoxide. The process by which this effect is achieved is by attributing a lower value to this step compared to that of the others (all values being set arbitrarily by Suzuki and Ford). It is true that in the situation they are simulating, using their chosen range of parameters, this approach works, and so the superoxide theory of oxygen toxicity is apparently demonstrated. If the value arbitrarily

assigned to the controlling step is changed, however, this theory is either demonstrated more dramatically, or is disproven, as can be seen in Figure 5.2 — situations that are not analyzed in Suzuki and Ford's paper. The response coefficients are a quantitative measure of the effect an external metabolite has on the flux through a pathway and as can be seen it is dependent on the value of the kinetic parameter defining the wheight of the interation of the metabolite with the pathway. In this case at the value set by Suzuki and Ford ($1.0 \times 10^7 M^{-1}s^{-1}$) the pathway will only respond to the presence of the radical anion superoxide, however if this value is decreased that response will be drastically reduced and at $1.0 \times 10^3 M^{-1}s^{-1}$ will be the same as for the hydroxyl radical.

The method Suzuki and Ford used to simulate the production of hydroxyl radical uses the Haber-Weiss reaction catalyzed by iron. They do not discuss this in any detail, adapting the kinetic parameter proposed by Walling [193] for the Fenton reaction. To emulate an oxidative stress event they simply increase the concentration of available iron to $0.3mM$ without making any changes to the reaction kinetics.

The values for the kinetic constant assigned to both Haber-Weiss and Fenton-type reactions have been widely used and discussed by many authors (see introduction). There are reasons to believe, however, that the concentration of free iron in normal conditions is almost zero and so there is no catalyst for the Haber-Weiss reaction, and as a result the overall kinetic parameter for this reaction is extremely low. Only in oxidative stress conditions can iron, or other metal ions, become available (concentrations ranging up to μM) possibly leading to chelation by cellular components and so also possibly affecting the kinetic parameter for the catalyzed Haber-Weiss reaction (depending on the situation these can increase by an order of magnitude). These considerations were not addressed in any way by Suzuki and Ford.

In the light of the foregoing argument I decided to translate this model into the SCAMP command language, and later into GEPASI, for further investigation, focussing in particular on the points mentioned above. While I was able to do much interesting work with this model (in preparation) it was ultimately found to be unfruitful for a detailed study of hydroxyl production. An important concept that Suzuki and Ford actually discuss in their paper is that of hydroxyl radical site-specific toxicity. Although they seem aware of its importance, it has not apparently been formulated in any way in their final model.

5.1.6 The study of the model proposed by Volkov and Lebedev

As with the other models, a generalized description of Volkov and Lebedev's model can be found in Chapter 3 and Table 3.6. The main feature of this work that is of interest is that they developed a model to study the possible implications of lipid peroxidation in controlling the cell cycle.

With one notable exception their model is extremely well thought out and implemented. Being aware of the wide range of kinetic parameters involved in the various processes under consideration they use the 'natural heirarchy of characteristic times of changes' (normalization technique) to reduce the size of the model. Babbs and Steiner had also tried a separation of their model based on the time scales of the processes involved. Volkov and Lebedev go a step further in that they do not just separate the model into two distinct parts, but their approach also reduces it, using the 'characteristic times' and 'normalization principles'. This is the correct approach to use when such a wide range of kinetic parameters is under consideration, and it should usually solve the problems associated with the stiffness of differential equations. The only drawback of this method is that, as with Babbs and Steiner, it masks mechanistic information.

Despite all the care that has gone into the formulation of this model a very important point has been overlooked. This is the production of hydroxyl radicals. Volkov and Lebedev, taking the same approach as Halliwell that is criticized in Chapter 3, have simply estimated a constant rate of hydroxyl radical production. For the reasons detailed in Chapter 3 this is incorrect and it obviously constitutes a major hindrance to any further investigation into their model in order to further my research.

5.1.7 Interim Conclusion

All the work reviewed above illustrates the variety of methodologies which have been used to tackle the modelling and simulation of systems involving free radical reactions in biochemistry. It also demonstrates that there are a variety of ways to overcome the technical difficulties (e.g. stiffness) associated with such models.

The models themselves can be designed with varying degrees of complexity ranging from that of Tappel *et al.* who use a simple spreadsheet approach, through the classical methodology of Babbs and Steiner, defining systems in terms of differential equations, to the sophisticated mathematical simplification underlying the work of Volkov and Lebedev.

While each approach is advantageous within the arena it was designed for, each model, as detailed above, has imprecisions in the way it is defined:

- Tappel *et al.* confuse the definition of processes and metabolites;
- Although the separation of variables in Babbs and Steiner's model is a valid way of tackling system stiffness, the separation they employ is partially implemented by simulation in an artificial way — not following a precise mathematical criteria — and does not reflect a real difference found *in vivo*;
- Suzuki and Ford's model is itself basically well-defined (despite some confusion about processes and metabolites); it is their simulation parameters that give cause for concern;
- Despite the mathematical correctness of Volkov and Lebedev's framework, one of the basic assumptions of the model is its fundamental flaw — the assumption concerning hydroxyl radical generation is an oversimplification and mathematically unsound.

Simulation strategies are highly dependent upon the type of model under investigation, and on the way in which that model is defined. In the light of this it can be appreciated that the models reviewed above employ different approaches according to the biological scenarios they were devised to study.

The model created by Tappel *et al* undergoes repeated simulation until the results match those obtained experimentally. Once a perfect fit is reached the model can then be used for the prediction of different biological situations (for example change in concentrations of the intervenient metabolites). The simulation approach can therefore be subdivided into two stages: the first being a 'fitting' technique; the second a more usual type of simulation where values are initialized then the model is run through an appropriate program to obtain results.

The simulation technique of Babbs and Steiner is that used in the second stage of Tappel *et al* model. In addition, because their model was not developed using the proper mathematical technique for variable separation, this was achieved by means of simulation. In their system of ODEs some are numerically solved, using a Runge-Kutta technique, while the other equations are set to zero and are solved using a Newton method. Although the resultant simulations seem to produce results comparable to the literature, the actual technique used is incorrect.

As has been mentioned earlier Suzuki and Ford have chosen to used a network thermodynamic approach. This seems to have overcome the stiffness problems, although the lack of discussion in their paper concerning this means that there is no explanation of how it has come about. While there are no problems apparent in the technique Suzuki and Ford have applied they did not present enough simulations using their model, and so they generalize a proof of the superoxide theory of oxygen toxicity from their results when, as I have shown, if their model is initialized with other (equally valid) parameter values this theory can be disproven.

Both Remacle *et al* and Volkov and Lebedev use their models to perform a stability analysis. As discussed previously, both models are based on systems of ODEs, but these are not solved numerically for a time analysis — instead they are solved as functions of predetermined variables. The aim is to construct phase diagrams to allow an analysis of the biological feasibility of systems.

A study of the strengths and weaknesses of all these approaches ultimately showed them to be inappropriate for the study of the different systems that can lead to the production of hydroxyl radical. They contained, however, important information in the form of kinetic parameters and reactions which could be used in developing new models to investigate the production of hydroxyl radical in biological systems.

5.2 The study of hydroxyl radical production

The controversy concerning the possible production of the hydroxyl radical *in vivo* is illustrated in Chapter 1 — and it exists not only at an experimental level but also at a theoretical level as exemplified earlier in this chapter. As yet no one has been able to ascertain whether hydroxyl radical actually is produced *in vivo* or not. Some of the researchers, including Walling [193], Halliwell and Gutteridge [70] and Koppenol and Butler [115] have developed simple experimental or, in the case of Koppenol and Butler [115], theoretical models. These models were intended to investigate the possibility of hydroxyl radical production. As mentioned previously, Walling is the only one of the above researchers who belives this process really occurs; Koppenol agrees, but only at the theoretical level, while Halliwell after a long period of study, seems to have come to a guarded acceptance.

As shown in the first part of this chapter modelling and simulation are helpful tools for the investigation of complex problems. However, the type and amount of information

they can give is directly attributable to the size and sophistication of the models themselves. More specifically it became apparent that it was impossible to determine hydroxyl radical metabolism precisely in the models examined above. Thus it was decided to adopt a different approach to this investigation. The revised strategy involves the successive modelling of:

- the Haber-Weiss reaction;
- the Haber-Weiss cycle;
- Fenton-type reactions;
- the Sulphenic acid hypothesis.

Only once a full understanding of these models has been attained will it be feasible to create a new model, incorporating more reactions and allowing the interconversion of the several varieties of free radical species.

5.2.1 The Haber-Weiss Reaction

To simulate this reaction a SCAMP command file was written according to Chapter 4 and its output is given in Figure 5.3. When a double check of the results obtained was considered desirable, the same model was simulated using Gepasi.

Inspection of Figure 5.3 will reveal, in the following order:

The type of simulation; the floating metabolite; the reactions included in the model and associated data (kinetic parameters and initial concentrations) and finally the type of output that was required from the simulation. When a time simulation was chosen an extra command was included at the end of the file specifying the amount of simulation time.

A wide range of simulations can be done using the Haber-Weiss reaction. The first type implemented involves the following metabolites: radical anion superoxide (O2_MIN); hydrogen peroxide (H2O2); the hydrogen ion (H_PLUS); singlet dioxygen (SO2); hydroxyl radical (OH) and water (H2O). All being designated internal, for a first set of simulations, and consequently having to be declared as floating metabolites. Then SCAMP, using the reactions provided, writes a system of ODEs with as many lines as floating metabolites (provided there are no conservation relationships).

```
Title Haber-Weiss Reaction ;
# Study of the reaction in isolation;

# specify simulation;
Simulate;

# declare the floating metabolites;
Dec
   SO2 , OH  ;

# define the reaction network;

Reactions;
        $O2_MIN + $H2O2  + $H_PLUS - SO2 + OH + $H2O /k1/;
eor;

Initialise;
     k1  = 1.0e-4;
     SO2    =0;
     O2_MIN =1.0e-11;
     H2O2   =3.0e-9;
     OH     =0;
     H_PLUS =1.0E-7;
     H2O    =55.5;
ei;

# Simulate up to the time point x;
timeend = 1000;

print_sim TIME,OH  (hwreact/2);

# And don't forget the 'END' statement;

END;
```

Figure 5.3: SCAMP command file for the simulation of the Haber-Weiss reaction. SO2 represents singlet oxygen, O2_MIN radical anion superoxide, H2O2 hydrogen peroxide, OH hydroxyl radical, H_PLUS the hydrogen ion and H2O water.

k $M^{-1}s^{-1}$	$[OH^{\cdot}]M$
1.0×10^{-4}	0
76	5.7×10^{-24}
4.7×10^{3}	3.5×10^{-22}

Table 5.4: Variation of the hydroxyl radical concentration for different rate constants of the

After choosing the type of simulation and metabolites the next the step is to initialise the kinetic parameters of the reactions and the initial concentrations. The required output was in the form of 'time into simulation' and 'concentration of all metabolites' (except water), however in subsequent simulations the output was reduced to the time and hydroxyl radical concentration (situation depicted in Figure 5.3). Initially the simulation time was arbitrarily set to 1000 seconds.

The initial concentrations of the products of the reaction (singlet oxygen, hydroxyl radical) were all considered to be zero, whereas 10^{-11} M for radical anion superoxide, $3 \times 10^{-9}M$ for hydrogen peroxide and $10^{-7}M$ for the hydrogen ion, simulating conditions with median pH of 7. The kinetic constant for this reaction, in agreement with values proposed in the literature was set to $10^{-4}M^{-1}s^{-1}$ [164]).

Further simulations with this model were performed. First the value of the kinetic constant was increased to $76M^{-1}s^{-1}$ and then to $4.7 \times 10^{3}M^{-1}s^{-1}$ to study the impact of a faster reaction upon hydroxyl radical production. Table 5.4 compiles the results obtained for the concentration of the hydroxyl radical. Subsequently the concentrations of radical anion superoxide and hydrogen peroxide were also increased to mimic oxidative stress conditions. These concentrations were increased by a factor of 10^{3} and the hydroxyl radical concentration obtained was $5.3 \times 10^{-15}M$.

There is a constant source of radical anion superoxide and hydrogen peroxide in a biological system, and the pH is also kept reasonably constant by buffering systems. In order to make the model reflect these facts it was necessary to transform these metabolites, taking them out of the floating metabolite list and adding a dollar ($) sign before their names within the reaction. SCAMP then treats these metabolites as external, and their concentration is kept constant throughout the simulation. Consequently the equations corresponding to these metabolites are removed from the system of ODEs. Another consequence of this transformation is that, whereas the original system is closed, this system will be open, so although the former system reaches equilibrium the modified system

attains steady-state — in biological terms a much more realistic model.

Having altered the model I reran the set of simulations previously utilized and investigated whether there was any change in the production of hydroxyl radical. For the simulations changing only the value of the kinetic parameter (mantaining the low values for the concentrations of the radical anion superoxide and hydrogen peroxide) there was no major change. When k was set at $1.0 \times 10^{-4} M^{-1} s^{-1}$ the hydroxyl radical concentration changed from zero (see Table 5.4) to $1.0 \times 10^{-28} M$ but in the other two situations (76 and $4.7 \times 10^{3} M^{-1} s^{-1}$) there was only an increase in the concentration by a factor of 10. Finally, when I reapeated the simulation mimicking an oxidative stress the concentration of hydroxyl radical hardly changed at all.

5.2.2 The Haber Weiss cycle

In the event of any hydroxyl radical being produced it is considered to react readily with any hydrogen peroxide present in the vicinity. For this reason it is more correct to consider the Haber-Weiss reaction as not occurring in isolation but rather comprising a set of two reactions which ultimately lead to the degradation of hydrogen peroxide to water and dioxygen. This has been termed the Haber-Weiss cycle [116], the whole process resembling a disproportionation [114]. It has been postulated that this cycle can take one of two forms, the first presented by Koppenol [114,116], the second proposed by Hill [59,84]. While the end effect is the same the constituent reactions are different. The reactions and their components can be inspected in the scamp command files from Figure 5.4 and 5.5.

To study the impact the addition of the second stage of the Haber-Weiss cycle has on the production of hydroxyl radical, two new SCAMP command files were written by editing the file in Fig 5.3 to give the new files in Figs 5.4 and 5.5. It is predicted (even before simulation) that the addition of the second stage of the Haber-Weiss cycle will lead to an even more marked decrease in concentration for both hydroxyl radical and hydrogen peroxide than that of the system considered in the previous section.

If the ODE systems for both Haber-Weiss reaction and cycle are analyzed, the equation defining the hydroxyl radical concentration for the Haber-Weiss reaction is found to have only one contributory factor, the

$$\frac{\delta[OH^{\cdot}]}{\delta t} = k[H_2O_2][O_2^{\cdot -}]$$

```
Title Haber-Weiss ( Koppenol ) ;

# specify simulation;
Simulation;

# declare the floating metabolites;
Dec
   SO2 , OH  ;

# define the reaction network;
Reactions;
        $O2_MIN + $H2O2  + $H_PLUS - SO2 + OH + $H2O /k1/;

        OH + $H2O2 - $H2O + $O2_MIN + $H_PLUS /k2/;
eor;

Initialise;
     k1  = 1.0e-4;
     k2  = 2.3e7;
     SO2    =0;
     O2_MIN =1.0e-11;
     H2O2   =3.0e-9;
     OH     =0;
     H_PLUS =1e-7;
     H2O    =55.5;
ei;

REPEAT LIST;
H_PLUS = 10E-11,10E-10,10E-9,10E-8,10E-7,10E-6,10E-5,10E-4,10E-3,10E-2;
REND;

# Simulate up to the time point x;
timeend = 1000;

print_sim OH,H_PLUS (habwe1ir/2);

END;
```

Figure 5.4: SCAMP command file for the simulation of the Haber-Weiss cycle

```
Title Haber-Weiss ( Hill ) irreversible;

# specify simulation;
Simulate;

# declare the floating metabolites;
Dec
    OH  ;

# define the reaction network;
Reactions;
        $O2_MIN + $H2O2  - $O2 + OH + $OH_MIN /k1/;

        OH + $H2O2 - $H2O + $O2_MIN + $H_PLUS /k2/;
eor;

Initialise;
     k1  = 1.3e-1;
     k2  = 2.3e7;
     O2    =1.0e-6;
     O2_MIN =1.0e-11;
     H2O2   =3.0e-9;
     OH     =0;
     H_PLUS =1e-7;
     H2O    =55.5;
     OH_MIN =1.0e-7;
ei;

REPEAT LIST;
H_PLUS = 10E-11,10E-10,10E-9,10E-8,10E-7,10E-6,10E-5,10E-4,10E-3,10E-2;
REND;

# Simulate up to the time point x;
timeend = 1000;

print_sim TIME,OH,H_PLUS (habwe2ir/2);

END;
```

Figure 5.5: SCAMP command file for the simulation of the Haber-Weiss cycle

.

With the cycle, however, there will be another term added to the previous equation, of the form:

$$\frac{\delta[OH^{\cdot}]}{\delta t} = k_1[H_2O_2][O_2^{\cdot -}][H^+] - k_2[OH^{\cdot}][H_2O_2]$$

. For the Koppenol variant, and:

$$\frac{\delta[OH^{\cdot}]}{\delta t} = k_1[H_2O_2][O_2^{\cdot -}] - k_2[OH^{\cdot}][H_2O_2]$$

for the Hill version, where k_1 and k_2 are the kinetic constants for the first and second reactions of the cycle. The effect that these equations will have on hydroxyl radical concentrations is: in the case of the model in the previous section, the concentration of the radical tended to increase continually (unless the system is closed); in the present form (considering the two reactions) it is not linear to predict what might be the behaviour of the hydroxyl radical.

The set of simulations for both of the command files presented in Figure 5.4 and 5.5 follow the same strategy as for the Haber-Weiss reaction apart from those treating all metabolites as internal. It was decided to carry straight on with the conditions where both hydrogen peroxide and radical anion superoxide are considered external, implying a constant biological source. The simulation time was set to 1000 seconds.

At this point, in order to gauge to what extent the reactions can be considered reversible, two more SCAMP command files were created, including the reversed reactions of those found in Figure 5.4 and 5.5. This created a problem as there were no kinetic constants available for the reversed reactions, and so these had to be calculated using, either the Nernst equation when the standard potentials are available,

$$\delta E^{\theta} = -\frac{RT}{nF} ln(K)$$

or,

$$\delta G^{\theta} = -RT ln(K)$$

when using standard free energy information. They were calculated based on data provided by Singh [179] and Koppenol [117] in the form of Gibbs free energies, electron potential and equilibrium constants. All the simulations done using these command files revealed

k $M^{-1}s^{-1}$	$[OH^{\cdot}]M$	
	Koppenol	Hill
1.0×10^{-4}	4.3×10^{-30}	4.3×10^{-23}
76	3.3×10^{-24}	3.3×10^{-17}
4.7×10^{3}	2.0×10^{-22}	2.0×10^{-15}

Table 5.5: Hydroxyl radical concentration at the end of a 1000s run at different settings for the rate constant of the first step of the Haber-Weiss cycle and for the two models presented above

that there was no difference between the two forms of reactions (reversible or irreversible) making a default choice of irreversibility when dealing with such reactions acceptable.

Table 5.5 summarises the results for the Haber-Weiss cycle. The difference in the concentration of hydroxyl radical between the two models is a factor of 10^7 that is due to the involvement of hydrogen ion in one of the models (the pH is 7). These results are also in the same order as those obtained for the Haber-Weiss reaction.

5.2.3 Fenton-type reactions

The problems associated with this type of reaction have been detailed in Chapter 1. These reactions were first proposed by Fenton [39] when he detected high reactivity in solutions containing both iron salts and hydrogen peroxide.

The role of iron salts has been claimed to be that of a catalyst for the Haber-Weiss reaction. The reactions detailed below illustrate the postulated role for the iron ions:

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

$$H_2O_2 + Fe^{2+} \longrightarrow OH^{-} + H_2O + Fe^{3+}$$

If the two reactions above are added together the Haber-Weiss reaction is obtained with the consequent elimination of the iron.

Two separate approaches can be used in developing a model for studying this process. The approach can include the action of a catalyst in an implicit way, or can describe it explicitly. The first option does not necessarily entail writing a new SCAMP file, and in some ways has already been dealt with while simulating the Haber-Weiss reaction. The effect of the catalyst on the reaction can simply be emulated by increasing the kinetic constant for the reaction. Some simulations were undertaken with the SCAMP command

```
Title Fenton Reaction ;
# Study of the reaction in isolation;

# specify simulation;
Simulate;

# declare the floating metabolites;
Dec
   OH, Fe_plus3, Fe_plus2 ;

# define the reaction network;

Reactions;
        $O2_MIN + Fe_plus3  - $O2 + Fe_plus2 /k1/;

        $H2O2  + $Fe_plus2 - OH + $H2O + Fe_plus3 /k2/;
eor;

Initialise;
     k1  = 1.0e-4;
     k2 =  76;
     O2     =1.0e-4;
     O2_MIN =1.0e-11;
     H2O2   =3.0e-9;
     OH     =0;
     H_PLUS =1.0E-7;
     H2O    =55.5;
     Fe_plus3=1.0E-6;
     CSUM1   =1.0e-6;
ei;

# Simulate up to the time point x;
timeend = 1000;

print_sim TIME,OH  (fenton/2);

END;
```

Figure 5.6: SCAMP command file for the simulation of the Fenton reaction

k $M^{-1}s^{-1}$	$[OH\cdot]M$
76	1.2×10^{-22}
4.7×10^{3}	6.6×10^{-21}
1.0×10^{4}	1.4×10^{-20}

Table 5.6: Hydroxyl radical concentration at the end of a 1000s run for different scenarios of the Fenton reaction (simulation of different catalysts)

file given in Fig 5.3.

In order to include the action of a catalyst explicitly in the model a new SCAMP command file had to be created. This is detailed in Figure 5.6 where the kinetic constant for the reaction involving Fe^{2+} in this case and hydrogen peroxide was initially set at $76M^{-1}s^{-1}$ to simulate the effect of isolated iron ions but this value subsequently altered according to the value presented in Table 5.6 to account for the effect of chelated iron or copper ions.

For reasons akin to those justifying the study of the Haber-Weiss cycle rather than the single step reaction, another reaction was added to this model — completing the process of disproportionation of hydrogen peroxide.

The results obtained simulating these models were not dissimilar to those obtained for the Haber-Weiss cycle. this is expected because the effect of adding a catalyst is just to sped up the process. While the overall behaviour is the same the difference lies in the level of concentrations of hydroxyl radical produced. Whereas the concentrations obtained for the uncatalysed Haber-Weiss cycle were invariably smaller than $1.0 \times 10^{-22}M$, those for the current model were in the region of 1.0×10^{-22}–$2.0 \times 10^{-20}M$ depending on which catalyst is used.

5.2.4 The sulphenic acid hypothesis

Some work undertaken while developing my own models, and discussions with Pinto (pers. comm.) led me to believe that there is one other process which could lead to the production of hydroxyl radical in biological systems. A very important metabolite, normally designated an antioxidant in biological systems is glutathione (GSH). As detailed in Chapter 3 this species has been detected in certain circumstances as having a dual role — being a pro-oxidant at the same time as being an antioxidant — exactly as is the case with ascorbic acid. Even though this dual behaviour has been reported the mechanisms which cause it have not been discovered Pinto has claimed, however, that during the chemical

```
Title SULPHENIC-ACID ( report ) ;

# specify simulation;
simulate;

# declare the floating metabolites;
Dec
   OH , GS , GSOH ;

# define the reaction network;

Reactions;
        $GSH + $H2O2 - GSOH + $H2O /k16/;

        GSOH + $O2_MIN - GS + OH + $O2 /k25/;
eor;

Initialise;
     k16 = 1.0e5;
     k25 = 1.0e4;
     O2     =1.0e-4;
     O2_MIN =1.0e-11;
     H2O2   =3.0e-9;
     OH     =1e-30;
     H2O    =66;
     GSH      =1.0e-3;
     GS       =1e-30;
     GSOH     =1e-30;
ei;

print_sim TIME,OH,GS,GSOH (sulf.res/2);

# And don't forget the 'END' statement;

END;
```

Figure 5.7: SCAMP command file for the simulation of the sulphenic acid hypothesis

disproportionation of hydrogen peroxide

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$$

he detected behaviour which could signify a mechanism involving the intermediate production of sulphenic acid. This mechanism would be of the form:

$$GSH + H_2O_2 \longrightarrow H_2O + GSOH$$

$$GSOH + GSH \longrightarrow GSSG + H_2O$$

He also pointed out that, in normal circumstances, the sulphenic acid produced in the above reaction would readily react with more glutathione leading to the production of oxidized glutathione (GSSG) and water.

The sulphenic acid hypothesis is based on the possible lability of the sulphenic molecule. It was proposed that this molecule could either undergo homolytic fission producing hydroxyl radical as:

$$GSOH \longrightarrow GS + OH$$

or react with increased concentrations of radical anion superoxide (occurring in the initial stage of oxidative bursts) emulating a Haber-Weiss type reaction of the form:

$$GSOH + O_2^{\cdot -} + H^+ \longrightarrow GS + OH^{\cdot} + OH^-$$

In the light of this hypothesis another SCAMP command file was written incorporating the reactions as shown in Fig 5.7.

Although the definition of the reaction posed no problems the same cannot be said for the associated kinetic constants. A thorough search through the literature did not reveal any information that could lead to the definition of this values. Consequently the kinetic constants had to be set arbitrarily, using the values shown in Fig 5.7.

With these conditions this system seems to be a lot more efficient in producing hydroxyl radical than the Haber-Weiss reaction. This result has to be analysed with care as not only the assumed rate constant values are already higher than those normally accepted for the Haber-Weiss reaction, but also the metabolites participating in these reactions are also present in much higher concentrations (normal glutathione levels in cells are in

the order of milimollar compared to nanomollar for hydrogen peroxide and even lower for the radical anion superoxide), contributing with a bigger factor when integrating the differential equations.

Notwithstanding the information gained from this model I still felt that, due to the lack of supporting experimental evidence, this line of research remains hypothetical.

5.3 Discussion

There is overwhelming evidence concerning the range of hydroxyl radical concentrations obtained from the simulations, namely that whatever the conditions and reactions simulated these concentrations were always very small (generally $< 10^{-12}M$).

In order to reflect two types of biological situations simulations were undertaken with a time interval either of 1000 seconds or of 1 second or less. The latter choice was intended to emulate the events at the beginning of an oxidative stress scenario. Whereas the concentration of hydroxyl radical can be found with a range from 10^{-20} to $10^{-12}M$ with a time interval of $1000s$ the concentration range for the shorter time simulations was always below $10^{-20}M$, in some cases less than $10^{-23}M$. At first sight the results indicate problems with the schemes proposed for $OH^{\cdot}$ generation, but, the very low values of the concentrations themselves cast doubt on any conclusions that could be drawn at this point.

These values have to be interpreted with care, taking into account the type of system, application or environment under study.

Both the concept of concentration and the deterministic approach used for this type of simulations are based on statistically representive populations, which means that how both reactions and concentrations are interpreted, is dependent on an average of the total amount of events that can occur as well as the actual amount of molecules present at any time in the system.

When the system being studied is of a biological size (simulation of events in cells, for example) the total volume available within the system is in the order of $10^{-12}dm^3$. When volumes of this size are under consideration the minimum concentration (corresponding to the presence of 1 molecule) of any substance possible is $10^{-13}M$. If a metabolite concentration falls below this value, it means that on average there is no molecule of that metabolite within the system — and in most of the simulations this was the case for the hydroxyl radical. One other possible implication of this result is that when the

concentration falls bellow the level of 1 molecule in the working volume this has to be considered as a 'time average' concentration, which creates a contradiction in the use of deterministic simulation. The method uses the 'time average' concentration to predict a continuous reaction and production of products, whereas this, based on the resulsts obtained, must of necessity be intermittent.

When the hydroxyl radical concentration falls below $10^{-13}M$ the numerical methods used ceased to be valid, and the same can be said of the deterministic approach in general. For these reasons a new type of mathematical approach needs to be utilized.

A new approach to simulating systems with such small concentrations will need to tackle individual events and molecules rather than concentrations. A methodology that can handle isolated rather than continuous events is more appropriate for the investigation of the steps leading to the production and subsequent interconversion of hydroxyl radical. Such an approach is presented in the following chapter.

Chapter 6

Monte Carlo modelling

The time evolution of a chemically reacting system is not a continuous process, as molecular population levels can only change by discrete integer amounts. However, it is a valid approach to consider chemical reactions as deterministic processes described by known chemical rate laws. This approximation is acceptable when dealing with systems containing molecular population levels which collapse to very low numbers and which can be considered homogeneous. Although spatial inhomogeneity might be adequately modelled by partial differential equations, this approach would not be valid under current scenario. Just as with other intensive variables such as temperature and pressure that are macroscopic representations of population averages, so with concentration the question is when is there enough molecules for it to make sense to define concentration? In a volume element small enough to molecules to collide and diffuse rapidly on the 'sampling' time scale (in this case the 'sampling' is by chemical reaction) there must be enough of the molecules for the variance to be smaller than the mean value.

The scale of concentrations encountered in these simulations leads to problems because the deterministic approach assumes the continuity of changes in chemical concentrations. It is therefore more appropriate to consider these systems with extremely low concentrations as discrete, because the number of molecules can only change by integral amounts, and study the changes in population levels rather than molar concentrations. The necessary approach for studying this kind of problem is stochastic because the random fluctuations are a significant component of the behaviour.

6.1 Choice of a suitable method

6.1.1 Simple Monte Carlo simulator

The first step in the process for adapting the models previously developed to a Monte Carlo simulation was to use a program already available. A computer program for the study of enzyme-enzyme interactions had been devised by Fell, "Interact" (personal communication), but no "off the shelf" stochastic reaction simulator were known to exist at this time. The program had been developed to simulate the random diffusional movement of the particles constituting a system, and when molecules approached closely enough a reaction could occur (depending on its probability). The system volume is discretised into a $3D$ grid, and, for a specific set of initial parameters (initial concentrations transformed into number of particles; kinetic constants; and diffusion coefficients), the procedure of simulation is:

1. assign a random spatial distribution of the molecules;
2. determine:
   - reaction probabilities;
   - diffusion probabilities;
3. run through the spatial grid and randomly move the molecules;
4. for each reaction: if any molecules of a particular reaction are in the same cell: make reactions occur randomly (if the random number generated at the time is bigger than the probability for the reaction);
5. increment the time step, and
6. repeat the last three items until the time chosen by the user is reached.

Problems

The first attempts to use the program for the *Haber-Weiss cycle* revealed some serious problems: with the same initial set of parameters as those used previously with SCAMP, the determined probability for the first reaction of the cycle was of the order of 10^{-12}, requiring a very long time for the simulation. However, it was later noticed that the

random number generator used could for numerical reasons never take a value lower than 10^{-7} , which in turn made the problem impossible to solve as the first reaction of the cycle would never occur.

Several other random number generators, such as those proposed by Sedgewick [174] and Press [157], were implemented and tested for the range of values they produced so that their usefulness for these problems could be established. A review of the field by Marsaglia [132] provided further information about the several types of random number generators available and possible means for testing their randomness. He also gave a table of results for the tests he applied to the different types of random number generators, showing how dangerous the use of such sequences can be without previous knowledge of their randomness.

None of the random number generators already available had a period large enough for the Haber-Weiss cycle to be studied. More specifically the period would have to include the possibility of generating a number between 1 and 10^{-12} for such reactions to be simulated. Other ways to deal with this kind of problem had to be devised. It was observed earlier, when using the Interact program, that the systems under study could be divided into two classes of reactions according to their calculated probabilities. One of the subgroups had quite high probability values (close to one) and corresponding frequencies, meaning that their ocurrence throughout the simulation was high. The other had very low probabilities (10^{-7} or lower), and as a result the chance of their occurring during the time of simulation was slight, indicating that, in order for at least one of them to occur, extremely long periods would have to be simulated. The problem is similar to that encountered with the deterministic integration of stiff ODEs — to accurately follow the progress of the fast reactions requires time steps so short that the number of iterations required to follow the slow processses becomes impossibly large. It was thought that, due to this difference between the two groups, both deterministic and stochastic approaches could be incorporated in the same program. Unfortunately this also proved difficult to achieve, as the set of decisions that had to be defined in the program for the interaction between the two approaches during the simulation would generate a source of ambiguity. Such decisions would have to include: how low a concentration of a metabolite would have to be in order to be interpreted as a number of particles and so dealt with by the stochastic method; how frequent, or how probable, a reaction would have to be for the

program to interpret it as stochastic or deterministic. There were also problems connected with reactions that had to be simulated with the stochastic approach, but for which the species involved had concentrations too high for this. This idea had to be abandoned and another way to approach the problem had to be found.

Several papers dealing either with problems of rapid bimolecular reactions or diffusion-controlled reactions proved to be too complex and unsuitable: the former are mainly directed towards the determination of rate constants for example Keiser [103] and the latter more suited for highly detailed model systems like those developed by Northrup [155].

6.1.2 The importance of random number generation

A new class of random number generators introduced by Marsaglia [133] solved the problem of the necessary period of random numbers. He transcribed the mathematical definition of the generator into the appropriate code and distributed it as a package named ULTRA, with instructions enabling the use of this implementation within different computer languages. This new random number generator has many useful properties lacking in other common generators, namely:

- extremely long period (more than 10^{356}, in other words more than 10^{270} numbers for each atom in the universe, in case someone wanted to simulate creation!);
- combines two different types of generators, to achieve a very thorough mixing;
- very fast;
- random bits, bytes, 16 or 32 bit words, single or double precision real numbers are all available;
- single precision reals (by far the most common) are guaranteed to have full precision in the fraction (mantissa);

Almost all generators produce reals by dividing a 32 (or 31) bit integer by 2^{32} (or 2^{31}). This means the smallest possible random reals are small multiples of 2^{-32}. ULTRA will produce random reals down to 2^{-50} or smaller with the proper frequencies. As a result it is impossible to get a 0, avoiding the rare, but irritating, program-stopping situation that arises from taking a logarithm of, or dividing by, zero.

The principal component of ULTRA is the Subtract-with-Borrow (SWB) generator that is described in the paper "A New Class of Random Number Generators" [133]. This uses a 148 byte seed array to obtain an astronomically large period, while satisfying all the usual theoretical and experimental tests for randomness.

The other component of ULTRA is the congruential generator with multiplier 69069 and base 2^{32}. This is a very well known, reliable (but short period) generator, tried and tested. It is, for example, the generator built into VAXs. The results of both of these generators are xored to provide the bytes which form the output of the ULTRA random number generator [133].

Some tests were performed before the implementation of ULTRA in Interact to check the production of numbers in the order of 10^{-12}. These were successful — for each 10 billion (10^{10}) calls to the generator a value smaller than 10^{-10} was presented. The initial hope of obtaining some useful results with this within Interact were soon dashed, as even when using large time periods nothing seemed to happen (the number or the concentration of all metabolites remained unchanged). The main problem was that the first reaction of the *Haber-Weiss cycle*, as already mentioned, has a probability below 10^{-10}, making the probability of its occurring only one in every 10 billion calls to the random number generator. These calls were being made not only to check the occurrence of reactions, but the movement of the molecules was also simulated on a random basis, so no one could know when the specific probability would be achieved. To avoid waiting for that reaction to occur, some alterations to the way the program started the simulation were added:

- the user was allowed the possibility of making a reaction with a low probability occur, either at the initial step ($t = 0s$) or at any step necessary;
- the possibility of making a certain molecule appear was allowed even if its concentration was initially set to zero.

Having chosen to have the first reaction of the *Haber-Weiss cycle* occur at the beginning of the simulation to try to set the cycle going still did not give any results apart from a temporary formation of hydroxyl radical which was quickly consumed in the following time steps. A similar result was obtained when initiating the simulation with a non-zero concentration of hydroxyl radical, which was consumed during the following steps of the process. While these results illustrate the high reactivity of dioxygen free radicals, and do

not in themselves constitute a problem, I felt that this simulation had shed no light on the main issue under study, namely: determining the initial source of hydroxyl radical.

One can probably say that the cycle as it is cannot be responsible for the production of OH radical, as even in the event of some being produced by the first reaction it is consumed as soon as it appears by the second reaction.

Even with the implementation of ULTRA in "Interact" this approach proved unsuccessful. The reasons for this were that the available time periods for simulation were far too short (only up to a millisecond). The system has to be able to simulate for much longer periods for the study of the *Haber-Weiss cycle* — and any other additional free radical reactions — to be feasible.

The problems with the time scale were much more pronounced when the cycle was coupled with the other six reactions that comprise the block of free radical interconversion. As at this stage the program did not allow the user to set a time for the simulation bigger than a millisecond, a thorough study of this system was ruled out.

6.1.3 Bunker and Gillespie's simulation method

In the light of the problems detailed in the previous chapter, and those specific to "Interact", as mentioned above, I began to consider a new method — the Bunker and Gillespie method — put forward in Moore [149] and revised by Edelson [34]. The method was developed by Bunker [15] and separately by Gillespie [53, 54]. It is much simpler to implement than the ODE technique, albeit expensive for large systems (longer time to converge to a solution implies higher computation costs) [34].

The main difference between this method and that used in "Interact" is that there is no spatial assignment of the molecules, and also no simulation of random movement. While "Interact" includes two levels of probability determination, namely simulation of movement and likelihood of reaction occurring, Bunker and Gillespie only utilise the latter. One could say that this would be a backwards step, as all the spatial information, and the explicit simulation of diffusion and movement, is lost, but this has to be balanced with gains in the speed and specifiable duration of the simulation.

The main features of the Bunker and Gillespie method as proposed in Bunker [15] are:

- very simple;
- very fast (compared with other stochastic methods);

- adjustable cost-resolution scale;
- no stability problems;
- time does not advance in prescribed steps;
- the calculation ends cleanly when zero reaction probability is attained.

While several of these features are self explanatory two of them are worthy of more detailed comment. Firstly — the adjustable cost-resolution scale means that the user, by controlling the number of particles present in the system and the number of events for the simulation (dependent on the duration of the simulation), will be able to control the precision of the method. In other words, the number of particles can be minimised in order to maximise the precision of the simulation, which ultimately decreases computational costs by shortening run time. Secondly, and the most striking difference between the stochastic and deterministic approaches, is the fact that the calculation ends cleanly when zero probability is attained. Put in another way — a reaction will only occur if all the reactants are present in the system. As soon as there are no molecules of any metabolite all reactions containing that metabolite will cease to function. If this were to happen for enough reactions in the system the simulation could stop before completing the specified simulation time, whereas a deterministic approach will always simulate for all the time specified. This is because a deterministic approach uses concentrations rather than number of particles, and a concentration can always increase or decrease by infinitesimal amounts - and therefore won't ever be considered to be zero. Obviously the Bunker and Gillespie approach is more true to life — if there are no molecules of a substance it cannot be involved in a reaction, and the Bunker and Gillespie method reflects this.

Development of a program

I had to develop a computer program as software integrating the Bunker and Gillespie approach did not exist. The algorithm is based on the assumption that there is a small reaction vessel, of volume V, containing a certain number of molecules (normally between hundreds and thousands) that are not spatially assigned (this latter point being one of the biggest differences between Bunker and Gillespie and the other stochastic approaches).

The program is based on the Bunker and Gillespie method the volume V is considered to typify the system as a whole, then:

$$X_i / \sum X_i = c_i / \sum c_i$$

$$V = \sum X_i / N . \sum c_i$$

where N is Avogadro's number, X_i the number of molecules of each species and c_i their respective concentrations. The instantaneous number of events for reaction j involving species i and k is then defined by:

$$a_j = k_j X_i X_k / (NV)^{s_j - 1}$$

where s_j is the order of the j^{th} reaction. The probability is defined as:

$$p_j = a_j / \sum a_i$$

the elapsed time, t, as:

$$t = (1 / \sum a_i) * \ln(1/r_2)$$

and the reaction number, m, of the next event to occur being determined by the inequality:

$$\sum_{i=1}^{m-1} p_i < r_1 < \sum_{i=1}^{m} p_i$$

where r_1 and r_2 are random numbers between 0 and 1.

My program works by initiating the values of reaction frequencies (probabilities) then by determining which reaction occurs (using the equations above) and finally updating the number of molecules present in the system with their new values. This process is repeated until the specified simulation time is reached or zero reaction probability is attained.

The necessary input for the program must be in the form of an itemised reaction list (nominated stoichiometry file[1]), kinetic information, metabolite concentrations (which are

[1]So called because it was originally a stoichiometry file, in order to make the new program compatible

transformed into numbers of molecules by the program, depending on the volume of the system), volume of reaction vessel and specified simulation time (nominated datafile).

The output information is presented as two files, both containing a copy of all the input data, initial reaction probabilities, molecule numbers and the stoichiometry matrix. The first file also comprises a list of the molecule numbers for each metabolite at specified time intervals (nominated concentration file) and the second includes the fluxes through all the reactions during the simulation (nominated flux file) — this last file can be easily altered to include more data if so desired. A serious hindrance was encountered in determining the specified time intervals, which the program calculates using the total simulation time and the initial number of events predicted to occur. However, the number of events due to occur is dependent upon the composition of the system which is changeable throughout the simulation, and so the number of events cannot be stated in advance nor the time intervals. One consequence of this was the difficulty in defining the output intervals so that no information would be lost during the simulation as some of the time intervals were obscuring events which had already happened. A solution to this has come to my attention recently through the internet in the form of a program provided by IBM Corporation (CKS – Chemical Kinetics Simulator) [26].

In the end the name chosen for my program was MCARLO and its general characteristics are:

- it was written using the non-standard Pascal language;
- it can be compiled with the Turbo Pascal compiler (version 6 or 7) provided by Borland International;
- it will run on any IBM PC or compatibles without any demands on specific processers.

The program will be made available through our local FTP site (bmsdarwin.brookes.ac.uk), although, because the development of the program was not the main goal of this reaserch, it does not have a user-friendly interface

```
R1:S1+S2=S3
R2:S3=S1+S2
R3:S3=S4+S1
```

Figure 6.1: Input file for MCARLO based on the Henri-Michaelis-Menten reaction scheme, where S1 is the enzyme, S2 the substrate, S3 the enzyme substrate complex and S4 the product.

```
Henri-Michaelis-Menten test
8.0e8
2.0e3
2.0e3
2.5e-8
4.0e-5
0
0
1.0E-15
4.0E-2
80
```

Figure 6.2: Data input file for MCARLO defining the necessary parameters for the reaction scheme presented in 6.1. the first line is the title for the simulations, the next three set the values for the rate constants, the following four the metabolite concentrations from S1–S4 and the last three lines define the volume, the time for the simulation and the number of output points required.

```
DATA OUTPUT FILE FROM:
                     testp.sto
                     testp.dat


MODEL DATA SUMMARY:
===================

STOICHIOMETRY MATRIX:
-1 1 1
-1 1 0
1 -1 -1
0 0 1

MATRIX IN:
-1 0 0
-1 0 0
0 -1 -1
0 0 0

KINETIC PARAMETERS:
             k           freq          prob
R[1]     2.0E+0008     7.7E+0007     6.7E-0001
R[2]     2.0E+0003     1.9E+0007     1.7E-0001
R[3]     2.0E+0003     1.9E+0007     1.7E-0001

INITIAL CONCENTRATIONS:
 2.5E-0006 9632
 4.0E-0005 154112
 2.5E-0006 9632
 0.0E+0000 0

Volume   1.0E-0015
Runtime  4.0E-0002
deltat   2.0E-0008
```

Figure 6.3: Sample of the header of the output file for the Henri-Michaelis-Menten model. The input files used for the generation of this header can be checked at the top of the figure, after which all the input data (topology and parameter values) is echoed alongside with the first calculations for the setting of the stochastic quantities (number of molecules, frequency of the process and initial probability)

Testing of the program

So that the performance of the Bunker and Gillespie program (and method) could be tested and compared accurately with that of "Interact" the simulations the author of "Interact" originally used to test his program with were utilised (Fell, personal communication). The model consists of the following three reactions:

$$Enzyme + substrate \longrightarrow Enzyme - substrate - complex \tag{6.1}$$

$$Enzyme - substrate - complex \longrightarrow Enzyme + substrate \tag{6.2}$$

$$Enzyme - substrate - complex \longrightarrow Enzyme + product \tag{6.3}$$

These reactions have already been mentioned in this thesis (Chapter 3) as a suitable model for testing the deterministic tools under study and represent the simplest Henri-Michaelis-Menten type of reaction between an enzyme and its substrate. This can easily be simulated with MCARLO by first writing the 'sto' file shown in Figure 6.1, and then creating another file containing all the necessary parameters, shown in Figure 6.2. When this information is passed on to the program, this will write two types of output files one containing the concentration changes and the other the flux change, however these two files contain the same type of header information as it is illustrated in Figure 6.3.

An exact solution for the V_{max} of this system can be determined and compared with the results from simulation. Using the method described above a simulation was performed for $4 \times 10^{-2}s$ with kinetic constants of $8 \times 10^{8}1/Ms$ for reaction 6.1, $2 \times 10^{3}1/s$ for reactions 6.2 and 6.3; initial concentrations of $2.5 \times 10^{-8}M$ and $4 \times 10^{-5}M$ for enzyme and substrate respectively. The exact solution for V_{max} at $v_{0.02}$ is $3.31 \times 10^{-5}M/s$, and the rate determined with the data from the simulation is in average the same. The program was also tested with the models proposed in Gillespie [54] and both the time profiles and the final numerical values correspond exactly to those obtained by Gillespie. This proves the accuracy and efficiency of the Bunker and Gillespie implementation and its suitability

with "Interact", so that files could be interchangeable, allowing genuine comparison. It was subsequently upgraded to its present form as an itemized reaction list both to make it more user friendly and to allow for the reaction lists to be interchanged with those of other deterninistic programs, for example SCAMP.

for simulating unknown systems.

One more test to the program was made by trying to repeat one of the simulations Gillespie had made. He claimed that Malek-Mansour and Nicolis had made a mistake when they proved that the following system of reactions:

$$X + Y \longrightarrow 2Y$$

$$2Y \longrightarrow Z$$

did not have a stable steady-state and that would be a proof that the stochastic approach had destroyed the stable solution of the deterministic system [54]. However, Gillespie presented a graph showing that even starting the simulation from two totally different initial conditions the same steady state would be reached [54]. As this result illustrates the suitability of the method I did the same simulations using my program (MCARLO) and did the same with a deterministic tool (SCAMP). By translating the results of the deterministic simulation into number of molecules it was possible to superimpose the results obtained with both approaches into a single graph shown in Figure 6.4.

Possible applications and criticisms

Other researchers have applied the Bunker and Gillespie method to a number of different problems, ranging from the study of evolution [49] through the analysis of very rapid kinetics [131, 120], to the study of molecule structure formation [64, 28]. In addition to the properties of the method listed by Bunker (detailed above) it is important to stress its suitability for analysing systems with large time and/or space fluctuations [201]. Edelson, in a review of the available numerical methods for simulating chemical reactions [34], presents the Bunker and Gillespie method as an exact stochastic simulation algorithm and points out its suitability to the "time dissipative structure" of coupled reactions. On the other hand Zhang [201] demonstrates the suitability of this method for analysing systems where diffusion is an important factor. However he also points out the limitations of the method — namely that simulating a model containing a large number of compartments becomes inefficient (takes a long time — although how long is never specified nor compared with other methods). This potential limitation was discussed by Gillespie in the original paper [53] where he warns that the algorithm was not originally built to deal with

Figure 6.4: Superimposition of two stochastic simulations (gill2a, gill2b) and two deterministic simulations (gill2as,gill2bs), starting from two different states. The initial conditions were as follows: $k_1 = 1.93 \times 10^9 M^{-1}s^{-1}$; $k_2 = 9.64 \times 10^6 M^{-1}s^{-1}$; $X = 2.59 \times 10^{-9} M$ (or 10 molecules; $Y = 0$ or $7.80 \times 10^{-7} M$ (0 or 3000 molecules); $Z = 0$; $t = 100s$ and $v = 6.4 \times 10^{-15}$.

Number	
1	$O_2^{\cdot -} + HO_2^{\cdot} + H^+ \longrightarrow {}^1O_2 + H_2O_2$
2	${}^1O_2 + {}^1O_2 \longrightarrow 2O_2$
3	${}^1O_2 + O_2^{\cdot -} \longrightarrow O_2 + O_2^{\cdot -}$
4	$H_2O_2 + H_2O_2 \longrightarrow {}^1O_2 + 2H_2O$
5	$H_2O_2 + O_2^{\cdot -} + H^+ \longrightarrow {}^1O_2 + OH^{\cdot} + H_2O$
6	$OH^{\cdot} + H_2O_2 \longrightarrow H_2O + O_2^{\cdot -} + H^+$
7	$O_2^{\cdot -} + H^+ + OH^{\cdot} \longrightarrow {}^1O_2 + H_2O$
8	$OH^{\cdot} + OH^{\cdot} \longrightarrow H_2O_2$
9	$HO_2^{\cdot} \longrightarrow O_2^{\cdot -} + H^+$
10	$LipidH + OH^{\cdot} \longrightarrow Lipid^{\cdot} + H_2O$
11	$Lipid^{\cdot} + O_2 \longrightarrow LipidOO^{\cdot}$
12	$LipidOO^{\cdot} + LipidH \longrightarrow Lipid^{\cdot} + LipidOOH$
13	$LipidOO^{\cdot} + LipidOO^{\cdot} \longrightarrow Lipid - Lipid$
14	$O_2^{\cdot -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$
15	$H_2O_2 + Fe^{2+} + H^+ \longrightarrow OH^{\cdot} + H_2O + Fe^{3+}$

Table 6.1: Set of reactions used for the simulations (see text for further information)

spatial inhomogeneities. This fact is not acknowledged by Zhang *et.al.* [201] suggesting that he probably did not read the original paper. Zhang goes on to describe a possible a possible modification to deal with the limitation, proposing an approximation to the original method, which itself results in increasing costs in computational terms.

6.2 Application to dioxygen free radical simulations

The simulations using Bunker and Gillespie's method followed the same development as that detailed in the previous chapter, namely: the study of the Haber-Weiss reaction, the cycle, the Fenton reaction, and, when appropriate, the interconversion block, and lipid peroxidation. The first reason behind echoing this development sequence was to determine whether the Haber-Weiss cycle could be responsible for the production of hydroxyl radical, and to what extent. The intention was then to investigate which other forms of free radical are predominant in the system, in particular the possible occurrence of singlet oxygen. Once the species of free radicals present in the system are established, it should be possible to determine which species is responsible for initiating lipid peroxidation.

Table 6.1 contains a list of the reactions used throughout the simulations. More specifically: reaction 5 is the Haber-Weiss reaction, reactions 5 and 6 are those which constitute

the Haber-Weiss cycle and were used for the first set of simulations; the reactions 1 to 9 comprise the interconversion block (second set of simulations); while peroxidation involves the full set (1 to 13); reactions 14 and 15 were used instead of reaction 5 when the effect of a metal catalyst (Fenton reaction) was simulated.

The kinetic parameters for all the reactions are the same as used for the deterministic simulations. Their values will be presented again with all the initial concentrationsduring the model development. The value for reaction 5 was chosen to be large enough to allow the process to occur at least once during the time specified for the simulations and so show which process competes most effectively for the hydroxyl radical.

In addition to the necessary procedure that is common with setting up a simulation model for the deterministic approach, there is in this case an extra parameter, volume V, that needs to be set for the stochastic approach. There are two reasons which explain this:

- the need to transcribe the concentrations normally used during the deterministic studies into numbers of particles (molecules, ions and radicals) – this number is obtained by multiplying the concentration for that species by the volume V of the system and by the Avogadro's number;
- the basis of the stochastic approach – when this technique is used the system under study will normally be of microscopic dimensions typifying an arbitrary reaction vessel, or in this project, the volume of a cell. The chosen value for V was of $10^{-12}l$ [12] and represents the average volume of a liver cell.

6.2.1 The Haber-Weiss reaction

In order to simulate reaction 5 (the Haber-Weiss reaction) using a Monte Carlo approach the following conditions were assumed: $10^{-9}M$ for H_2O_2; $10^{-11}M$ for O_2^-; $10^{-7}M$ for H^+; the initial kinetic constant was set at $10^{-4}lmol^{-1}$ and 10^6s was the designated simulation time. With the above conditions this reaction automatically has a probability of 1 (reaction probability is defined in the section describing the method), making this event possible within the chosen time period. Another aspect of the simulation is that the reaction occurs only once because of its associated frequency of 1.2×10^{-12}. In order to make this event occur more than once it is necessary to increase the simulation time to a value greater than $10^{12}s$ — the simulation time would have to be extended one million times for the reaction to occur a second time, which demonstrates how rare this event is. Repeated

k ($M^{-1}s^{-1}$)	Number of events
1.0×10^{-4}	7.8×10^{-7}
1.0×10^{-3}	7.8×10^{-6}
1.0×10^{-2}	7.8×10^{-5}
0.1	7.8×10^{-4}
1.0	7.8×10^{-3}
1.0×10^{1}	7.8×10^{-2}
1.0×10^{2}	0.78
1.0×10^{3}	7.8

Table 6.2: Effect the rate constant for the Haber-Weiss reaction has on the possible number of events during the simulation

simulations with the same conditions showed hydroxyl radical being produced at different points in time, demonstrating the value of the stochastic approach.

So that changes to the reaction, such as the action of a catalyst, for example, could be emulated, a set of simulations were run with kinetic constant values increasing to $10^3 lmol^{-1}$. The results obtained by this are summarized in Table 6.2.

Only when the kinetic constant was set to 10^3 were more than three hydroxyl radicals produced in a simulation — in fact six were produced. This meant that all the radical anion superoxide in the system was consumed. Despite the calculated number of events for the simulation being 7.8 the lack of further reactants limited the actual events to six. This result is therefore an important illustration of the efficacy of the Monte Carlo approach.

The results illustrate the possibility of the production of hydroxyl radical by the Haber-Weiss reaction at a random moment in time — even with the kinetic constant set to the lower value 10^{-4} proposed in the literature. Questions remain, however: to what extent is this result biologically significant; how far can hydroxyl radical diffuse within the system and how effective will it be in initiating other processes?

There is no dount that the stochastic simulation suggests that production of a hydroxyl radical wull be a rare and very transient event in an average cell.

6.2.2 The Haber-Weiss cycle and the production of $OH^{\cdot}$

The Monte Carlo method was next applied to the Haber-Weiss cycle, keeping all the initial parameters as they were before (summarized in Table 6.3. Having two reactions rather than one at first suggests different calculated probabilities to those of the Haber-Weiss reaction. While this is apparently a logical expectation, the initial concentration

Metabolite	Concentration ($moll^{-1}$)
O_2^-	1.0×10^{-11}
H_2O_2	3.0×10^{-9}
H^+	1.0×10^{-7}
$OH^\cdot$	0
H_2O	0
$^1O_2$	0
Reaction	k ($M^{-1}s^{-1}$)
5	1.0×10^{-4}
6	2.3×10^7
Volume	$1.0 \times 10^{-12} dm^3$
Time	$1.0 \times 10^6 s$

Table 6.3: Initial concentrations and kinetic parameters for the study of the Haber-Weiss cycle and hydroxyl radical production

k ($M^{-1}s^{-1}$)	pH	Approx time for $OH^\cdot$ (s)
1.0×10^{-4}	4	1.0×10^{15}
	7	1.0×10^{18}
	10	1.0×10^{21}
10	4	1.0×10^{10}
	7	1.0×10^{13}
	10	1.0×10^{16}
100	4	1.0×10^9
	7	1.0×10^{11} - 1.0×10^{12}
	10	1.0×10^{14} - 1.0×10^{16}
10^3	4	1.0×10^7 - 1.0×10^7
	7	1.0×10^{10}
	10	1.0×10^{13}

Table 6.4: Rate constant for the first reaction of the cycle (5), pH and the necessary time for producing the first $OH^\cdot$ during the simulation.

of hydoxyl radical being zero means that the second reaction probability begins by being zero. Consequently the first event to occur within the simulation is always the first reaction — therefore having the same calculated probability as the Haber-Weiss reaction (which is one).

After the occurence of the initial event the probability values change as hydroxyl radical enters the system, invariably yielding a probability very close to one for reaction two — and so the disproportionation is always the second reaction within the cycle.

The differences encountered in comparison with the Haber-Weiss reaction were that the time interval needed for production of hydroxyl radical was in the order of $10^{18}s$, making the first reaction of the cycle the only event ever to occur within the simulation,

and when more than one event occured in any simulation there was always consumption of hydroxyl radical rather than accumulation. In each simulation a single hydroxyl radical was always produced and this fact can be translated into a concentration of 1.6×10^{-12}.

I then investigated what effect changing either the medium pH, or the kinetic constant, for the first stage of the Haber-Weiss cycle would have on hydroxyl radical production. The results obtained are summarized in Table 6.4 and are much as predicted: increased pH slows down production of hydoxyl radical while a pH decrease speeds it up.

Half-life study

In order to make study of events subsequent to hydroxyl reaction production possible, I extended the timescale for the simulations to 10^{20} seconds. The increased timescale simulations confirmed the prediction that hydroxyl radical is always consumed after its production. The question which now arises is how long it takes for the radical to be consumed. Two methods can be used to determine this: the first is based on the observation of stochastic time intervals; the second uses the deterministic equation defining the half-life of a species. During the simulations the ΔT for the occurrence of the second event varied between 0.1 seconds and around 50 seconds, giving a mean half-life of 20 seconds for the hydroxyl radical.

Considering that the concentration of hydrogen peroxide is much bigger than that of hydroxyl radical, being, in fact 1875 times greater, this reaction can be assumed to be first order with respect to the hydroxyl radical: $t_{\frac{1}{2}} = \frac{\ln 2}{k'} = \frac{0.69}{2.3\times10^{7}\times3.0\times10^{-9}} = 10$ seconds, being the deterministic half-life of the hydroxyl radical, and k' being the pseudo-first order kinetic constant for the second reaction of the Haber-Weiss cycle. Although this value has been calculated using deterministic conditions it agrees with the values obtained stochastically.

The diffusion constant for the hydroxyl radical is given as $2.3 \times 10^{-9} m^2 s^{-1}$ [31] and the distance any particle can travel is defined as:

$$l = \sqrt{6 \times D \times t}$$

where l is length, D is diffusion constant and t is the time. Given the above then

$$l = \sqrt{6 \times 2.3 \times 10^{-9} \times 10} = 3.7148 \times 10^{-4} m = 371 \mu m$$

This value is around 37 times bigger than the dimensions of the side of the cubic liver cell, which I have set at $10\mu m$.

The deterministic method, however, becomes non-linear when simulations using a different constant for the first part of the Haber-Weiss cycle were chosen. Namely, when k took values of 76 and 4.7×10^3 in order to account for the presence of catalysts. In these conditions the number of events producing hydroxyl radical during the simulation increased, contributing to a sharp decrease in hydrogen peroxide concentration that eventually becomes zero. Assuming pseudo-first order kinetics are no longer valid a different equation for the half-life calculation has to be used:

$$t_{\frac{1}{2}} = \frac{1}{k} \times \frac{1}{conc}$$

With $k = 2.3 \times 10^7$ and $conc = 1.6 \times 10^{-12}$ (assuming equimolar concentrations for both reactants), $t_{\frac{1}{2}} = 2.7174 \times 10^4$ seconds. Consequently l is $10^4 \mu m$. This value is a thousand times bigger than the given side dimensions for the simulations.

6.2.3 The interconversion block of dioxygen free radicals

The addition of reactions to the Haber-Weiss cycle was based on my previous model development, which was derived from Koppenol [115]. This set of reactions was studied to investigate the interconversion of the different radicals, that is those that could be responsible for the initiation of biological damage.

The initial simulations, using the conditions described in Table 6.5, showed that the Haber-Weiss reaction is unlikely to function as a source of hydroxyl radicals. The kinetic parameter for this reaction was varied between 1×10^{-4} $lmol^{-1}s^{-1}$ and 4.7×10^3 $lmol^{-1}s^{-1}$ to simulate the situations where no catalyst is present or when iron and copper are available.

Until now all the metabolites in the previous models were considered to vary during simulation, but it was necessary at this point to test two situations: at first all metabolites were considered internal but then, as a consequence of the first set of these simulations, some metabolites had to be considered to be external.

In a situation where the system is closed (i.e. all metabolites are internal) the timescale of all the events in the system made it extremely difficult to simulate long enough periods of time for any hydroxyl radical to be produced. Timescale for hydroxyl radical production

Metabolite	Concentration ($moll^{-1}$)
O_2^-	1.0×10^{-11}
H_2O_2	3.0×10^{-9}
H^+	1.0×10^{-7}
$^1O_2$	0
$OH^\cdot$	0
$HO_2^\cdot$	1.0×10^{-8}
O_2	1.0×10^{-4}
Reaction	k ($M^{-1}s^{-1}$)
1	8.5×10^7
2	1.0×10^{12}
3	3.6×10^7
4	1.0×10^{-10}
5	1.0×10^{-4}
6	2.3×10^7
7	1.0×10^{10}
8	5.5×10^9
9 direct	2.0×10^{-5}
9	reverse 1
Volume	$1.0 \times 10^{-12} dm^3$
Time	$1.0 \times 10^6 s$

Table 6.5: Initial concentrations and kinetic parameters for the simulation of the model containing the interconversion block of reactions

varies between 1×10^{11} and 1×10^{18} seconds and all the other events were observed as never having time intervals bigger than 1×10^4 seconds. Because of this the simulation would have to have $\frac{1 \times 10^{11}}{1 \times 10^4}$ events, equal to 10 million, before eventually producing hydroxyl radical. Another implication of this is that metabolites run out long before anything like that number of events had occurred. Therefore when all metabolites were considered internal an equilibrium state was reached when no hydroxyl radical could be produced within the timescale of the simulations.

The metabolites selected to be external were radical anion superoxide, hydrogen peroxide and hydrogen ion. The reasons for this are that it was necessary to maintain the pH at 7, and there are constant sources of both radical anion superoxide and hydrogen peroxide. Only when the kinetic parameter for the Haber-Weiss reaction was set to 1×10^6 $lmol^{-1}s^{-1}$ could any production of hydroxyl radical be detected during the simulation time. This value is even bigger than that normally considered when copper is catalysing the reaction (possibly also corresponding to a situation where iron is chelated to EDTA [72]).

Even though there is more than one reaction competing for hydroxyl radical (reactions

6 and 8 from table 6.1) the only process possible is that of reaction 6 because there need to be two hydroxyl radicals available for reaction 8 to occur, and this never happened. This result can not be stressed enough as one of the major differences between the stochastic approach used here and the deterministic simulation used in the previous chapter. The deterministic simulation would allow half a hydroxyl radical to react with the other half to produce half a H_2O_2 (reaction 8).

The current model illustrates the continuous production of radical anion superoxide and its transformation into hydrogen peroxide, which sometimes disproportionates to water and dioxygen with an intermediate production of hydroxyl radical and singlet dioxygen. However, this model is not sufficient to investigate the possibility of hydroxyl radical initiating biological damage, so some more reactions had to be added to the system where competition for the hydroxyl radical can be simulated. This means that there has to be one more reaction that could cause the initiation process so that simulations can be run to determine whether the initiation process occurs.

6.2.4 Lipid peroxidation

To simulate lipid peroxidation reactions 10 – 13 from Table 6.1 were added to the previous set and a similar simulation strategy was employed (the only difference was that the new system was never considered to be closed). The initial conditions for the simulations can be found in Table 6.6. It is important to note that the model at this point was assumed to be homogeneous and the lipid was designated an external metabolite (its concentration is four orders of magnitude bigger than any of the other metabolites, justifying this choice).

In the light of the problems encountered in simulating the production of hydroxyl radical, and also because I now intended to investigate which events compete to consume hydroxyl radical, I decided to set the kinetic parameter for the Haber-Weiss reaction to $1 \times 10^6\ lmol^{-1}s^{-1}$. Similarly to the previous model the simulations evolved with relatively large time increments until the occurence of a hydroxyl radical within the system. When this radical appeared it always initiated the peroxidation and consequently the time increments decreased more than 10,000 fold. This process competes efficiently for the hydroxyl radical — in fact in every simulation it consumes hydroxyl radical. This and the fact that the radical is always produced at different points in time for each simulation, demonstrating the randomness of the process, are illustrated in Figure 6.5.

Metabolite	Concentration ($moll^{-1}$)
$O_2^{\cdot -}$	1.0×10^{-11}
H_2O_2	3.0×10^{-9}
H^+	1.0×10^{-7}
$^1O_2$	0
$OH^{\cdot}$	0
$HO_2^{\cdot}$	1.0×10^{-8}
O_2	1.0×10^{-4}
$LipidH$	1.0×10^{-3}
$Lipid^{\cdot}$	0
$LipidOO^{\cdot}$	0
$LipidOOH$	0
$Lipid - Lipid$	0
Reaction	k ($M^{-1}s^{-1}$)
1	8.5×10^7
2	1.0×10^{12}
3	3.6×10^7
4	1.0×10^{-10}
5	1.0×10^{-4}
6	2.3×10^7
7	1.0×10^{10}
8	5.5×10^9
9 direct	2.0×10^{-5}
9	reverse 1
10	5.0×10^8
11	1.0×10^8
12	1.0×10^5
11	2.0×10^8
Volume	$1.0 \times 10^{-12} dm^3$
Time	$1.0 \times 10^6 s$

Table 6.6: Initial concentrations and kinetic parameters for the simulation of the lipid peroxidation model

Figure 6.5: The randomness of the onset of lipid peroxidation. Overlapping of 5 different runs with the model containing all 1–13 reactions from Table 6.1 with the initial conditions shown in Table 6.6. The simulations were all run up to the time point where 500 molecules of lipid peroxide (Lipid-OOH) had accumulated.

The change of process for hydroxyl radical consumption coupled wih a biologically more meaningful model meant that the hydroxyl radical half life, $t_{\frac{1}{2}}$, had to be recalculated. The new value is 1.38×10^{-6} seconds, decreasing the possible radius of hydroxyl radical action to $0.14\mu m$ which is a tenth of the given cell dimensions. These results are more biologically realistical, although the radius of action still seems quite large.

6.2.5 The two compartment model

It is not correct to consider a system with apolar substances homogeneous. For this reason, and based on Gillespie's proposal [53], I devised a process separating the lipid and aqueous phases, and including another step in the model to simulate the possible diffusion of hydroxyl radical from one phase to another.

The frequency of such processes are defined as [53]:

$$a_u = D_i A_{ll'}[X_{l,i}/V_l - (X_{l',i}/V_{l'})]/d_{ll'}$$

where D_i is the diffusion constant of species X_i (the hydroxyl radical), $d_{ll'}$ is the centre to centre distance between subvolumes V_l and $V_{l'}$, and $A_{ll'}$ is the interface area between these two volumes. The area of interface between the aqueous and lipid compartments was set as $10^{-8}m^2$ for a simulated aqueous volume of $10^{-15}m^3$, to represent the membrane content of liver cells [12]. The thickness of the aqueous layer was therefore $10^{-7}m$, which together with an assumed lipid layer thickness of $10^{-8}m$ gives a value for $d_{ll'}$ of $5.5 \cdot 10^{-8}m$.

As an approximation $X_{l',i}/V_{l'}$ was considered to be zero (all the hydroxyl radical is produced in the cytoplasm) and so the equation can be rewritten in the form:

$$a_u = K X_{l,i}$$

where

$$K = \frac{D_i A_{ll'}}{V_l d_{ll'}} = x.y \cdot 10^6 s^{-1}$$

During the simulations, whenever the Haber-Weiss reaction occurred, the next processes to occur were invariably the diffusion to the membrane followed by the initiation of the lipid peroxidation. One reason is that reaction 8 is intrinsically improbable since there is only one hydroxyl radical in the reaction volume for extremely brief periods relative to the time for which there is none at all. The other hydroxyl–consuming reactions have large

rate constants but also involve one other species, such as superoxide or hydrogen peroxide, whose concentration is low. Thus none of the other hydroxyl–consuming reactions in the aqueous phase of the model are fast enough to prevent the radical diffusing to the lipid compartment and reacting there.

6.3 Discussion

The results obtained from the Monte Carlo type simulations indicate that hydroxyl radical is accumulated when only one reaction is under consideration. However, in biological systems there are always several reactions competing for this radical, and so models containing one or more reactions that consume it are more realistic. In such a scenario whenever one hydroxyl radical is produced it is invariably consumed in the next event. It can be seen that the low concentrations of hydroxyl radical predicted by deterministic simulation correspond to a time average, with hydroxyl radical absent within a cell for far, far longer than it is present.

Depending on the model considered, hydroxyl radical is readily consumed by one of the following:

- during the second stage of the Haber-Weiss cycle;
- by the abstraction of a hydrogen atom from a lipid molecule for the initiation of lipid peroxidation;
- by the movement of hydroxyl radical out of the aqueous phase into the membrane compartment for lipid peroxidation.

A direct consequence of these results is that the number of radicals present in the system is only ever zero or one. As the model considers the volume of a liver cell to be $1 \times 10^{-15}\ l$ the number of radicals in the system translates to $0M$ or $1.661 \times 10^{-12}\ M$. These results demonstrate the discontinuity of the problems under study, and that certain reactions — however fast — can be eliminated from consideration in intracellular conditions because their reactants are effectively never simultaneously present.

These simulations also illustrate how many molecules of hydroxyl radical are necessary to initiate chain reactions — one.

The stochastic approach has proven to be both appropriate and successful in examining the detailed mechanisms leading to the production of hydroxyl radical, and its consequences. It gives an entirely different perspective on free radical reactions, illustrating the rarity and randomness with which hydroxyl radical will appear within a cellular volume.

The hydroxyl radical can survive long enough to diffuse to a lipid membrane in these simulations. Of course, water-soluble targets for $OH^{\cdot}$ have not yet been included (protein-SH, water-soluble antioxidants), and it would be interesting to investigate the relative frequency of consumption of $OH^{\cdot}$ by lipids compared with other cellular materials

Chapter 7

Conclusion

The work for this thesis has illustrated problems relating to the creation of models of dioxygen free radical reactions under biologically relevant conditions and their simulation. These include:

- the reaction, concentration and time scale values being spread across a wide range;
- the model structure having serious implications for the choice and efficacy of mathematical methods;
- the development of the models, and their complexity, having to correlate directly to the specific aims and objectives of the work in hand, making it extremely difficult to utilize models designed for other applications;
- the choice of methodology, for both model and simulation, having to reflect the scale of the investigation.

A deterministic approach implies continuity of change within the system under study, and is therefore only appropriate for the investigation of metabolite concentration profiles with statistically meaningful values. In other words the concentration changes are neither discrete nor unpredictable, instead being continuous over a period of time. For these reasons this approach was found to be inappropriate for the models developed for this thesis to study the initiation and consumption of hydroxyl radical.

A stochastic approach is based on the description of the fluctuations of molecular populations. This means that this is the method *par excellence* for the study of any system involving both large and very sparse concentrations at the same time. As sets of reactions

involving dioxygen free radicals can have concentrations ranging between 1×10^{-6} and 1×10^{-15} M within the same system, the stochastic approach is the only suitable method.

When a deterministic approach was used, the concentrations of hydroxyl radical encountered had to be carefully analysed so that their biological significance could be taken into account. Considering that only one radical in a liver cell means that the radical concentration is 1.661×10^{-12} M, any values smaller than this obtained during deterministic simulation of such a system have no biological meaning. It is true that methods exist to overcome this problem, but they are artificial and mask attempts to clarify which processes really occur, and under which conditions. Once a stochastic approach is used, however, these criticisms no longer apply.

Within this thesis, using the Monte Carlo method, I have demonstrated that:

- the change in the concentration (defined as number of molecules per unit volume) of hydroxyl radical within the systems under study is either zero (no change) or one, never taking non-integer values;
- when the reactants of some processes are not available these processes do not occur (a situation which is not correctely represented by a deterministic approach);
- the production of hydroxyl radical is always possible, however if there is no catalyst available it is highly improbable;
- one hydroxyl radical is enough to initiate biologically damaging oxidative processes, even if it has to diffuse into a membrane to do so.

The ongoing controversy concerning the biological importance of hydroxyl radicals stems from the polarization of the debate into two schools of thought: one which claims their chemical features preclude a biological impact, and the other which postulates a significant role, until now without a firm theoretical basis. This research resolves these apparently irreconcilable differences by showing that they are the product of inadequate modelling — resulting principally from the tendency of the deterministic approach to average a small number of highly significant events into an undetectably low background level. The modelling framework proposed here, however, presents a much truer picture, allowing the study of those catastrophic events which are otherwise hidden because of their extremely low frequency. Only through adopting this approach can scientists hope to study

the mechanisms by which extremely rare events, such as the generation of hydroxyl radical, initiate processes whose effects are so profoundly deleterious to biological systems.

Bibliography

[1] B.J. Alder, D.M. Ceperly, and E.L. Pollock. Quantum mechanical simulation of liquids. *Acc. Chem. Res.*, 18:268–273, 1985.

[2] RC Allen. Phagocytic leukocyte oxygenation activities and chemi-luminescence - a kinetic approach to analysis. *Methods in Enzymology*, 133:449–493, 1986.

[3] O.I. Aruoma, B. Halliwell, B.M. Hoey, and J. Butler. The antioxidant action of taurime, hypotaurine and their metabolic precursors. *Biochem. J.*, 256:251–255, 1988.

[4] C. F. Babbs and M. G. Steiner. Simulation of free radical reactions in biology and medicine: a new two-compartment kinetic model of intracellular lipid peroxidation. *Free Radical Biol. & Med.*, 8:471–485, 1990.

[5] B.R. Bacon, R.S. Britton, R. O'Neil, S.C. Y Li, and Y. Kobayashi. *Free radicals: from basic science to medicine*, chapter Hepatotoxicity of experimental hemochromatosis, pages 242–260. Birkhäuser Verlag, Basel, 1st edition, 1993.

[6] B.A. Barshop, R.F. Wrenn, and C. Frieden. *Anal. Biochem.*, 130:134–145, 1983.

[7] A.F. Bartholomay. A stochastic approach to statistical kinetics with application to enzyme kinetics. *Biochemistry*, 1(2):223–230, 1962.

[8] L. Bergendi and M. Ferencik. The role of reactive oxygen intermediates in intracellular killing of microorganisms. *Biologia*, 37(12):1187–1194, 1982.

[9] B.H.J. Bielski and R.L. Arudi. Preparation and stabilization of aqueous/ethanolic superoxide solutions. *Analytical Biochemistry*, 133:170–178, 1983.

[10] B.H.J. Bielski, D.E. Cabelli, R.L. Arudi, and A.B. Ross. Reactivity of $HO_2^{\cdot}/O_2^{-}$ radicals in aqueous solution. *J. Phys. Chem. Ref. Data*, 14(33):1041–1099, 1985.

[11] J. Bland. Biochemical effects of excited state molecular oxygen. *J. Chem. Edu.*, 53:274–279, 1976.

[12] R. P. Bolender, D. Paungartner, G. Losa, D. Mueller, and E. R. Weibel. Integrated stereological and biochemical studies on hepatocytic membranes: 1. membrane recoveries in subcellular fractions. *J. Cell Biol.*, 77:565–583, 1978.

[13] W. Bray. *J. Am. Chem. Soc.*, 60:82–86, 1938.

[14] J. Brummer and F. Wilkinson. Chemical kinetics of singlet oxygen in solution. *J. Phys. Chem. Ref. Data*, 10(4):809–999, 1981.

[15] D.L. Bunker, B. Garret, T. Kleindienst, and G.S. Long III. Discrete simulation methods in conbustion kinetics. *Combustion and Flame*, 23:373–379, 1974.

[16] J. A. Burns, A. Cornish-Bowden, A. K. Groen, R. Heinrich, H. Kacser, J. W. Porteous, S. M. Rapoport, T. A. Rapoport, J. W. Stucki, J. M. Tager, R. J. A. Wanders, and H. V. Westerhoff. Control analysis of metabolic systems. *Trends Biochem. Sci.*, 10:16, 1985.

[17] G.W. Burton and K.U. Ingold. β-carotene: an unusual type of lipid antioxidant. *Science*, 224:569–573, 1984.

[18] G.V. Buxton, C.L. Greenstock, W.P. Helman, and A.B. Ross. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals ($HO^{\cdot}/O^{\bar{\cdot}}$) in aqueous solution. *J. Phys. Chem. Ref. Data*, 17(2):513–781, 1988.

[19] E. Cadenas. Biochemistry of oxygen toxicity. *Ann. Rev. Biochem.*, 58:79–110, 1989.

[20] B. Chance, H. Sies, and A. Boveris. Hydroperoxide metabolism in mammaliam organs. *Physiological reviews*, 59(3):527–595, 1979.

[21] S.H. Chiou. *J. Biochem.*, 94:1259, 1983.

[22] M.R. Clemens and H.D. Waller. Lipid peroxidation in erythrocytes. *Chem. Phys. Lipids*, 45:251–268, 1987.

[23] G. Cohen. Oxidative stress in the nervous system. In H. Sies, editor, *Oxidative Stress*, chapter 16, pages 383–402. Academic Press, Orlando, 1985.

[24] S.D. Conte and C. de Boor. *Elementary numerical analysis: an algorithmic approach*. McGraw-Hill, New York, 1980.

[25] A. Cornish-Bowden and J.-H.S. Hofmeyr. Metamodel: a program for modelling and control analysis of metabolic pathways on the ibm pc and compatibles. *CABIOS*, 7:89–93, 1991.

[26] IBM Corporation. Chemical kinetics simulator 1.0: user's manual, 1995.

[27] F.S. Dainton and J. Rowbottom. *Trans. Faraday Soc.*, 49:1160–1173, 1953.

[28] J.S. Davis. Myosin thick filaments and subunit exchange - a stochastic simulation based on the kinetics of assembly. *Biochemistry*, 32(15):4035–4042, 1993.

[29] D.A. Dixon and R.A. Shafer. Computer simulation of kinetics by the Monte Carlo technique. *J. Chem. Edu.*, 50(9):648–650, 1973.

[30] J.L. Doob. *Stochastic processes*. John Wiley and Sons, Toronto, 1990.

[31] L. M. Dorfman and G. E. Adams. Reactivity of the hydroxyl radical in aqueous solutions. Technical Report NRSDS-NBS-46, National Bureau of Standards, Government Printing Office, Washington, 1973.

[32] J. Doussiere and P.V. Vignais. Immunological properties of $ho_2^{\cdot}/o_2^{\cdot-}$ generating oxidase from bovine neutrphils. *FEBS lett.*, 234(2):362–366, 1988.

[33] H.-J. Duchstein and H.-J. Gurka. Activated species of oxygen: A challenge to mordern pharmaceutical chemistry. *Archiv der Pharmazie*, 325:129–146, 1992.

[34] D. Edelson. Computer-simulation in chemical-kinetics. *Science*, 214(24):981–986, 1981.

[35] M Ehlde and G Zacchi. Mist - a user-friendly metabolic simulator. *CABIOS*, 11(2):201–207, 1995.

[36] H. Esterbauer, G. Striegl, H. Puhl, S. Oberreither, M. Rotheneder, M. Elsaadani, and G. Jurgens. The role of vitamin-e and carotenoids in preventing oxidation of low-density lipoproteins. *Annals of the New York Acad. of Sciences*, 70(EC):254–267, 1989.

[37] J.A. Fee. A comment on the hypothesis that oxygen toxicity is mediated by superoxide. In *Oxygen and life*, pages 76–97, London, 1980. Royal Society of Chemistry.

[38] J.A. Fee. Is superoxide important in oxygen poisoning? *TIBS*, 7:84–86, 1982.

[39] H.J.H Fenton. Oxidation of tartaric acid in the presence of iron. *J. Chem. Soc.*, 65:899–903, 1894.

[40] L. Flohé and R. Zimmermann. GSH-induced swelling of isolated mitochondria. In *Glutathione*, pages 241–247, Tubingen, 1973.

[41] K-L. Fong, P.B. McCay, J.L. Poyer, H.P. Misra, and B.B. Kelle. *Chem. Biol. Interac.*, 15:77–89, 1976.

[42] C. S. Foote. Mechanisms of sensitized oxidations. *Science*, 162:963–970, 1968.

[43] C.G. Fraga, Leibovitz, and A.L. Tappel. Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: characterization and comparison with homogenates and microsomes. *Free Radical Biol. & Med.*, 4:155–161, 1988.

[44] I. Fridovich. Oxygen radicals, hydrogen peroxide, and oxygen toxicity. In William A. Pryor, editor, *Free Radicals in Biology*, pages 239–277. Academic Press, New York, 1976.

[45] I. Fridovich. Biological effects of the superoxide radical. *Arch. Biochem. Biophys.*, 247:1–11, 1986.

[46] I Fridovich. Superoxide dismutases. *Adv. in Enz. and Rel. Areas of Molec. Biol.*, 58:61–97, 1986.

[47] I Fridovich. Superoxide dismutases: an adaptation to a paramagnetic gas. *J. Biol. Chem.*, 14:7761–7764, 1989.

[48] I Fridovich. Superoxide radical and superoxide dismutases. *Ann. Rev. Biochem.*, 64:97–112, 1995.

[49] A. Garciatejedor, J.C. Sanznuno, J. Olarrea, F.J. Delarubia, and F. Montero. Influence of the hypercycle on the error threshold - a stochastic approach. *J. of Theor. Biol.*, 134(4):431–443, 1988.

[50] D.A. Garfinkel and J.J. Rutledge, J.D. Higgins. *Commun. Assoc. Comput. Mach.*, 4:559–562, 1961.

[51] D. Garfinkerl. A machine-independent language for the simulation of complex chemical and biochemical systems. *Computers Biomed. Res.*, 2:31–44, 1968.

[52] J.M. Gebicki and B.H.J. Bielski. Comparison of the capacities of the perhydroxyl and the superoxide radicals to initiate chain oxidation of linoleic acid. *J. Am. Chem. Soc.*, 103:7020–7022, 1981.

[53] D.T. Gillespie. A general method for numerically simulating the stochastic time evolution of coupled chemical reactions. *J. Comp. Phys.*, 22:403–434, 1976.

[54] D.T. Gillespie. Exact stochastic simulation of coupled chemical reactions. *J. Phys. Chem.*, 81(25):2340–2361, 1977.

[55] A.W. Girotti. Mechanisms of lipid peroxidation. *Free Radical Biol. & Med.*, 1:87–95, 1985.

[56] A.A. Gorman and M.A.J. Rodgers. Singlet molecular oxygen. *Chem. Soc. Rev.*, 10:205–231, 1981.

[57] T.E. Graedel and R. McGill. Graphical presentation of results from scientific computer models. *Science*, 1215:1191–1198, 1982.

[58] G. W. Grams, K. Eskins, and G. E. Inglett. Dye-sensitized photooxidation of α-tocoferol. *J. Amer. Chem. .Soc.*, 94:866–868, 1972.

[59] M.J. Green and H.A.O. Hill. Chemistry of dioxygen. *Methods in Enzymology*, 155:3–21, 1984.

[60] M. C. Gutteridge. Reactivity of hydroxyl and hydroxyl-like radicals discriminated by the release of thiobarbituric acid-reactive material from deoxy sugars, nucleosides and benzoate. *Biochem. J.*, 224:761–767, 1984.

[61] M. C. Gutteridge. Superoxide dismutases inhibit the superoxide-driven fenton reaction at two different levels. *FEBS Letters*, 185(1):19–23, 1985.

[62] M. C. Gutteridge and Bannister J.V. Copper+zinc and manganese superoxide dismutases inhibit deoxyribose degradation by superoxide-driven fenton reaction at two different stages. *Biochem. J.*, 234:225–228, 1986.

[63] F. Haber and J. Weiss. The catalytic decomposition of hydrogen peroxide by iron salts. *Proc. R. Soc. London. Ser. A*, 147:332–351, 1934.

[64] A.T. Hagler and J. Moult. Computer simulation of the solvent structure around biological macromolecules. *Nature*, 272:222–226, 1978.

[65] B. Halliwell. The superoxide dismutase activity of iron complexes. *FEBS Letters*, 56:34–38, 1975.

[66] B. Halliwell. An attempt to demonstrate a reaction between superoxide and hydrogen peroxide. *FEBS Letters*, 72:8–10, 1976.

[67] B. Halliwell. Superoxide and superoxide-dependent formation of hydroxyl radicals are important in oxygen toxicity. *TIBS*, 7:270–272, 1982.

[68] B. Halliwell and J. M. C. Gutteridge. Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy. *Lancet*, ii:1396–1397, 1984.

[69] B. Halliwell and J. M. C. Gutteridge. Oxygen free radicals in relation to biology and medicine: Some problems and concepts. *Arch.Biochem.Biophys.*, 246:501–514, 1986.

[70] B. Halliwell and J.M.C. Gutteridge. The deoxyribose assay: an assay both for 'free' hydroxyl radical and for site-specific hydroxyl radical production. *Biochem. J.*, 253:932–933, 1988.

[71] B. Halliwell and J.M.C. Gutteridge. *Free radicals in Biology and Medicine*, chapter Oxygen is poisonous - an introduction to oxygen toxicity and free radicals, pages 1–21. Clarendon press, Oxford, 2nd edition, 1989.

[72] B. Halliwell and J.M.C. Gutteridge. The role of free radicals and catalytic metal ions in human disease: An overview. *Methods in Enzymology*, 186:1–85, 1990.

[73] B. Halliwell and M. C. Gutteridge. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.*, 219:1–14, 1984.

[74] B. Halliwell, M.A. Murcia, S. Chirico, and O.I. Aruoma. Free radicals and antioxidants in food and *in vivo*: what they do and how they work. *Critical Reviews in Food Science and Nutrition*, 35(1 & 2):7–20, 1995.

[75] N.M. Hamers and D. Roos. Oxidative stress in human neutrophilic granulocytes: host defence and self-defence. In H. Sies, editor, *Oxidative Stress*, chapter 15, pages 351–381. Academic Press, Orlando, 1985.

[76] R.W. Hamming. *Digital filters*. John Wiley and Sons, New Jersey, 1989.

[77] J.E. Harrison, B.D. Watson, and J. Schultz. Myeloperoxidase and singlet oxygen: a reappraisal. *FEBS Letters*, 92(2):327–332, 1978.

[78] N. Haugaard. Cellular mechanisms of oxygen toxicity. *Physiological reviews*, 48:229–240, 1968.

[79] N Hayashi, K.and Sakamoto. *Dynamic Analysis of Enzyme Systems, an introduction*. Japan Scientific Societies & Springer-Verlag, Tokyo, first edition, 1986.

[80] R. Heinrich, S. M. Rapoport, and T. A. Rapoport. Metabolic regulation and mathematical models. *Prog. Biophys. Molec. Biol.*, 32:1–82, 1977.

[81] R. Heinrich and T. A. Rapoport. A linear steady–state treatment of enzymatic chains; general properties, control and effector strength. *Eur. J. Biochem.*, 42:89–95, 1974.

[82] R. Heinrich and T. A. Rapoport. A linear steady state treatment of enzymatic chains. *Eur. J. Biochem.*, 42:97–105, 1974.

[83] A.J. Hide. The history of free radicals and moses gomberg's contributions. In *Free radicals in solution: international symposium on free radicals in solution*, pages 1–13, London, 1967. Butterwoths.

[84] H.A.O. Hill. The chemistry of dioxygen and its reduction products. In D.W. Fitzsimmons, editor, *Oxygen Free Radicals and Tissue Damage*, pages 5–11, Amsterdam, 1979. Excerpta Medica.

[85] T.L. Hill and Y.D. Chen. Interacting enzyme systems at steady state: Further Monte Carlo calculations on two-state molecules. *Proc. Natl. Acad. Sci. USA*, 75:5260–5263, 1978.

[86] C.G. Hill Jr and R.A. Grieger-Block. Kinetic data: Generation, interpretation and use. *Food Technol.*, pages 56–66, 1980.

[87] A.C. Hindmarsh. LSODE and LSODI, two new initial value ordinary differential equation solvers. *ACM SIGNUM Newsletter*, 15(Dec):4, 1980.

[88] A.C. Hindmarsh. Current methods for large stiff systems. *IMACS Num. Math. Applic.*, pages 135–144, 1986.

[89] H.G. Holzhütter and A. Colosimo. Simfit: a microcomputer software toolkit for modelistic studies in biochemistry. *CABIOS*, 6:23–28, 1991.

[90] A.A. Horton and S. Fairhurst. Lipid-peroxidation and mechanisms of toxicity. *CRC Critical Rev. in Toxicol.*, 18(1):27–79, 1987.

[91] A.A. Horton and Fairhurst S. Lipid peroxidation and mechanisms of toxicity. *Critical Reviews in toxicology*, 18(1):27–79, 1987.

[92] F.E. Jr. Hunter, A. Scott, P.E. Hoffsten, J.M. Gebicki, J. Weinstein, and A. Schneider. Studies on the mechanism of swelling, lysis, and disintegration of isolated liver mitochondria exposed to mixtures of oxidized and reduced glutathione. *J. Biol. Chem.*, 239:614–621, 1964.

[93] F.E. Jr. Hunter, A. Scott, P.E. Hoffsten, F. Guerra, J. Weinstein, A. Schneider, B. Schutz, J. Fink, L. Ford, and E. Smith. Studies on the mechanism of ascorbate-induced swelling and lysis of isolated liver mitochondria. *J. Biol. Chem.*, 239:604–613, 1964.

[94] F.E. Jr. Hunter, A. Scott, J. Weinstein, and A. Schneider. Effects of phosphate, arsenate and other substances on swelling and lipid peroxide formation when mitochondria are treated with oxidized and reduced glutathione. *J. Biol. Chem.*, 239:622–630, 1964.

[95] D.H. Irvine, E.O. Voit, and M.A. Savageau. Analysis of complex dynamic networkswith essyns. a program for the efficient solution of non-linear differential equations in s-sytem form. In E.O. Voit, editor, *Canonical non-linear modelling. S-system approach to understand complexity.*, pages 133–141. van Nostrand Reinhold, New York, 1st edition, 1991.

[96] C.J. Jachimowski, D.A. McQuarrie, and M.E. Russel. A stochastic approach to enzyme-substrate reactions. *Biochemistry*, 3(11):1732–1736, 1964.

[97] D.P. Jones. The role of oxygen concentration in oxidative stress: hypoxic and hyperoxic models. In H. Sies, editor, *Oxidative Stress*, chapter 8, pages 151–185. Academic Press, Orlando, 1985.

[98] H. Kacser and J. A. Burns. The control of flux. *Symp. Soc. Exp. Biol.*, 27:65–104, 1973.

[99] J.R. Kanofsky, H. Hooglnad, R. Wever, and S.J. Weiss. Singlet oxygen production by human eosinophils. *J. Biol. Chem.*, 263:9692–9696, 1988.

[100] J.R. Kanofsky, H. Sugimoto, and D.T. Sawyer. Singlet oxygen production from the reaction of superoxide ion with halocarbons in acetonitrile. *J. Am. Chem. Soc.*, 110:3698–3699, 1988.

[101] D.R. Kearns. Physical and chemical properties of singlet molecular oxygen. *Chemical Reviews*, 71(4):395–427, 1971.

[102] J.P. Keene. Pulse radiolysis of ferrous sulphate solution. *Radiat. Res.*, 22:14–20, 1964.

[103] J. Keizer. Theory of rapid bimolecular reactions in solution and membranes. *Acc. Chem. Res.*, 18:235–241, 1985.

[104] A. U. Khan. Theory of electron tranfer generation and quenching of singlet oxygen [1g+ and 1g] by superoxide anion.the role of water in the dismutation of O_2^-. *J.Amer.Chem.Soc.*, 99:370–371, 1977.

[105] A. U. Khan. Activated oxygen: Singlet molecular oxygen and superoxide anion. *Photochem.Photobiol.*, 28:615–627, 1978.

[106] M.R. Kibby. Stochastic method for the simulation of biochemical systems on a digital computer. *Nature*, 222:298–299, 1969.

[107] S.J. Klebanoff. Bactericidal effect of fe^{2+}, caeruloplasmin, and phosphate. *Arch. Biochem. and Biophys.*, 295(2):302–308, 1992.

[108] A. Knowles. The flavin-sensitized photooxidation of nucleotides-ii. the participation of the flavin triplet state. *Photochem.Photobiol.*, 13:225–236, 1971.

[109] A. Knowles. A mechanism for the methylene blue sensitized oxidation of nucleotides. *Photochem.Photobiol.*, 13:473–487, 1971.

[110] A. Knowles and G. N. Mautner. The flavin- sensitized photooxidation of nucleotides-iii. the participation of oxigen. *Photochem.Photobiol.*, 15:199–207, 1972.

[111] E.I. Kolkov and P.N. Lebedev. Lipid peroxidation as cell cycle timer. In *unknown*, Pau, 1992. submited to Proceedings of the III Conference on Math. Popul. Dynamics.

[112] Y. Kono and I. Fridovich. Superoxide radical inhibits catalase. *J. Biol. Chem.*, 257:5751–5754, 1982.

[113] J.M. Kootsey, M.C. Kohn, M.D. Feezor, G.R. Mitchell, and P.R. Fletcher. SCoP: an interactive simulation control program for micro- and minicomputers. *Bull. Math. Biol.*, 48(34):427–441, 1986.

[114] W. H. Koppenol. Reactions involving singlet oxygen and the superoxide anion. *Nature*, 262:420–421, 1976.

[115] W. H. Koppenol and J. Butler. Mechanisms of reactions involving singlet oxygen and the superoxide anion. *FEBS Letters*, 83:1–6, 1977.

[116] W. H. Koppenol, J. Butler, and J. W. van Leeuwen. The Haber-Weiss cycle. *Photochem. Photobiol.*, 28:655–660, 1978.

[117] W.H. Koppenol. Thermodynamics of reactions involving oxyradicals and hydrogen-peroxide. *Bioelectrochemistry and Bioenergetics*, 18(1-3):3–11, 1987.

[118] A. Kornhauser, N. I. Krinsky, P.-K. Huang, and D. C. Clagett. A comparative study of photodynamic oxidation and radiofrequency- discharge-generated $^1O_2$ oxidation of guanosine. *Photochem.Photobiol.*, 18:63–69, 1973.

[119] I. Kraljic and V.A. Sharpatyi. Determination of singlet oxygen rate constants in aqueous solutions. *Photochem.Photobiol.*, 28:583–586, 1978.

[120] M. Kraus and B. Wolf. Model formation in biology - a structured analysis of intracellular calcium oscillations in nonelectrically-excitable cells. *Naturwissenschaften*, 79(7):289–299, 1992.

[121] N.I. Krinsky. Mechanisms of action of biological antioxidants. *Proc. of Soc. Exp. Biol. & Med.*, 200:248–254, 1992.

[122] M. Kubicek and M. Marek. *Computational methods in bifurcation theory and dissipative structures*. Springer Verlag, New York, 1983.

[123] M.E. Lacy. Systems theory as a conceptual and organizational framework for computational and inferential chemistry. *J. Chem. Edu.*, 63(5):392–396, 1986.

[124] S.M. Leake, D.S. amd Rankin. The oxidative modification of low-density lipoproteins by macrophages. *Biochem. J.*, 270:741–748, 1990.

[125] A.L. Lehninger and M. Schneider. Mitochondrial swelling induced by glutathione. *J. Biophys. Biochem. Cytol.*, 5:109–1116, 1959.

[126] M.K. Lenz and D.B. Lund. Experimental procedures for determining destruction kinetics of food components. *Food Technol.*, pages 51–56, 1980.

[127] P. Linda. Automatic selection of methods for solving stiff and nonstiff systems of ordinary differential equations. *SIAM J. Sci. Stat. Comput.*, 4(1):136–148, 1983.

[128] E.M. Link. Inflamation and a mechanism of hydrogen peroxide cytotoxicity. In G. Poli, E. Albano, and M.U. Dianzani, editors, *Free radicals: from basic science to medicine*, Molecular and Cell Biology Updates, pages 113–122. Birkhauser Verlag, Basel, first edition, 1993.

[129] D.A. MacQuarry. *Stochastic approach to chemical kinetics*. Methuen, London, 1968.

[130] B.G. Malmström. Enzymology of oxygen. *Ann. Rev. Biochem.*, 51:21–59, 1982.

[131] M. Mareschal and A. Dewit. Microscopic simulation of a chemical-instability. *J. of Chem. Phys.*, 96(3):2000–2004, 1992.

[132] G. Marsaglia. A current view of random number generators. In 16^{th} Symposium of the interface, editor, *Computer scince and statistics*, North-Holland, Amsterdam, 1985.

[133] G. Marsaglia and A. Zaman. A new class of random number generators. *The Annals of applied Prob.*, 1(3):462–480, 1991.

[134] MathSoft Inc (Cambridge Massachusetts). Mlab: mathematical modelling system, 1992.

[135] The MathWorks. Matlab, 1990.

[136] E. A. Mayeda and A. J. Bard. The production of singlet oxigen in electrogenerated radical ion electron transfer reactions. *J.Amer.Chem.Soc.*, 95:6223–6226, 1973.

[137] G.J. McClune and J.A. Fee. Stopped flow spectrophotometric observation of superoxide dismutation in aqueous solution. *FEBS Letters*, 67:294–298, 1976.

[138] J. M. McCord and I. Fridovich. Superoxide dismutase: An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.*, 244:6049–6055, 1969.

[139] J.M. McCord. Free radicals and inflamation: protection of synovial fluid by superoxide dismutase. *Science*, 185:529–531, 1974.

[140] J.M. McCord and E.D. Day Jr. Superoxide-dependent production of hydroxyl radical catalyzed by iron-edta complex. *FEBS Letters*, 86(1):139–142, 1978.

[141] P. Mendes. GEPASI: a software package for modelling the dynamics, steady states and control of biochemical and other systems. *CABIOS*, 9(5):563–571, 1993.

[142] P. Mendes. Biochemistry by numbers: simulation of biochemical pathways with Gepasi 3. *TIBS*, 22:361–363, 1997.

[143] P. B. Merkel, R. Nilsson, and D. R. Kearns. Deuterium effects on singlet oxygen lifetimes in solutions: a new test of singlet oxygen reactions. *J.Amer.Chem.Soc.*, 94:1030–1031, 1972.

[144] C. Michiels, O. Toussaint, and J. Remacle. Comparative study of oxigen toxicity in human fibroblasts and endothelial cells. *J. of Cell. Physiol.*, 144:295–302, 1990.

[145] J.H. Miller, W.E. Wilson, C.E. Swenberg, L.S. Myers Jr, and D.E. Charlton. Stochastic model of free radical yields in oriented dna exposed to densely ionizing radiation at $77k$. *Int. J. Radiat. Biol.*, 53(6):901–907, 1988.

[146] J.M. Misra and I. Fridovich. The generation of the superoxide radical during the autoxidation of hemoglobin. *J. Biol. Chem.*, 247:6960–6962, 1972.

[147] W. D. C. Moebs. *Math. Bio. Sci.*, 22:113–120, 1974.

[148] J. P. Moniz-Barreto. Analise dinamica de um sistema. Technical report, Faculdade de Ciencias da Universidade de Lisboa, 1989.

[149] J.W. Moore. *Kinetics and Mechanism.* John Wiley and Sons, New York, 1981.

[150] F. Morel, J. Doussiere, and P.V. Vignais. The superoxide-generating oxidase of phagocytic cells: Physiological, molecular and pathological aspects. *Eur. J. Biochem.*, 201:533–546, 1991.

[151] A. Naqui and Chance B. Reactive oxygen intermediates in biochemistry. *Ann. Rev. Biochem.*, 55:137–166, 1986.

[152] P. Neta, R.E. Huie, and A.B. Ross. Rate constants for reactions of inorganic radicals in aqueous solutions. *J. Phys. Chem. Ref. Data*, 17(3):1027–, 1988.

[153] P. Neta, R.E. Huie, and A.B. Ross. Rate constants for reactions of peroxyl radicals in fluid solutions. *J. Phys. Chem. Ref. Data*, 19(2):412–513, 1990.

[154] R. Nilsson, P. B. Merkel, and D. R. Kearns. Unambigous evidence for the participation of singlet oxigen ($^1\triangle_g O_2$) in photodynamic oxidation of amino acids. *Photochem.Photobiol.*, 16:117–124, 1972.

[155] S.H. Northrup, S.A. Allison, and J.A. McCammon. Brownian dynamics simulation of diffusion-influenced bimolecular reactions. *J. Chem. Phys.*, 80(4):1517–1524, 1984.

[156] G. Poli, E. Albano, and M.U. Dianzani, editors. *Free radicals: from basic science to medicine.* Molecular and Cell Biology Updates. Birkhauser Verlag, Basel, first edition, 1993.

[157] W. H. Press, B. P. Flannery, S. A. Teukolsky, and W. T. Vetterling. *Numerical Recipies in C. The Art of Scientific Computing.* Cambridge University Press, Cambridge, 1988.

[158] W. A. Pryor. The formation of free radicals and the consequences of their reactions in vivo. *Photochem. Photobiol.*, 28:787–801, 1978.

[159] W.A. Pryor. The role of free radical reactions in biological systems. In William A. Pryor, editor, *Free Radicals in Biology*, pages 1–49. Academic Press, New York, 1976.

[160] B. Rabinovitch. The Monte Carlo method: Plotting the course of complex reactions. *J. Chem. Edu.*, 46(5):262–268, 1969.

[161] J. G. Reich and E. E. Selkov. *Energy Metabolism of the Cell.* Academic Press, London, 1981.

[162] J. Remacle, D. Lambert, M. Raes, E. Pigeolet, and C. Michiels. Importance of various antioxidant enzymes for cell stability: Confrontation between theoretical and experimental data. *Biochem. J.*, 286:41–46, 1992.

[163] C.A. Rice-Evans, A.T. Diplock, and M.C.R. Symons. *Techniques in free radical research*, volume 22 of *Laboratory techniques in biochemistry and molecular biology.* Elsevier, 1991.

[164] A. Rigo, R. Stevenato, A. Finazzi-Agro, and G. Rotilio. An attempt to evaluate the rate of the Haber-Weiss reaction by using $OH^{\cdot}$ radical scavengers. *FEBS Letters*, 80:130–132, 1977.

[165] B.D. Ripley. *Stochastic simulation.* John Wiley and Sons, New York, 1987.

[166] G. Roman and D. Garfinkerl. Biossim: structured machine-independent biological simulation language. *Computers Biomed. Res.*, 11:3–15, 1978.

[167] J.R. Rowland. *Linear control systems: modeling, analysis and design.* Prentice-Hall, New York, 1986.

[168] I. Saguy and M. Karel. Modeling of quality deterioration during food processing and storage. *Food Technol.*, pages 78–84, 1980.

[169] D. Samuel and F. Sterkel. The physico-chemical properties of molecular oxygen. In Hayaishi O., editor, *Molecular oxygen biology: topics in molecular oxygen research*, pages 1–32, Amsterdam, 1974. North-Holland pub. company.

[170] H.M. Sauro. SCAMP: a metabolic simulator and control analysis program. *Mathl. Comput. Modelling*, 15(12):15–28, 1991.

[171] M. A. Savageau. Biochemical systems analysis: I. some mathematical properties of the rate law for the component enzyme reactions. *J. Theor. Biol.*, 25:365–369, 1969.

[172] M. A. Savageau. Biochemical systems analysis: II. the steady state solutions for an n–pool system using a power–law approximation. *J. Theor. Biol.*, 25:370–379, 1969.

[173] R.: Real Software (Soquel California) Schafly. Mercury v 2.00, 1990.

[174] R. Sedgewick. *Algorithms*, chapter 35, pages 509–520. Addison-Wesley, 1988.

[175] A.W. Segal and A. Abo. The biochemical basis of the NADPH oxidase of phagocytes. *TIBS*, 18:43–47, 1993.

[176] H. Sies, editor. *Oxidative Stress*. Academic Press, Orlando, 1985.

[177] H. Sies. Stratagies of antioxidant defense. *Eur. J. Biochem*, 215:213–219, 1993.

[178] A. Sikorski and J. Skolnick. Dynamic Monte Carlo simulations of globular protein folding. *J. of Mol. Biol.*, 215:183–198, 1990.

[179] A. Singh. Introduction: Interconversion of singlet oxygen and related species. *Photochem. Photobiol*, 28:429–433, 1978.

[180] A. Singh. Chemical and biochemical aspects of superoxide radicals and related species of activated oxygen. *Can. J. Physiol. Pharmacol.*, 60:1330–1345, 1982.

[181] J. Skolnick, A. Kolinski, and R. Yaris. Monte Carlo studies of the long-time dynamics of dense polymer systems. the failure of the reptation model. *Acc. Chem. Res.*, 20:350–356, 1987.

[182] G.A. Soffen. Priestley lecture: mars, biogenesis and the role of oxygen. In *Oxygen and life*, pages 1–18, London, 1980. Royal Society of Chemistry.

[183] Civilised Software. Mlab: mathematical modelling system, 1992.

[184] G. Stark. The effect of ionizing radiation on lipid membranes,. *Biochim. Biophys. Acta,*, 1071:103–122, 1991.

[185] H.J. Stoffers, E.L.L. Sonnhammer, G.J.F. Blommestijn, N.J.H. Raat, and H.V. Westerhoff. Metasim: object-oriented modelling of cell regulation. *CABIOS*, 8:443–449, 1992.

[186] Y.J. Suzuki and G.D. Ford. Mathematical model supporting the superoxide theory of oxygen toxicity. *Free Radical Biol. & Med.*, 16(1):63–72, 1994.

[187] A. Szent-Gyorgy. The electronic theory of cancer. *Int. J. Quant. Chem., Quant. Biol. Symp.*, 3:45–50, 1976.

[188] A. Szent-Gyorgy. The living state and cancer. *Int. J. Quant. Chem., Quant. Biol. Symp.*, 7:217–222, 1980.

[189] B. Tadolini and L. Cabrini. On the mechanism of $OH^{\cdot}$ scavenger action. *Biochem. J.*, 253:931–932, 1988.

[190] L. Takács. *Stochastic processes: Problems and solutions*. Chapman an Hall, London, 1966.

[191] A. L. Tappel, A. A. Tappel, and C. G. Fraga. Application of simulation modelling to lipid peroxidation processes. *Free Radical Biol. & Med.*, 7:361–368, 1989.

[192] P. Todd, T. Nishidai, L. Révész, and M. EDgreen. Chemical competition in target radical reactions: numerical simulation of the theory and comparisons with mesured oxygen effect on dna damage in cells. *Int. J. Radiat. Biol.*, 50(6):1023–1037, 1986.

[193] C Walling. Fenton's reagent revisited. *Acc. Chem. Res.*, 8:125–131, 1975.

[194] R.H. Weiss and R.W. Estabrook. The mechanism of cumene hydroperoxide-dependent lipid peroxidation: the significance of oxygen uptake. *Arch.Biochem.Biophys.*, 251:336–347, 1986.

[195] R.H. Weiss and R.W. Estabrook. The mechanism of cumene hydroperoxide-dependent lipid peroxidation: the function of cytochrome p450. *Arch.Biochem.Biophys.*, 251:348–360, 1986.

[196] S.J. Weiss. The role of superoxide in the destruction of erythrocyte targets by human neutrophils. *J. Biol. Chem.*, 255:9912–9917, 1980.

[197] R. L. Wilson. Hydroxyl radicals and biological damage *in vitro*: what relevance *in vivo*? In D.W. Fitzsimmons, editor, *Oxygen Free Radicals and Tissue Damage*, pages 19–34, Amsterdam, 1979. Excerpta Medica.

[198] R.L. Wilson. *Free radicals: from basic science to medicine*, chapter Trevor Slater, free radical redox chemistry and antioxidants: From NAD^+ and vitamin C to CCL_4 and vitamin E, to thiols, myoglobin and vitamins A and D., pages 2–17. Birkhäuser Verlag, Basel, 1st edition, 1993.

[199] C.C. Winterbourn, J.M.C. Gutteridge, and B. Halliwell. Doxorubicin-dependent lipid peroxidation at low partial pressures of O_2. *Free Radical Biol. & Med.*, 1:43–49, 1985.

[200] B.P. Yu. Cellular defenses against damage from reactive oxygen species. *Physiological reviews*, 74(1):139–162, 1994.

[201] H.-D. Zhang, J.-M. Lu, and L.-L. Yang. Sample duplication method for monte carlo simulation of large reaction-diffusion system. *Science in China*, 37(4):385–394, 1994.

[202] C.T. Zimmerle and C. Frieden. Analysis of progress curves by simulations generated by numerica integration. *Biochem. J.*, 258:381–387, 1989.